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I am very grateful to Professor Luca Rastrelli, Dipartimento di Scienze Farmaceutiche e Biomediche, University of Salerno, 84084 Fisciano (SA), Italy, for organizing this issue, originating from the XIX SILAE (Società Italo-Latinoamericana di Etnomedicina) Congress, which was held at Cagliari, Italy, from September 6th-10th, 2010, and attended by a large number of participants from Latin America and the European Union. The present issue highlights some significant aspects of ethnomedicine. The editors join me in thanking Professor Rastrelli, the authors and the reviewers for their efforts that have made this issue possible, and to the production department for putting it into print.

Pawan K. Agrawal
Editor-in-Chief
Editorial

The Italo-Latin American Society of Ethnomedicine (SILAE, www.silae.it) is an international non-profit organization dedicated to advancing science around the world by serving as an educator, leader, spokesperson and professional association. The fundamental objective of SILAE is to promote research and development into the use of medicinal and food plants in different countries of the World. SILAE welcomes and actively seeks opportunities to work cooperatively, activating and intensifying scientific relations between countries and between SILAE members. Since SILAE was founded (1990) its objective has been set to contribute to the close examination of the themes of great interest and actuality in the context of the relationships between Latin America and the European Union. In addition to this, SILAE aimed to individualize new ways of collaboration between its member countries and other European as well as Asiatic countries to sign accords with intergovernmental organizations. SILAE proposes to establish contacts with Scientific Communities, Universities, and Research Centres for the pursuit of medicinal and food plants knowledge. Moreover SILAE_live, the one-to-one live Chat and Messenger on our website (www.silae.it), is the first scientific chat on the web and is a developed tool to engage the interest and imagination of the public and for helping non-scientists to understand and enjoy scientific discoveries and the scientific processes. In addition to organizing membership activities, SILAE publishes the SILAE Special Issues, as well as many scientific newsletters, books and reports, and spearheads programs that raise the bar of understanding for science worldwide.

Natural Product Communications is publishing a special issue that contains a selection of papers that were presented at the XIX SILAE Congress (Cagliari, Italy, September, 6-10, 2010). For the Conference, 292 papers from authors coming from 19 different countries were accepted and published in the Proceedings of the SILAE 2010 (Abstract book ISBN: 88-8160-218-0). The most promising 60 submissions were proposed for publication in the special issue of Natural Product Communications in October 2010, each of which was reviewed by at least two anonymous referees. Following the review, 31 papers from different universities of Argentina (7), Brazil (8), Colombia (2), Cuba (1), Honduras (1), Ireland and Serbia (1), Italy (6), Mexico (2) and Venezuela (3) were selected for publication in this Special Issue. They are original papers on all aspects of natural products including isolation, characterization, spectroscopic properties, biological activities, synthesis, analytical methods and tissue culture; several are collaborative works between two or more countries.

Ten papers present the compositions of essential oils from different aromatic plants using analytical techniques such as GC, GC/MS, GC/MS-LRI, esGC, GC-C-IRMS (1, 2, 4, 7, 8, 11, 14, 16, 22, 28) and also NMR spectroscopy (22). Although essential oils have been used therapeutically for centuries, there is little published research on many of them. Bonaccorsi et al. (1) report in their article on samples of Egyptian neroli oils, obtained from the flowers of bitter orange (Citrus aurantium, Rutaceae). For all the samples the composition was determined by GC/FID and by GC/MS-LRI; the samples were also analyzed by esGC to determine the enantiomeric distribution of twelve volatiles and by GC-C-IRMS for the determination of the δ13CVPDB values of some mono and sesquiterpene hydrocarbons, alcohols and esters. The analytical procedures allowed the quantitative determination of 86 components (1). Radulović et al. (7) identify 109 constituents from an essential oil sample obtained from dry leaves of Nepeta × faassenii Bergmans, a hybrid species produced by crossbreeding N. mussinii Spreng. with N. nepetella L. The chemical composition of the oil was compared, using multivariate statistical analyses (MVA), with those of the oils of other Nepeta taxa, in particular N. mussinii and N. nepetella. The authors also report the chemical composition dissimilarity relationships of 36 Nepeta essential oil samples. Some authors report the in vitro activity of the essential oil against bacteria (2, 11, 16, 28). Bruno et al. (2) record the results obtained with the oil from the aerial parts of Salvia verbenaca (Labiatae) against Bacillus subtilis, Staphylococcus aureus, S. epidermidis, Streptococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris and Pseudomonas aeruginosa; Perez et al. (11), the essential oil of Chrysactinia mexicana (Astaraceae) against Staphylococcus pneumonia; Rios Tesch et al. (16), Lantana camara var. moritziana (Verbenaceae) leaf essential oil against Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, and Pseudomonas aeruginosa; and Mora et al. (28), Phthiriusa adunca (Loranthaceae) against Salmonella typhi, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli and Klebsiella pneumonia. Results confirm that many essential oils possess in vitro antimicrobial activity against pathogens when compared with reference drugs. Martini et al. (8) report the chemical composition of two Lamiaceae, Ocimum selloi and Hesperoyzigis myrtoides, widely used in Brazilian traditional medicine. The authors report, for the first time, the chemical composition of the essential oil from H. myrtoides, a very aromatic small bush found in the region of Aiuruoca (Minas Gerais State, Brazil); this plant is also used in the preparation of a drink with “cachaca”, the Brazilian sugar cane spirit, where the plant is soaked in the bottle’s spirit and buried for one year before being consumed. The essential oils showed activity against Candida albicans C. glabrata, C. krusei, C. parapsilosis and C. tropicalis. Oliva et al. (14) also report the activity against Candida yeasts (C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. guilliermondii, C. parapsilosis and C. tropicalis) of an essential oil obtained from Aloysia triphylla (Verbenaceae), a promising alternative from the Argentinean flora for the treatment of candidiasis.
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Veloso et al. report the application of dioxirane chemistry to essential oils in order to generate modified compounds with potential uses in several areas of medicine and industry (22). Polyphenols are among the most widespread class of metabolites in nature, and their distribution is almost ubiquitous.

**Nine papers** describe the isolation and structure elucidation, as well as the bioactivity, of polyphenolic compounds. Interesting biological properties are reported, such as antioxidant (17, 18, 26, 29), antifungal (13), antimicrobial (17), antiplatelet (20), antiangiogenic (25), chemopreventive and apoptotic activities (21). Arevalo et al. (3) report a new polyphenolic apiosyl derivative, two uncommon apiosyl derivatives, and known phenyl propanoids and flavonoids from *Martillina obovata* (Bignoniiaceae) collected in Honduras, and used by indigenous peoples to treat various eye ailments, including inflammation and conjunctivitis. It is well known that the consumption of polyphenol-rich products, mainly due to their antioxidant properties, is beneficial for human health, Salvador et al. (18), as well as Aislan et al. (29), report the antioxidant capacity and polyphenolic organic acids and flavonoids content of Myrtaceae plants of the south of Brazil. The article by Mendiondo et al. (17) presents the antioxidant and antimicrobial activity of the methanolic extract of *Chuquiraga straminea* (Asteraceae). The extract was also active against ten methicillin resistant and sensitive *S. aureus* strains isolated from nosocomial infection; kaempferol and quercetin glycoside derivatives seem to be responsible for some of the observed biological activity of the extracts. Derita and Zacchino (13) dedicate their article to the bio-guided fractionation of the active dichloromethane extract of *Polygonum persicaria* L. (Polygonaceae). This genus is represented in Argentina by 21 species and some of them have been used in traditional medicine of that country to treat complaints related to fungal infections, such as skin ailments and vaginal disease. Isolated sesquiterpene dialdehydes and flavonoids showed activity against yeasts, *Aspergillus* spp. and dermatophytes with MICs between 3.9 - 250 µg/mL. The results validate the popular use of this plant.

Data from the literature show that some flavonoids and other polyphenolic substances have the property to interfere with the platelet system. A diet rich in polyphenolic compounds may favorably contribute to reducing the risks of cardiovascular diseases through several mechanisms. Douglas et al. (20) assessed the inhibitory activity toward clotting formation and platelet aggregation of an aqueous extract of leaves from *Petroselinum crispum*, an aromatic herb from the Apiaceae family that has been employed in the food, pharmaceutical, perfume and cosmetic industries. The active principles, cosmosrin (apigenin 7-O-glucoside) and apigenin, showed in vitro antiplatelet aggregation activity. Angiogenesis is a crucial step in many pathological conditions like cancer, inflammation and metastasis formation; on this basis, the search for antiangiogenic agents has widened. In order to identify new compounds able to interfere with the Vascular Endothelial Growth Factor Receptor-1 (VEGFR-1), Lepore et al. (25) investigated the extract of *Calyclossus moritzianus* (Myrtaceae) leaves by a competitive ELISA-based assay. Phytochemical and pharmacological investigation of the active fractions led to the isolation of flavonoids and terpenes. The authors hypothesized that the inhibitory activity of PIgf and VEGF interaction with Flt-1 receptor by the *C. moritzianus* CHCl3 extracts and fractions may be due to the presence of a combination of compounds acting synergistically or as vehicles enhancing the biological activity. These results suggest that the use of *C. moritzianus* extract is preferable to that of a purified single compound. Torrenegra et al. (21) evaluated the activity of 3,5-dihydroxy-7-methoxy-flavanone, 3,5-dihydroxy-7-methoxyflavone and 3,5,7-trihydroxy-6-methoxyflavone present in *Chromolaena leviensis* (Asteraceae) on cell viability, cell cycle distribution, mitochondrial membrane depolarization and viability of peripheral blood mononuclear cells and fibroblasts. 3,5-Dihydroxy-7-methoxyflavone showed activity on mitochondrial membrane, whereas both 3,5-dihydroxy-7-methoxyflavone and 3,5-dihydroxy-7-methoxyflavone slightly increased the proliferation of peripheral blood mononuclear cells either with phytohemagglutinin or without it, and the proliferation of fibroblasts.

The chemical composition of naturally grown herbs may vary according to climatic conditions, harvest time, storage condition, and so on. As such, the same type of herb may vary in its composition and concentrations of chemical constituents from batch to batch. These variabilities can result in significant differences in pharmacological activity. Therefore, the identification and extraction of active ingredients from a medicinal plant represent a new approach to the development of natural product based drugs. Picerno et al. (26) report on the evaluation of polyphenol components and antioxidant properties of fresh bergamot juice (*Citrus bergamia*, Rutaceae), as well as on the production and characterization of powders obtained by loading the fresh juice onto maltodextrins as a carrier (BMP) for spray-drying. Moreover, a formulation study to develop tablets containing BMP for oral administration has been performed. The characteristics of the tablets were evaluated in terms of disintegration time and the release of the active compounds into water and simulated biological fluids.

**Five papers** deal with the evaluation of pharmacological activity of crude extracts from plants used in Mexican (6), Argentinean (9, 10 and 31) and Cuban (12) traditional medicine. Pazos et al. (6) investigated the effect of *Morinda citrifolia* (Rubiaceae) seed (noni oil) on serum lipid levels in normolipidemic and hyperlipidemic induced mice. They found that administration of noni oil causes a reduction in total cholesterol and triglyceride levels in both models. GC-MS analysis of the fatty acid methyl esters indicated the presence of five major fatty acids. The mean linoleic acid content of crude noni seed oil was 67.8%; these results indicate that noni seeds may be a useful new source of vegetable oil. Few medicinal plants have been...
scientifically evaluated for their safety, efficacy and potential benefits, despite the great public interest in these herbs. Sabini et al. (10) evaluated the cytotoxic and genotoxic activities of a cold aqueous extract obtained from *Achyrocline satureioides* (Asteraceae) using the *Allium cepa* test, whereas Escobar et al. (31) assessed the genotoxic and cytotoxic activities of a methanolic extract of *Verbascum thapsus* (Scrophulariaceae) using a micronucleus test in mouse bone marrow. Numerous investigations have reported bioactive properties for both medicinal plants and the results obtained in these present studies allow the conclusion to be made that the aqueous extract of *A. satureioides* and the methanolic extract of *V. thapsus* do not contain genotoxic and cytotoxic compounds. Cytotoxicity, antiviral and virucidal activities of aqueous extracts of *Baccharis articulata* were also evaluated by Cristina Vanesa Torres et al. (9). Extracts exhibited more than 95% virucidal activity against *Herpes suis* virus type 1. These findings support the potential application of these extracts as a disinfectant or antiseptic consistent with ancient ethnomedicinal thinking. In Cuba, alcoholic extracts of propolis are popular as a homemade remedy. Three main types of Cuban propolis directly related to their secondary metabolite classes were described: brown Cuban propolis (BCP), rich in polyisoprenylated benzophenones, red Cuban propolis (RCP), containing isoflavonoids as the main constituents, and yellow Cuban propolis (YCP) with a variety of triterpenoids as the major chemical components. Monzote et al. (12) assessed the activity of Cuban propolis extracts (brown, red and yellow type) on *Leishmania amazonensis* and *Trichomonas vaginalis*. All propolis samples caused inhibition of growth of the *Leishmania* parasite. RCP was the most active and the most cytotoxic. Only five propolis extracts showed activity against *T. vaginalis* and in this case YCP samples were the most active.

According to the World Health Organization (WHO), more than 80% of the world's people, mostly in poor and less-developed countries, depend on traditional medicine for their primary healthcare requirements. They use medicinal plants not only for themselves but also for their domestic animals. Traditionally, people collected the ingredients for their medicines from forests. However, due to rapid and extensive deforestation, accompanied by uncontrolled over-exploitation, the wild populations of medicinal plants are disappearing very fast.

**Three papers** deal with biodiversity and nature protection. The *cerrado*, a vast tropical savanna ecoregion of Brazil, particularly in the states of Goiás and Minas Gerais, is characterized by an enormous range of plant and animal biodiversity. The cerrado is one of the world's threatened biodiversity hotspots. About 60% of its vegetation has already been removed and the remaining areas are isolated in forest fragments. Due to the devastation, many natural compounds with potential biological activities have been lost. Soares et al. (23) compared the basal cytotoxicity of active compounds extracted from plants of the Brazilian “cerrado”. Those with low toxicity were subjected to further anti-inflammatory assays as natural products with low cytotoxicity constitute an excellent alternative source for complementary treatments for inflammatory disease. The viability was assayed using the neutral red uptake assay in Mac Coy cells after 24 h of exposure. The dose evaluated was 50µg/µL. The test substances were: cinnamic acid, *p*-coumaric acid, chlorogenic acid, syringic acid, vanilic acid, homogentisis acid, scandenin, palustric acid, diosgenin, and cabraleone. From 1975 until the beginning of the 1980s, many governmental programs have been launched with the intent of stimulating the development of the "cerrado" region, through subsidies for agriculture. As a result, there has been a significant increase in agricultural and cattle production. On the other hand, urban pressure and rapid establishment of agricultural activities in the region have rapidly reduced the biodiversity of the ecosystems. Camargo et al. (30) tried to diagnose the current public programs focused on herbal medicines in Brazil from 1985 to 2006.

Sharry et al. (19) dedicated their article to the establishment of vegetative propagation systems for three native forest species widely used in Argentinean folk medicine: *Erythrina crista-galli* (Fabaceae), *Acacia caven* (Mimosaceae) and *Salix humboldtiana* (Salicaceae). In the last few years the use of in vitro culture techniques for trees has facilitated the cloning of selected phenotypes, leading to the preservation and manipulation of vegetal material. The authors are able to support the conservation of native forest resources for medicinal use by means of vegetative propagation techniques: macro and micropropagation and somatic embryogenesis.

**The last four papers** are quite different from the others, each one dealing with its own subject of great importance. Marques et al. (5) report for the first time the isolation of six aristolactams from *Ottonia anisum* (Piperaceae). Aristolactams belong to a large and important group of naturally occurring alkaloids that possess a phenantherene lactam skeleton with a phenolic hydroxy function. They constitute an important alkaloid group due to their unique structural features and potent biological activities, such as anti-inflammatory, anti-arthritis, anti-PAF, anti-mycobacterial, and neuro-protective. Aristolactams have been reported from plants of the Annonaceae, Monimiaceae, Menispermaceae, Piperaceae and Saururaceae families. Nicoletti (15) analyzed, first by HPTLC and later by isolation and analysis of spectroscopic data, the presence of a sildenafil derivative (thiosildenafil) in herbal products. Quality assurance has enabled health professionals to prescribe safely herbal medicines that the population has been taking for quite a long time. The presence of synthetic drugs in the formulation of herbal products in order to improve the efficacy has been reported in several cases.
Viegi et al. (24) revised the use of toxic or potentially toxic plants for the treatment of ailments in livestock and pets in ethnoveterinary practice in Italy. More than 250 of the entities used (81% for curative purposes) can be toxic unless dosed appropriately. The species belong to 71 families, among which the Fabaceae predominates. Drugs derived from natural sources are usually produced by harvesting the natural source or through semi-synthetic methods: semisynthesis is usually used when the precursor molecule is too structurally complex, too costly or too inefficient to be produced by total synthesis. It is also possible that the semisynthetic derivative outperforms the original biomolecule itself with respect to potency, stability and safety. Usubillaga et al. (27) undertook the isomerization of kaurenic acid to obtain ent-kaurenic acid, a tetracyclic diterpene that has been reported to have antimicrobial, antiparasitic and cytotoxic activity. The occurrence of kaurenic acid, which has an exocyclic double bond at Δ16, is widespread in the plant kingdom, while the occurrence of its isomer ent-kaur-15-en-19-oic acid is rare.

The congresses of SILAE are international events whose organizations are submitted to an International Organizer Committee composed of professors from Italian and Latin America Universities. The Italo-Latin American Congress of Ethnomedicine arose from the necessity to evaluate the important potentialities of little known medicinal and alimentary plants, typical and traditional plants of the Latin American continent and to provide connections between Italian and other European and Latin-American researchers, with common objectives of research in the areas in which the projects will be articulated. Traditional medicine is used by 85% of the World’s population and is of great importance in developing countries. In accordance with the requirements of the World Health Organization, a scientific basis and proof for the use of medicinal plants is required and so the organization of such a Congress provides an important exchange of such information and coordination of scientific activity. This Natural Product Communications special issue provided an opportunity for publication of original, peer-reviewed, full-length articles on new research on medicinal plants used in Latin America; this will serve to stimulate the studies in these areas that are extremely important for academia and industry.

The Guest Editor would like to thank the contributors who gave so generously of their time and experience and who made this publication a valuable tool for scientists in the field of natural products chemistry and biology. Thanks are also due to the referees for their valuable comments and for the very detailed and accurate review of manuscripts; their comments certainly helped to improve the papers. I am also grateful to my staff who lent their considerable talents to the project: Annalisa Piccinelli, Florence Somma, Luca Campone and the webmaster of SILAE, Vincenzo Barbarulo. I thank all of them for their commitment, continued support and friendship.

I am also very grateful to the Editorial Board of Natural Product Communications for embracing this project with interest and enthusiasm, and for the opportunity to publish this Special Issue. I hope that this will be the first of a long series in this attractive and interesting Journal. Finally, I would like to thank the Editor-in-Chief, Pawan Agrawal, for his valuable input and for careful supervision. Thank you Pawan!

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Use of Dimethyldioxirane in the Epoxidation of the Main Constituents of the Essential Oils Obtained from *Tagetes lucida*, *Cymbopogon citratus*, *Lippia alba* and *Eucalyptus citriodora*

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Dimethyldioxirane (DMDO), a widely used oxidant in organic synthesis is considered an environmentally friendly oxygen transfer reagent because acetone is the only byproduct formed in its oxidation reactions. This work describes the isolation of the main constituents (terpenes) in the essential oils obtained from *Tagetes lucida*, *Cymbopogon citratus*, *Lippia alba* and *Eucalyptus citriodora*, their epoxidation with DMDO in acetone solution and the characterization of the resulting epoxides by GC-MS (EI) and NMR. This is one of the first reports involving the application of dioxirane chemistry to essential oils in order to generate modified compounds with potential uses in several areas of medicine and industry.

**Keywords:** dimethyldioxirane, epoxidation, estragole, citral, carvone, citronellal.

The use of dimethyldioxirane (DMDO), a member of a new class of three-membered ring peroxide containing oxidants, has increased notably in recent years due to its ability to do oxygen atom transfer reactions with a wide range of substrates, including C=C bonds [1], C-H insertions in hydrocarbons [2], as well as oxidations of atoms containing lone pairs of electrons, such as sulfides [3] and primary and secondary amines [4]. DMDO is also considered to be an environmentally friendly oxygen transfer reagent, and is attractive from the “green chemistry” [5] point of view due to its lack of toxic or harmful metals. DMDO’s reactions occur under extremely mild and neutral conditions, and the only byproduct of its reactions is acetone, which can be easily removed. Thus, DMDO reactions do not require complicated purification procedures to obtain pure products.

This electrophilic oxygen transfer reagent is the reagent of choice for the epoxidation of both conjugated and unconjugated double bonds containing other functional groups, such as hydroxyls or carbonyls. Isolated double bonds are selectively and easily oxidized under mild conditions (0-25°C and neutral pH) [6]. On the other hand, allylic alcohols are oxidized frequently with tert-butyl hydroperoxide (TBHP) [7] in the presence of transition metals, which are necessary to initiate the reaction. Typically, enones are epoxidized with alkaline hydrogen peroxide (H$_2$O$_2$) [8], but in some cases the hydrolytically-sensitive epoxide products open via a side reaction to form a diol. The three types of olefins mentioned above have been successfully oxidized with DMDO in high regio- and stereoselectivity.

Several methods for the generation of epoxides from alkenes have been developed, such as the Sharpless [9] and Jacobsen [10] catalytic epoxidations, the use of alternative routes involving the formation of intermediate halohydrins by using trichloroisocyanuric acid in aqueous acetone [11] and the use of peracids to generate both mono- and bisepoxides, as well as cleaved oxidation products. A system involving a hydrogen peroxide-dinuclear manganese (IV) complex and a carboxylic acid has been successfully employed for the efficient epoxidation of terpenes such as limonene, citral, carvone and linalool, while other sterically hindered terpenes (i.e., citronellal, α- and β-pinene) were epoxidized in low yields [12]. The selective oxidation of monoterpenes (i.e., limonene, γ-terpinene, neryl acetate, geranyl acetate, citral and geranyl nitrile) with H$_2$O$_2$ catalyzed by peroxotungstophosphate under biphasic conditions produced mono- and bisepoxides in good yields [13]. While alkene epoxidation has found widespread use in the
total synthesis of natural products, there are very few reports in the literature of regioselective epoxidations of natural products with DMDO. Therefore, in this work, we have carried out the DMDO epoxidation of the main terpene constituents in the essential oils extracted from Tagetes lucida, Cymbopogon citratus, Lippia alba and Eucalyptus citriodora, which makes this one of the first reports where dioxirane chemistry has been applied to isolated components of essential oils.

*Tagetes lucida* (Asteraceae) is an endemic plant that is widely distributed in Central and South America, and is known in Colombia as “Estragón de invierno” [14]. Its extracts are reported to possess bactericidal [15], antymycotic [16] and antioxidant [17] activities, but this plant has not been well studied from the chemical point of view. In Colombia, the essential oil extracted from this plant contains a large amount (93.8%) of a phenylpropanoid, estragole (1-allyl-4-methoxybenzene), which is present in many vegetables as a flavoring [18].

*Cymbopogon citratus* (Poaceae) is known worldwide as the herb lemongrass, and is widely used in folk medicine due to its antimicrobial [19], anti-inflammatory, antispasmodic [20] and antifungal [21] activities. The essential oil from lemongrass has been widely used in the perfume and cosmetics industries. It has also been used in chemical synthesis, due to its high citral (3,7-dimethyl-2,6-octadien-1-one) content. Citral exists as a natural mixture of two isomeric aldehydes, geranial and nerol. Recently, some citral derivatives with potential bactericidal activity were generated using “green” thiol conjugate additions [22]. Citral is a polyfunctional molecule that mediates a wide number of chemical transformations, mainly epoxidation reactions.

*Lippia alba* (Verbenaceae) is a shrub widely distributed from Mexico to South America [23] and is known in Colombia as “Pronto alivio”. *L. alba* essential oil and some organic and polar extracts have shown analgesic, anti-inflammatory, antifungal [25] and antibacterial [26] properties. In Colombia, the essential oil from this plant is distinguished by its high content of S-carvone [2-methyl-5-(1-methylethenyl)-2-cyclohexene-1-one] (40-57 %), a substance with a high-value for the perfume and cosmetics industries and as a starting material for fine organic synthesis [27].

*Eucalyptus citriodora* (Myrtaceae) is an aromatic specie known as “lemon scented gum”. Water extracts from dried leaves of *E. citriodora* are traditionally used as antipyretics, anti-inflammatory and analgesic [28]. *E. citriodora* essential oil has been shown to contain high concentration (70-80%) of citronellal (3,7-dimethyl-6-octen-1-al) [29], which is effective against bacterial and fungal infections [30]. Besides, this compound is attractive from the stereochemical point of view since it can be used in an efficient way to introduce a new stereoegenic center in more complex structures [31].

As an important application of dioxirane chemistry to essential oils, this work describes the epoxidation of the main constituents of the essential oil from *Tagetes lucida*, *Cymbopogon citratus*, *Lippia alba* and *Eucalyptus citriodora* with acetone solutions of DMDO. In general, the importance of the derivatives generated is due to their polyfunctionality and the high reactivity of the oxirane ring. Such terpene epoxides can be readily transformed into alcohols, aldehydes, ketones and heterocycles, which give these terpene epoxides a promising position in terpene chemistry with future applications in industry and medicine.

The chemical composition and identity of the main constituents of the essential oils obtained from *Tagetes lucida*, *Cymbopogon citratus*, *Lippia alba* and *Eucalyptus citriodora* were determined by GC-MS (EI). The main constituent of the oil from *T. lucida* was estragole, which had a relative abundance of 93.8% and a retention time of 7.7 minutes. Citral was the main component of the oil from *C. citratus*. It had a relative abundance of 74% and retention times of 8.1 and 8.3 minutes for the isomers. Carvone was the main constituent of the oil from *L. alba*, with a relative abundance of 42% and a retention time of 7.9 minutes. Citronellal was the main constituent of the oil from *E. citriodora* with a relative abundance of 74% and a retention time of 6.7 minutes. All of the compounds were isolated in high purity (95%), which was confirmed by GC-MS.

The epoxidation of estragole, citral, carvone and citronellal with DMDO in an acetone solution afforded epoxyestrargole 1, 6,7-epoxycitral 2a and 2b, 8,9-epoxy-carvone 3 and 6,7-epoxycitronellal 4 (Figure 1), with greater than 95% conversion (confirmed by GC-MS). The resulting epoxides were stored at room temperature and evaluated at several points during the course of one year and found to be stable (data not shown).

![Figure 1: Epoxides obtained from the DMDO reaction.](image-url)
Dimethyldioxirane epoxidation of essential oils

Dimethyldioxirane epoxidation of cycloalkenes, were epoxidized with aqueous H₂O₂ and a polystyrene-supported triphenylarsine reagent, the moderately unstable epoxide products were isolated in poor yield [32].

Kim et al. [33] synthesized trans-anethole oxide from trans-anethole (an isomer of estragole) using an acetone solution of DMDO as the oxidizing reagent. This oxide was stable for one year, and reaction’s yield was >95%. This yield was much better than that obtained by Mohan and Whalen [34] and Greca et al. [35], who used m-CPBA to oxidize the same substrate, but obtained low yields (38%) that were complicated by the presence of m-CPBA’s acid byproduct. These results and estragole’s structural characteristics provide evidence supporting the high reactivity of DMDO. Therefore, it is clear that this oxygen transfer reagent is the reagent of choice for the epoxidation of double bonds, including electron deficient olefins. The use of this oxidant offers a variety of advantages; it is generated from readily available reagents, it reacts to generate epoxides under mild conditions and its only byproduct is acetone and prevents the need for work-up conditions that can cause the opening of the oxirane ring.

Another substrate chosen for DMDO epoxidation was citral, which is widely distributed in nature as a mixture of E/Z isomers. This substrate can undergo a wide range of reactions due to its multiple functional groups, including two trisubstituted double bonds, one of which is adjacent to a carbonyl group. One benefit of using this substrate is that the regioselectivity in the epoxidation of the 6,7 double bond versus the 2,3 double bond provides a measure of the relative rate of reaction of the two olefins that can be read from the ratio of the two possible monoepoxides (i.e., the 6,7 and 2,3 epoxides) because this is a simple intramolecular competition experiment. Although the two double bonds are trisubstituted, the inductive electron-withdrawing effect of the carbonyl group lowers the nucleophilicity of the 2,3 double bond, and if electronic properties are the decisive factor in the reactivity, then preferential epoxidation of the 6,7 double bond would be expected.

The 6,7-epoxycitral 2a, 2b was obtained as a mixture of diastereoisomers with an E/Z ratio of 50:50 which corresponds to the content in essential oil and in 87% yield (92 mg). The preferential epoxidation of the 6,7 double bond is probably due to its greater nucleophilicity, which is a consequence of its electron-donating alkyl substituents. Conversely, the reactivity of the 2,3 double bond is drastically reduced due to the electron-withdrawing effect of the conjugated carbonyl group, which results in much lower nucleophilicity. This result shows the electrophilic character of DMDO and its preference for electron-rich double bonds. In addition, the high conversion to the 6,7 monooxepoxide indicates that the reaction’s rate increases with the nucleophilic character of citral’s terminal double bond due to the presence of its electron-donating substituents. The exclusive formation of the monoepoxide shows that the oxidation of the aldehyde function to a carboxylic acid did not occur under the reaction conditions employed, which indicates that DMDO promotes the epoxidation of the double bonds in preference to oxygen insertion.

Other studies have shown that under the same conditions used in our epoxidation of citral, the oxidation of geraniol (the allylic alcohol corresponding to the reduction of citral) with DMDO produces the 2,3 and 6,7 epoxides, as well as the bis-epoxide [36]. Clearly, the nature of the substrate is an important factor in determining the regioselectivity of the epoxidation reaction. In the case of geraniol, the formation of the 2,3 epoxide has been explained in terms of the stability gained from an intermolecular hydrogen bond formed by the hydroxyl and the DMDO molecule in the epoxidation transition state. The lack of 2,3 epoxyxiranyl formation can be explained by the inductive electron-withdrawing effect of the carbonyl group, which lowers the nucleophilicity of the adjacent double bond. The resonance effect of the α,β-unsaturated aldehyde also contributes to the decreased reactivity of the double bond. Finally, in the case of citral, the formation of a hydrogen bond between the substrate and the DMDO molecule is not possible.

The treatment of citral with H₂O₂ in an alkaline medium in the presence of a phase-transfer catalyst gives rise to the formation of the 2,3 epoxide and 6-methyl-5-hepten-2-one, which forms as a result of the epoxide’s decomposition [37]. Alternatively, treating citral with peracetic acid gives the 6,7-epoxy derivative and the (E/Z)-2,6-dimethyl-5,6-epoxy-1-heptenyl isomers resulting from a Baeyer-Villiger side reaction [38]. The formation of the 6,7 derivative has also been reported in moderate yield (43%) by Woitiski [12] by reacting citral with a H₂O₂-dinuclear manganese (IV) complex of oxalic acid.

Carvone is another substrate chosen for DMDO epoxidation, which contains two electron-poor double bonds, the exocyclic double bond due to the low number of electron-donating alkyl substituents and the endocyclic bond due to the bearing electron-withdrawing effect of the carbonyl group. These carvone’s structural characteristics allow to compare the reactivity of the two double bonds in an intramolecular competition experiment with this cyclic substrate. The results show the regioselectivity in the reaction of carvone with DMDO and the formation of the exocyclic monoepoxide 3 as the only product of the reaction in 80% yield (102 mg) and 50:50 diastereoselectivity. The selective epoxidation of the carvone exocyclic double bond corroborates that DMDO is a powerful reagent for the epoxidation of electron-poor double bonds. The lack of endocyclic oxirane formation demonstrates the low nucleophilicity of the enone double bond due to the carbonyl group electron-withdrawing effect.
In the epoxidation of carvone under oxygen using Nickel(II) acetylacetonate as catalyst, (R)-(-)-carvone exhibited low reactivity due to long times reaction to obtain the exocyclic monooxide [39]. When the anhydrous hydrogen peroxide/alumina system is used, a high regioselectivity of the exocyclic monooxide (93.8%) and a low substrate conversion (8.7%) were observed, however, yields decreased to 0.8% in the absence of alumina catalyst [40].

Citronellal is an aliphatic olefin which contains an aldehyde group and a terminal trisubstituted double bond. This substrate can be used as a chemoselectivity probe for DMDO epoxidation in terms of the aldehyde oxidation to carboxylic acid versus the double bond epoxidation. The results show the double bond oxidation leading to the formation of the diastereomeric epoxides in 84% yield (272 mg) and 50:50 ratio, confirming the high reactivity and chemoselectivity of DMDO to nucleophilic olefins. Similar results were obtained for the epoxidation of Citronellal with tert-butyl hydroperoxide (TBHP) and molybdenum catalyst in nonpolar and aprotic solvents, giving a mixture of diastereomeric epoxides with a 50:50 ratio and 71% yield [41].

Essential oils are an important source of terpenes that can be modified chemically to generate a wide variety of polyfunctional compounds. From the chemical point of view, epoxidation of the main constituents of Tagetes lucida, Cymbopogon citratus, Lippia alba and Eucalyptus citriodora with DMDO generates oxirane derivatives and provides evidence for the high reactivity of this new oxygen transfer reagent. Although a large number of compounds have been epoxidized with DMDO, to our knowledge, this is the first report of the synthesis of estragole, citronellal and citral epoxides using DMDO. Epoxide stability is an important characteristic when carrying out tests of biological activity.

The high conversion of estragole and the good yield of epoxystragole indicate that unactivated olefins can be efficiently oxidized with DMDO. The epoxidation of citral proved to be regioselective, providing only 6,7 epoxide as a result of the higher nucleophilicity of the 6,7 double bond relative to the 2,3 double bond, which is electron deficient due to the electron-withdrawing effect of the conjugated carbonyl group. In addition, the use of citral shows that DMDO oxidizes double bonds in preference to oxidizing aldehydes to carboxylic acids. For carvone, the nucleophilicity of the double bond adjacent to the carbonyl group is quite low due to the electronic-withdrawing effect of this group, which is an important reactivity factor. The DMDO epoxidation of citronellal confirms the chemoselectivity of this oxidant agent in terms of the aldehyde oxidation to carboxylic acid versus the double bond epoxidation. Our results indicate that it is important to consider both the regio and chemoselectivity of the DMDO in the epoxidation of natural compounds.

Experimental

General: Gas Chromatography-Mass Spectrometry (GC-MS) analyses were performed using a Shimadzu GC-2010 gas-chromatograph coupled to a selective mass detector (Shimadzu QP-2010) and equipped with an Rtx-5Sil-MS column (30 m x 0.25 mm i.d., 0.25 μm film thickness) operating in electronic ionization mode at 70 eV. Helium was used as the carrier gas. All data processing was done on the Shimadzu Labsolutions software (GCMS Solution version 2.5), which includes the Wiley Registry of Mass Spectral Data, 7th Edition (Wiley Interscience, New York). 1H NMR spectra were acquired on a Bruker Avance DRX400 (400.13 MHz) spectrometer at 25 °C using CDCl3 as the solvent and TMS as an internal standard.

Essential oils extraction: Essential oils were obtained from plant leaves by microwave-assisted hydrodistillation (MWHD) at CENIVAM (Centro Nacional de Investigaciones para la Agroindustrialización de Especies Vegetales Aromáticas y Medicinales Tropicales), Universidad Industrial de Santander, Bucaramanga (Colombia).

Terpenes isolation: The essential oils from T. lucida (800 mg) and L. alba (812 mg) were fractionated over a silica gel column eluting with n-hexane-chloroform (90:10) and increasing the polarity of the solvent to 50:50. Estragole (119 mg) and carvone (128 mg) were obtained with a purity of 95% as determined by GC-MS. The structure of each compound was identified by comparison of their mass spectrum (Wiley Registry of Mass Spectral Data) and was further confirmed by comparison to 1H NMR data reported in the literature.

The essential oils from C. citratus (3 mL) and E. citriodora (3 mL) were distilled under reduced pressure (60°C/550 mm Hg and 90°C/550 mm Hg, respectively). Citral (106 mg) and citronellal (324 mg) were obtained with a purity of 95% as determined by GC-MS. The structure of each compound was confirmed by comparison of their mass spectrum (Wiley Registry of Mass Spectral Data) and was further confirmed by comparison to 1H NMR data reported in the literature.

Preparation of DMDO in acetone solution: A concentrated solution of DMDO in acetone (0.11 M) was prepared using Oxone (Caroate, 2KHSO5.KHSO4.K2SO4), acetone and NaHCO3 according to a previously reported literature procedure [42]. The DMDO concentration was determined by UV/Vis spectrophotometry.

General procedure for the epoxidation of terpenes with DMDO: Estragole, citral, carvone and citronellal were dissolved in acetone (1 mL) and 1.0-1.2 equiv. of DMDO (0.11 M solution in acetone) was rapidly added at 25 °C. The solution was stirred at this temperature and monitored by TLC and GC-MS until the peroxide test (KI/HOAc) was negative (total reaction time 0.5-1 h). The solvent was...
removed under reduced pressure to give the respective epoxides in high purity. The structure of the epoxides was confirmed with spectral data and comparison with spectral data reported in the literature of 1 [43], 2a, 2b [44], 3 [45] and 4 [44].

Epoxysteragole (1)

1H-NMR (400 MHz, CDCl3): \( \delta 7.16 \) (2H, \( d, J=8.0 \) Hz, H-5, H-9), 6.84-6.87 (2H, m, H-6, H-8), 3.79 (3H, s, H-10), 3.09-3.11 (1H, m, H-2), 2.74-2.89 (3H, m, H-I, H-3), 2.52 (1H, dd, \( J=2.8 \) Hz, \( J=5.0 \) Hz, H-3).

6,7-Epoxyctitral

1H-NMR (400 MHz, CDCl3) 2a epogyperanial: \( \delta 10.0 \) (1H, d, \( J=8.0 \) Hz, H-1), 5.91-5.93 (1H, m, H-2), 2.71-2.77 (3H, m, H-4, H-6), 2.01 (3H, s, H-9), 1.64-1.83 (2H, m, H-1), 1.28 and 1.31 (6H, s, H-I, H-10).

2b epoxyneral: \( \delta 9.97 \) (1H, d, \( J=8.0 \) Hz, H-1), 5.91-5.93 (1H, m, H-2), 2.71-2.77 (1H, m, H-6), 2.29-2.48 (2H, m, H-4), 2.20 (3H, s, H-9), 1.64-1.83 (2H, m, H-5), 1.28 and 1.31 (6H, s, H-I, H-10).

8,9-Epoxycarvone (3)

1H-NMR (400 MHz, CDCl3) Isomer A: \( \delta 6.72-7.66 \) (1H, H-3), 2.71 (1H, d, \( J=4.4 \) Hz, H-9), 2.03-2.57 (6H, m, H-4, H-5, H-6, H-9), 1.77-1.80 (3H, m, H-7), 1.33 (3H, s, H-10).

Isomer B: \( \delta 6.72-7.66 \) (1H, H-3), 2.68 (1H, d, \( J=4.8 \) Hz, H-9), 2.03-2.57 (6H, m, H-4, H-5, H-6, H-9), 1.77-1.80 (3H, m, H-7), 1.32 (3H, s, H-10).

6,7-Epoxyctitrone (4)

1H-NMR (400 MHz, CDCl3): \( \delta 9.77 \) (1H x 2, m, H-1), 2.68-2.71 (1H x 2, m, H-6), 2.39-2.46 (2H, m, H-2'), 2.24-2.30 (2H, m, H-2), 2.08-2.18 (1H x 2, m, H-3), 1.52-1.57 (4H x 2, m, H-4, H-5), 1.27, 1.31 (6H x 2, s, H-8, H-10), 1.00 (3H x 2, d, \( J=6.8 \) Hz, H-9).

Supplementary data: 1H-NMR spectra for compounds 1, 2a, 2b, 3 and 4 are contained in the supplementary data.

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Validation of the Ethnopharmacological Use of Polygonum persicaria for its Antifungal Properties

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Polygonum L. genus (Polygonaceae) is represented in Argentina by 21 species and some of them have been used in the traditional medicine of our country to treat affections related with fungal infections, such as skin ailments and vaginal diseases. With the aim of contributing to the correct ethnopharmacological use of this genus, in the present work we describe the antifungal properties of P. persicaria (species not studied up to now) and the bio-guided isolation of the main active compounds. Results showed that dichloromethane extracts was the most active with MICs (Minimum Inhibitory Concentrations) between 31.2 – 1000 µg/mL, validating the ethnopharmacological use of P. persicaria to treat affections related with fungal infections in the Argentinean traditional medicine.

Keywords: Validation, Ethnopharmacological use, Polygonum, Antifungal activity.

Since the early 1980s, fungal infections have emerged as major causes of morbi-mortality, mainly among immunocompromised patients. The majority of deaths were associated with species of Candida, Aspergillus and Cryptococcus [1]. Instead, dermatophytes such as Trichophyton and Microsporum spp. produce superficial infections (tineas) which are usually not threatening but dramatically diminish the quality of life of human beings [2]. Although it appears to be an array of antifungal agents (polyenes, azoles, allylamines and the recent echinocandins) there are, in fact, few therapeutic options. Decreased susceptibilities of yeasts to the currently available antifungal agents [3] added to the increase in the number of reported cases of resistance [4], have led to a general consensus that new efforts for detecting novel antifungal entities remain a priority. In this context, the study of plants with history of ethnopharmacological use for ailments related to fungal infections, can serve two goals: validation of the use of traditional medicines and finding new leads [5].

Polygonum L. genus (Polygonaceae) is represented in Argentina by 21 species and some of them have been used to treat affections related with fungal infections, such as skin ailments and vaginal diseases [6]. Previous studies of this genus reported that P. punctatum possessed antifungal properties against yeasts and dermatophytes [7]. With the aim of contributing to the correct ethnopharmacological use of this genus, in a previous work we described the antifungal properties of P. acuminatum [8] and in this work we describe those of P. persicaria (species not studied up to now) and the bio-guided isolation of two sesquiterpene dialdehydes: polygodial (1), isopolygodial (2), and three flavonoids: pinostrobin (3), flavokawin B (4) and cardamonin (5) (Figure 1).

Figure 1: A) Sesquiterpene [polygodial (1) and isopolygodial (2)] and B) flavonoids [pinostrobin (3), flavokawin B (4) and cardamonin (5)] isolated from P. persicaria DCM extract.
[14] and results are shown in Table 2. They were active against yeasts, *Aspergillus spp.* and dermatophytes with MICs between 3.90 - 250 µg/mL.

As it can be observed in Table 2, the five compounds isolated from *P. persicaria*, drimanes as well as flavonoids, all showed antifungal activity. Among them, polygodial (1) showed the best activity against yeasts and dermatophytes with MICs between 3.9 to 62.5 µg/mL and it was almost inactive against species of *Aspergillus* genus. Its epimer, isopolygodial (2), showed a lower antifungal activity (MICs between 31.2 to 250 µg/mL), suggesting that the C-9 configuration plays an important role in the antifungal activity, as we have been found in a previous paper [8].

Regarding flavonoids 3-5, there is not a clear difference among the antifungal activities of them against yeasts and *Aspergillus* spp. Nevertheless, chalcone 5 showed a high antifungal activity against *T. rubrum* and *T. mentagrophytes* with MICs = 15.6 µg/mL, eight times higher than the activity showed by chalcone 4 against the same strains. This striking difference in activity against *Trichophyton* spp. could be attributed to the phenolic OH present in compound 5 which is absent in 4.

These results show that the antifungal activity of *P. persicaria* could be attributed to polygodial but it is clear that the rest of the isolated compounds could contribute to the antifungal behavior of this traditional used species. In addition, these results validate the ethnopharmacological use of *P. persicaria* to treat affections related to fungal infections in the Argentinean traditional medicine and add a new evidence that the ethnopharmacological approach is useful in guiding the discovery of antifungal compounds against dermatophytes, as it was demonstrated in a recent survey among seven Latinamerican countries [15].

**Experimental**

Extracts preparation and compounds isolation: Air-dried aerial parts of each species (100 g) were powdered and successively macerated (3×24 h each) with Hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) with mechanical stirring to obtain the corresponding extracts, after filtration and evaporation. Bioassay-guided fractionation of DCM extract allowed us to isolate the compounds responsible for the antifungal activity. 1.1 g of *P. persicaria* DCM extract were submitted to column chromatography using mixtures of Hex: AcOEt in increasing polarity as elution solvents. We obtained 10 fractions; three of them were active (fractions 6-8). From 150 mg of fraction 6, by repeated column chromatography, we obtained 55 and 30 mg of compounds 1 and 2 respectively. From 170 mg of fraction 7, by repeated column chromatography, we obtained 50, 46 and 25 mg of compounds 3, 4 and 5 respectively. Additionally, from 70 mg of fraction 8, we obtained 10 mg of compound 5. All the compounds were characterized by UV-visible, IR, 1H NMR and 13C NMR spectroscopy.

**Antifungal assay:** For the antifungal evaluation, strains from the American Type Culture Collection (ATCC, Rockville, MD, USA) and Centro de Referencia en Micología, CEREMIC (C, Faculty of Biochemical and Pharmaceutical Sciences, Suipacha 531 (2000)-Rosario, Argentina) were used: *Candida albicans* (Ca) ATCC 10231, *Saccharomyces cerevisiae* (Sc) ATCC 9763, *Cryptococcus neoformans* (Cn) ATCC 32264, *Aspergillus*...
flavus (Af) ATCC 9170, Aspergillus fumigatus (Af) ATCC 26934, Aspergillus niger (An) ATCC 9029, Trichophyton rubrum (Tr) C 110, Trichophyton mentagrophytes (Tm) ATCC 9972 and Microsporum gypseum (Mg) C 115. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, and subcultured every 15 days to prevent pleomorphic transformations. Inocula of cell or spore suspensions were obtained and quantified following reported procedures (CLSI).[14]

Minimum Inhibitory Concentration (MIC) of each extract or compound was determined by using broth microdilution techniques according to the guidelines of CLSI for yeasts: document M27-A2 and for filamentous fungi, M38A. For the assay, stock solutions of extracts or pure compounds (100 µL) were two-fold diluted with the culture medium. A volume of 100 µL of inoculum suspension [adjusted to 1–5 × 10^4 cells/spores as Colony Forming Units (CFU/mL)] was added to each well with the exception of the sterility control where sterile water was added to the well instead. Ketoconazole (Sigma Chem. Co., St. Louis, MO), Terbinafine (Novartis) and Amphotericin B (Sigma) were used as positive controls.

Acknowledgments - CONICET, ANPCyT, UNR, ERASMUS MUNDUS, UNIBO.

References

On the Isomerization of ent-Kaurenic Acid

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Kaurenic acid (1a) is a tetracyclic diterpene that has an exocyclic double bond at \( \Delta 16 \). Isokaurenic acid (2a) has an endocyclic \( \Delta 15 \)double bond. This compound has been isolated from *Espeletia tenore* (Espeletiinae), a resinous plant from the Venezuelan Andes, but its occurrence is rare. In order to obtain a larger amount of 2a, the isomerization of 1a, which is easily obtained from other Espeletiinae, was tried. Kaurenic acid methyl ester (1b) was treated with dil. HCl in CH\(_2\)Cl/EtOH, after 6 h under reflux a yield of 41.5\% isokaurenic acid methyl ester (2b) was obtained but 35.7\% 16\-\alpha\-ethoxy-kauran-19-oic acid methyl ester (3b) had formed as a byproduct. Treating 1b with CF\(_3\)COOH in refluxing CH\(_2\)Cl\(_2\) permitted to obtain a yield of 66.6\% of 2b in 4 h and only traces of 16\-\alpha\-hydroxy-kauran-19-oic acid methyl ester (3a) as a byproduct. Both isomers were separated on a silica gel column impregnated with 20\% AgNO\(_3\). Treating 2b with KOH in refluxing DMSO yielded pure isokaurenic acid, no back isomerization was observed.

**Keywords:** Ent-kaur-16-en-19-oic acid, ent-kaur-15-en-19-oic acid, CF\(_3\)COOH, methyl esters, silver nitrate chromatography.

Ent-kauranic acid (1a, Figure 1) is a tetracyclic diterpene that has been reported to have antimicrobial [1] and antiparasitic activity [2]. It also shows cytotoxicity against several cancer cell lines [3]. The occurrence of kaurenic acid, which has an exocyclic double bond at \( \Delta 16 \), is widespread in the plant kingdom, while the occurrence of its isomer ent-kaur-15-en-19-oic acid (2a), also called isokaurenic acid, is rare. This substance (2a) was first obtained by Ekong and Ogan by lithium reduction of Xylopic acid [4a], but 2a has been isolated as a natural product from *Espeletia tenore*, a midget Espeletiinae from the Venezuelan Andes [4b].

Since the quantity of isokaurenic acid that could be obtained from *E. tenore* is small, isomerization of kaurenic acid was studied in order to obtain it in sufficient quantity to explore its biological properties. Double bond migration in olefins could be base-catalyzed or acid-catalyzed [5a,5b]. Base-catalyzed isomerization can be effected in homogeneous solution or in the presence of basic heterogeneous catalysts. Isomerization of 1b was tried with sodium on alumina according to Shabtai and Gil-Av [6], but no reaction was observed after 24 hours. Isomerization was also attempted treating 1b with iodine in benzene solution under reflux, according to Barnes and MacMillan [7] but only 10.6\% of 2b was obtained after 6 hr.

Since it had been observed that, during isolation of the acidic fraction of some Espelettinae, treatment with HCl produced traces of isokaurenic acid, isomerization of 1b in CHCl\(_3\)/EtOH solution was tried adding 5 drops of HCl:H\(_2\)O (10:1) at room temperature. The course of the reaction was followed by gas chromatography. As it is shown on Table 1, after one hr of reaction 35.4\% of the original amount of 1b had isomerized into 2b, while the relative concentration of 1b had diminished to 55.6\%, but at the same time 9.0\% of ent-16\-\alpha\-ethoxy-kauran-19-oic acid (3b) had formed as a by-product. After 4 hr of reaction a maximal yield of 2b was achieved (46.4\%), but after 6 hr the relative concentration of 2b had diminished and settled at 41.4\%. At the same time the by-product (3b) represented 35.7\% of the reaction mixture.

Since formation of 3b was undesirable, isomerization was tried using CF\(_3\)COOH in dry CH\(_2\)Cl\(_2\). The course of the reaction is shown on Table 2. After 1.0 hr of reaction...
After 72 h under reflux the yield of the yield was 63.5%. After 4.0 hr the reaction had arrived to equilibrium where relative concentration of 2b was 66.6% and concentration of 1b was down to 33.3%. But after 4.0 hr it was observed that 0.1% of 3a had been formed, probably caused by the presence of traces of water.

Table 1: Acid-isomerization of ent-kaurenic acid using HCl in CHCl3/MeOH.

<table>
<thead>
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<th>Time (hr)</th>
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<th>Time (hr)</th>
<th>% 2b</th>
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<td>4</td>
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<td>33.3</td>
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Figure 2 shows the increase of 2b as a function of time and the abatement of kaurenic acid methyl ester concentration (1b) to end up with a constant concentration mixture made up of 66.6% 2b and 33.3% 1b. Absolute exclusion of water would hinder the formation of 16α-hydroxy-kauren-19-oic acid methyl ester (3a). But from a practical point of view two hours of reaction is enough to obtain a good yield (63.5%) of the desired product. Separation of both isomers is accomplished over a silica gel column impregnated with 20% silver nitrate. Taking into consideration that silver nitrate chromatography is required to separate both isomers, the methyl ester of kaurenic acid (1b) was used instead of the free acid (1a). Isokaurenic acid (2a) was recovered refluxing 2b with KOH in DMSO solution.

According to Hubert and Reimlinger [5b] the acid catalyzed isomerization takes place via carbonium ions. In the case of kaurenic acid, presence of an acid causes the formation of a carbonium ion at C-16. The catalyst acts as a proton donor and acceptor. Migration of the double bonds occurs because a proton is transferred to the C-17 exocyclic methylene moiety, which becomes a methyl group, and at the same time, a proton is lost from C-15 generating a Δ15 double bond. But this process is reversible and the reaction proceeds until it reaches a thermodynamic equilibrium. In this case a Δ15 double bond is thermodynamically more stable and therefore its formation is enhanced leading to the migration of 2/3 of the original exocyclic double bond to a Δ15 position.

The rate of formation of 3a increases with reaction time. After 72 h under reflux the yield of 3a was 13%.

Table 2: Acid-isomerization of ent-kaurenic acid using CF3COOH.

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<th>Time (hr)</th>
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<th>% 1b</th>
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<td>4</td>
<td>66.6</td>
<td>33.3</td>
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t : traces

Figure 2 shows the increase of 2b as a function of time and the abatement of kaurenic acid methyl ester concentration (1b) to end up with a constant concentration mixture made up of 66.6% 2b and 33.3% 1b. Absolute exclusion of water would hinder the formation of 16α-hydroxy-kauren-19-oic acid methyl ester (3a). But from a practical point of view two hours of reaction is enough to obtain a good yield (63.5%) of the desired product. Separation of both isomers is accomplished over a silica gel column impregnated with 20% silver nitrate. Taking into consideration that silver nitrate chromatography is required to separate both isomers, the methyl ester of kaurenic acid (1b) was used instead of the free acid (1a). Isokaurenic acid (2a) was recovered refluxing 2b with KOH in DMSO solution.

According to Hubert and Reimlinger [5b] the acid catalyzed isomerization takes place via carbonium ions. In the case of kaurenic acid, presence of an acid causes the formation of a carbonium ion at C-16. The catalyst acts as a proton donor and acceptor. Migration of the double bonds occurs because a proton is transferred to the C-17 exocyclic methylene moiety, which becomes a methyl group, and at the same time, a proton is lost from C-15 generating a Δ15 double bond. But this process is reversible and the reaction proceeds until it reaches a thermodynamic equilibrium. In this case a Δ15 double bond is thermodynamically more stable and therefore its formation is enhanced leading to the migration of 2/3 of the original exocyclic double bond to a Δ15 position.

The rate of formation of 3a increases with reaction time. After 72 h under reflux the yield of 3a was 13%.

Experimental

General procedures: Melting points were determined on a Fisatom 430 D apparatus and are uncorrected. IR spectra were measured on a Shimadzu Affinity instrument as KBr discs. NMR spectra were recorded with a Bruker Avance 400 MHz instrument for solutions in CDCl3, 1H, 13C, DEPT, H-H COSY, HMQC, and HMBC experiments were performed. GC was made on a Perkin Elmer Autosystem gas chromatograph equipped with FID detector. A 5% phenylmethyl polysiloxane capillary column was used (30 m, 0.25 mm i.d., film thickness 0.25 μm). The oven temperature was programmed from 250°C to 300°C at 10°C/min., and kept isothermal at the higher temperature for 10 min. The injector and detector temperatures were 200°C and 300°C respectively. The carrier gas was helium at 0.9 mL/ min. The samples (1.0 μL) were injected using a split ratio of 1:10. For mass spectrometry an Agilent MSD 5973 instrument equipped with a DB-5MS capillary column (30 m, 0.25 mm, 0.25 μm film). The oven temperature program was the same used for GC analysis. Injector temperature, carrier gas, and sample injection conditions were also the same but a split ratio of 1:50 was used. Analytical TLC was performed on Merck aluminum-backed silica gel foils (F254). Flash chromatography was performed on Merck silica gel Grade 9385 (230-400 mesh) by gradient elution with hexane-EtOAc or hexane-diethyl ether mixtures. Kaurenic isomers were separated on a silica gel column impregnated with 20% AgNO3.

Isolation of kaurenic acid (1a): *Espeletia semiglobuta* was collected at Paramo de Piedras Blancas in February 2009. A voucher specimen (AU-30) was deposited at the MERF Herbarium. The leaves (10 Kg) were air dried, ground and extracted with a hexane-diethyl ether mixture (3:1) at room temperature. The extract was shaken with 0.5 N NaOH solution. The aqueous layer was made acidic by addition of dil. HCl and shaken with hexane to recover the acid fraction. Kaurenic acid was purified by flash chromatography over silica gel using hexane and hexane-diethyl ether (9:1) as solvent. Chromatographic fractions were inspected by TLC, fractions containing pure kaurenic acid were combined and crystallized Pure kaurenic acid...
Isomerization of Kaurenic acid

**Isomerization of 1b with diluted HCl in CHCl₃/EtOH:** A solution of kaurenic acid (1a, 20 mmol) was dissolved in Et₂O and mixed with freshly distilled diazomethane (about 30 mmol) obtained from nitrosomethylurea [9a,9b]. After 24 h at room temperature the solvent and excess diazomethane were distilled off at low pressure. Kaurenic acid methyl ester (1b) crystallized from hexane, MP 75°C. GC retention time 3.80 min. MS (EI, 70 eV): m/z (% = 316.2 (51), 302 (35), 284 (62), 257 (199), 241 (79), 213 (33), 187 (2), 121 (45), 91 (47).

**Attempted isomerization of 1b with sodium on alumina:** The catalyst was prepared according to Shabtai and Gil-Av [6]. Alumina was heated at 300°C during 24 h. Pretreated alumina (5.0 g) was mixed with 1.0 g of sodium at 140°C with stirring under argon atm. The catalyst was cooled at 2-3°C and a solution of 1b (500 mg) in dry hexane was added. Samples were taken after continuous stirring at 1.0 h, 6.0 h, and 24 h, and analyzed by GC-MS after 1.0 h, 6.0 h, and 24 h, but no isomerization occurred and only the peak of 1b, with a retention time of 3.79 min., was observed on the gas chromatogram.

**Isomerization of 1b with iodine in benzene solution:** Kaurenic acid methyl ester (1b, 300 mg) was dissolved in dry benzene containing 20 mg of iodine and it was heated under reflux for 6 h. The solution was cooled and shaken twice with aqueous sodium thiosulphate. The organic layer was taken to dryness. A 5 mg sample of the solid was dissolved in diethyl ether and analyzed by GC. It was observed that after six hours of reaction only 10.6% of 1b had isomerized into 2b which had a retention time of 3.55 min.

**Isomerization of 1b with diluted HCl in CHCl₃/EtOH:** Kaurenic acid methyl ester (2.0 mmol) was dissolved in a mixture 50 mL of CHCl₃ and 10 mL of EtOH. After addition of 5 drops of 10% HCl, the mixture was heated under reflux. Aliquot samples (5 mL) were taken at 1h, 2h, 4h, and 6 h, washed with dil. NaHCO₃, and with H₂O. The organic layer was dried over Na₂SO₄ and evaporated to dryness. Samples (5 mg) were dissolved in Et₂O and analyzed by GC. Results are shown on table 1.

**Isolation of ent-16α-ethoxy-kauran-19-oic acid methyl ester (3b):** The rest of the solution (40 mL) of the previous isomerization reaction was washed with dil. NaHCO₃ and H₂O. The chlorofom layer was treated with dry Na₂SO₄, filtered, and mixed with 1 g of silica gel. CHCl₃ was evaporated under vacuum and the silica gel containing the product of isomerization was added to the top of a flash column charged with 12 g of silica gel. The column was eluted with hexane which yielded a mixture of 1b and 2b.

When both isomers had eluted the column was treated with hexane-EtOAc (10:1) to elute 3b.

MP: 140°C.
IR (cm⁻¹): 2901, 2832, 1754, 1724, 1646, 1466, 1243, 1157, 1068, 987.

**Isolation of 1b with CF₃COOH in CH₂Cl₂:** To a solution of kaurenic acid methyl ester (5.0 mmol) in dry CH₂Cl₂ (100 mL) 10 drops of trifluoroacetic acid were added and the mixture was heated under reflux in an argon atmosphere. Aliquot samples (5 mL) were taken at 1h, 2h, and 4h. They were washed with H₂O; the organic layer was dried over Na₂SO₄ and evaporated to dryness. Samples (5mg) were dissolved in Et₂O and analyzed by gas chromatography. Table 2 shows the results of this reaction as a function of time. To recover the isoakaurenic acid methyl ester the rest of the reaction mixture was washed with H₂O, dried over Na₂SO₄, filtered, and the solvent was evaporated to dryness. The reaction product was dissolved in hexane and chromatographed on a column of silica gel impregnated with 20% of AgNO₃. The column was eluted with hexane; 100 mL fractions were taken and inspected by GC. Fractions 6-12 eluted pure 2b (722 mg). MP 74-75°C. GC retention time 3.55 min. It was identical to an authentic sample obtained by methylation of 2a isolated from *Espeletia tenore* [4b] (IR, ¹H NMR, MS). Fractions 13-18 eluted a mixture of 1b and 2b (330 mg), and fractions 19-27 pure 1b (275 mg). To a solution of 500 mg of 2b in 50 mL of DMSO 300 mg of KOH were added and the mixture heated under reflux for 2 h. The solution was cooled; 50 mL of H₂O was added, and taken to pH 3.0 by addition of dil. HCl. The solution was then shaken with 100 mL of hexane. The hexane layer was shaken twice with 20 mL of H₂O, dried over Na₂SO₄, and the solvent distilled under vacuum. The isoakaurenic acid (455 mg) was crystallized from hexane, MP 169-171°C. Identical to 2a isolated from *Espeletia tenore* (IR, ¹H NMR )[4b]. A sample (5 mg) was methylated and examined by GC. Only one peak was observed at 3.55 min (100%).
Isolation of ent-16α-hydroxy-kauran-19-oic acid methyl ester (3a): To a solution of kaurenic acid methyl ester (2.0 mmol) in CH₂Cl₂ five drops of trifluoroacetic acid were added and the mixture was heated under reflux. After 72 h, the reaction mixture was cooled and shaken with H₂O. The organic layer was dried over Na₂SO₄, filtered, and evaporated to dryness. A 5 mg sample was dissolved in Et₂O and inspected by GC. The gas chromatogram showed peaks at 3.54 (2b, 60%), 3.79 (1b, 27%), and 4.94 min (3a, 13%). Flash chromatography over a silica gel column afforded 42 mg of 3a. MP: 159-160°C. IR (KBr, cm⁻¹): 3412, 2982, 2953, 2868, 1728, 1424, 1157, 925. ¹H NMR (400 MHz, CDCl₃): 0.77 (1H, dt, J = 4; 14 Hz, H-1a), 0.80 (3H, s, H-20), 0.96 (1H, m, H-9), 1.01 (1H, m, H-5), 0.97 (1H, m, H-3a), 1.15 (3H, s, H-18), 1.35 (3H, s, H-17), 1.42 (1H, m, H-14a), 1.5 (2H, m, H-11), 1.53 (2H, m, H-15), 1.57 (1H, m, H-7a), 1.58 (1H, m, H-14b), 1.75 (2H, m, H-6), 1.80 (1H, bs, H-13), 1.82 (1H, m, H-2b), 1.84 (1H, m, H-1b), 1.89 (1H, m, H-7b), 3.63 (3H, s, OCH₃). ¹³C NMR (100 MHz, CDCl₃): 174.3 (COO), 79.7 (C-16), 58.1 (C-15), 57.3 (C-5), 56.3 (C-9), 51.4 (OCH₃), 49.2 (C-13), 45.6 (C-8), 44.1 (C-4), 42.4 (C-7), 41.0 (C-1), 39.8 (C-10), 38.4 (C-3), 37.9 (C-14), 29.0 (C-18), 27.1 (C-12), 24.8 (C-17), 22.4 (C-6), 19.4 (C-2), 18.6 (C-11), 15.7 (C-20). MS (EI, 70 eV): m/z (%): 334 (11), 316 (100), 302 (26), 276 (67), 257 (73), 217 (30), 180 (32), 121 (85).

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References

Aristolactams from roots of *Ottonia anisum* (Piperaceae)

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The Piperaceae species are known worldwide for its medicinal properties and its chemical compounds. In Brazil, many species of this family are distributed mainly in Amazon Region and in the Atlantic Forest. The genus *Ottonia* is known as source of amides, flavonoids, arylpropanoids and terpenes with record biological activities. Six aristolactams, including, aristolactam BII, piperolactam C, goniothalactam, stigmalactam, aristolactam AII and aristolactam BIII were isolated from roots of this species. GC-MS, 1H NMR and NOESY techniques were used to characterize these compounds. This is the first report about the occurrence of aristolactams in the *Ottonia anisum* Sprengel.

**Keywords:** Piperaceae, *Ottonia anisum*, aristolactams, alkaloids.

The family Piperaceae is composed approximately by 2000 species with wide tropical and subtropical distribution and great representation in Central and South Americas, occurring in Mexico, Panama, Peru, Costa Rica, Argentina and from North to Southern Brazil. The *Piper* species are mostly tropical plants of worldwide occurrence and are represented by ca. 700 species. In Brazil, 260 species are distributed mainly in Amazon Region and in the Atlantic Forest of the country [1a-c]. The phytochemical investigation of *Piper* has led to the isolation of a large number of bioactive compounds such as alkaloids, amides, propanoylephensols, piperolides, flavonoids, chromenes, prenylated benzoic acid derivatives and lignoids. Furthermore, literature records registered the occurrence of interesting secondary metabolites in species of the genus *Peperomia* (benzoic acid derivatives and seconelignans), *Ottonia* (amides, flavonoids and arylpropanoids) and *Pothomorphe* (catechol derivatives) [2a-c]. The chemistry of Piperaceae from Rio de Janeiro State, Brazil, has been addressed with great success. Previous phytochemical investigations of Piperaceae species from Brazilian Atlantic Forest performed by our research group led to the isolation of several compounds, such as kaplanin, lhotzchromene and blandachromenes I and II [3a-c]. In order to continue with the phytochemical studies of native Piperaceae species from Southeast Brazil, *Ottonia anisum* have been successfully studied [4]. The *O. anisum* root extracts were chemically investigated to afford six aristolactams. This is the first work regarding the presence of aristolactams in *Ottonia* species. Aristolactams belong to a large and important group of naturally occurring alkaloids that possess the phenanthrene lactam skeleton [5]. The phenanthrene chromophore group is found in the Aristolochiaceae together with the aristolochic acids and 4,5-dioxoaporphines alkaloids. Aristolactams have been reported from plants of the Annonaceae, Monimiaceae, Menispermaceae, Piperaceae and Saururaceae families [6a,b]. The phenanthrene lactam core is frequently found in biologically active natural products [7a-d]. Among them, aristolactams and aporphines constitute an important alkaloid group due to their unique structural features and potent biological activities, such as anti-inflammatory, to treat arthritis, gout, rheumatism, anti-PAF, antimycobacterial, and neuro-protective [8a-e].

Major secondary compounds from non polar root extracts of *O. anisum* were isolated by chromatographic techniques and identified as 3,4-dimethoxy-aristolactam (aristolactam BII, 1), 2,3,4-trimethoxy-aristolactam (piperolactam C, 2), 6-hydroxy-3,4-dimethoxy-aristolactam (stigmalactam, 3), 6-hydroxy-2,3,4-trimethoxy-aristolactam (goniothalactam, 4), 3-hydroxy-4-methoxy-aristolactam (aristolactam AII, 5), 3,4,6-trimetoxy-aristolactam (aristolactam BIII, 6) (Figure 1). These compounds 1-6 were identified by comparison of physical and spectroscopic data (MS and NMR) with literature records [9a-c]. Compound (1) was obtained as a yellow needle crystalline solid. The molecular formula was confirmed by GC-MS measurement.
m/z [M+] 279, suggesting the C$_7$H$_7$NO$_3$. The $^1$H NMR spectrum of compound (1) showed signals related to six aromatic protons, a broad D$_2$O-exchangeable proton at δ 10.67 and protons of two methoxy groups attached to aromatic ring at δ 4.06 (3H, s) and δ 4.04 (3H, s). The signal registered at δ 9.17 (1H, dd, $J = 6.0, 3.0$ Hz) was assigned to H-5, and the signals at δ 7.52 (2H, m) and δ 7.94 (1H, dd, $J = 6.0, 3.0$ Hz) corresponding to H-6, H-7 and H-8 respectively (Table 1). A singlet was identified at δ 7.86 (1H, s) corresponding to H-2. The NOESY spectrum of compound (1) showed correlations between H-2/CH$_3$O-3, CH$_3$O-3/CH$_3$O-4, CH$_3$O-4/H-5, as well as between H-8/H-9 (Figure 1). Significant correlations between H-5 (δ 9.17) and H-6 (δ 7.58), H-7 (δ 7.58) and H-8 (δ 7.94) were also observed. Considering these data as well as literature records, the structure of compound (1) was identified as aristolactam BII. The NMR analysis of previous analyzed compounds and allowed to confirmed (3) as goniothalactam. Compound (4) was isolated as brownish yellow needles. The mass spectrum m/z [M+] 325 established the molecular formula C$_{18}$H$_{15}$NO$_5$. The $^1$H NMR spectrum of compound (4) showed signals related to four aromatic protons, a broad D$_2$O-exchangeable proton at δ 10.79 and three methoxy groups attached to aromatic ring at δ 3.93 (3H, s), δ 4.04 (3H, s) and δ 4.38 (3H, s). The signal at δ 8.58 (1H, d, $J = 3$ Hz) assigned to H-5, and the signals at δ 7.08 (1H, dd, $J = 6.0, 3.0$ Hz) and 7.79 (1H, d, $J = 6.0$, Hz) corresponding to H-7 and H-8 respectively. The NOESY spectrum was quite similar to the compound (2), showing the spatial correlation between the methoxyl groups at C-2, C-3 and C-4. These data are in accordance with stigmalactam, 4. The compound (5) was isolated as a pale yellow powder. The same aristolactam skeleton was observed by the MS and NMR analysis. The mass spectrum m/z [M+] 265 established the molecular formula C$_{16}$H$_{11}$NO$_3$. The $^1$H NMR spectrum of compound (5) showed signals related to six aromatic protons, a broad D$_2$O-exchangeable proton at δ 10.69 and a single methoxy group attached to aromatic ring at δ 4.03 (3H, s). The signal at δ 9.25 (1H, dd, $J = 6.0, 3.0$ Hz) assigned to H-5, the signals at δ 7.96 (1H, dd, $J = 6.0, 3.0$ Hz) corresponding to H-8 and the signals at δ 7.54 (2H, m) corresponding to H-6 and H-7. Spatial correlations were found between the hydroxyl group at C-3 and the H-2 and CH$_3$O-4. NOESY correlations between H-5, H-6, H-7 and H-8 were also confirmed, as well as between H-8/H-9. Analysis of GC-MS, $^1$H NMR and NOESY spectral data showed chemical shifts very similar to the analogous previous analyzed compounds and allowed to confirmed (5) as aristolactam AII. Compound (6) was isolated as a yellow green powder with m/z [M+] 265 obtained from GC-MS. The $^1$H NMR spectrum of compound (6) showed signals related to five aromatic protons, a broad D$_2$O-exchangeable proton at δ 7.67 and three methoxy groups attached to aromatic ring at δ 3.96 (3H, s), δ 4.18 (3H, s) and δ 4.45 (3H, s). The signals at δ 8.65 (1H, d, $J = 3$ Hz) was assigned to H-5, and the signals at δ 7.15 (1H, dd, $J = 6.0, 3.0$ Hz) and δ 7.77 (1H, d, $J = 6.0$, Hz) corresponding to H-7 and H-8, respectively. A singlet observed at δ 7.80 was assigned to H-2. NOESY correlations between the methoxyl group at δ 3.96 and H-5 and H-7 was confirmed. This is in agreement with the aristolactam skeleton and suggested that the signal at δ 3.96 refers to the methoxyl group located at position C-6, confirming compound (6) as aristolactam BIII.

Nowadays, many communities in the North of Brazil still remain using O. anisum popularly in the treat toothache...
Aristolactams from *Ottonia anisum*

**Extraction and Isolation:** Air-dried and powdered roots (650 g) were extracted with MeOH. The solvent was evaporated to dryness under reduced pressure to give the MeOH extract (15 g) which was partitioned successively between n-hexane, followed by dichloromethane, ethyl acetate and n-butanol. Hexane and dichloromethane fractions were combined providing about 2 g that were subjected to a column chromatography on Sephadex LH 20, eluted with a MeOH/CHCl₃ (7:3) system furnishing 30 fractions. The last 10 fractions were combined furnishing 200 mg of a very strong UV fluorescence fraction. This sample was subjected to preparative normal phase TLC eluted with hexane/ethyl acetate (3:2) yielding six fractions with different UV fluorescence color. Aristolactam 1 (17.0 mg), 2 (7.0 mg), 3 (4.0 mg), 4 (2.0 mg), 5 (4.5 mg) and 6 (3.0 mg) were isolated and analyzed by GC-FID, GC-MS and ¹H and NOESY NMR techniques.

**GC-FID analysis:** Qualitative analyses were carried out on a GC 2010 Shimadzu apparatus with a DB-1MS fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness). The operating temperatures used were: injector 260°C, detector 290°C and column oven 60°C up to 290°C (10°C min⁻¹). Hydrogen at 1.0 mL min⁻¹ was used as carrier gas.

**GC-MS analysis:** Qualitative analysis was carried out on a GC-MS QP 5000 Shimadzu machine with a ZB-5MS fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness) using the same experimental conditions reported for GC-FID analysis. The aristolactams mass spectra were matching with WILEY 275 and National Institute of Standards and Technology (NIST 3.0) libraries provided with the computer controlling the GC–MS system. The results were also confirmed by comparison of data of the isolated compounds with mass and fragmentation reported in the literature [9a-c].

**NMR Spectroscopy:** The pure six constituents obtained were analyzed by ¹H NMR and NOESY recorded on a Varian VNMRS 500 spectrometer. Chemical shifts were determined in DMSO-d₆ and CDCl₃, using TMS as internal standard. The signals of NMR analysis were compared with laboratory data [9a-c].

**Acknowledgments** – This work was supported by CNPq.

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**Table 1: ¹H NMR spectroscopic data of compounds 1-5 in DMSO-d₆ and compound 6 in CDCl₃, δH (Hz).**

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**Experimental**

**Plant material:** Branches and roots of *Ottonia anisum* Sprengel were collected in Duque de Caxias, Rio de Janeiro, RJ, Brazil in April of 2007. A Voucher specimen was identified by Dr. Elsie Franklin Guimarães and is deposited at the RB Herbarium of Jardim Botânico do Rio de Janeiro, under the number RB393494.

**Chromatographic materials:** Sephadex LH-20 (Pharmacia) was used for column chromatographic separation. Silica gel PF254 (Merck) was used for TLC preparative/purification. All compounds were visualized on analytical TLC under UV light (254 and 365 nm) and by spraying with ceric sulfate solution followed by heating.

and as local anesthetic, even if a survey of the literature data shows that some of natural compounds have carcinogenic and nephrotoxic properties. Aristolactams are known to be nephrotoxic, carcinogenic and mutagenic [8b,d] [10a-f]. However, naturally occurring aristolactams such as cepharanone B (aristolactam BII), aristolactam BIII, piperolactam A and goniola lactam have shown potent inhibitory activity against human cancer cells. For example, aristolactam BII inhibits T and B lymphocyte proliferation as well as shows cytotoxic activity, while aristolactam FI (piperolactam A) displays inhibitory effects on NO generation by R AW264 [5]. Several synthetic aristolactam derivatives exhibited potent antitumor activities against a broad array of cancer cell lines with submicromolar range and some are equally potent toward multidrug resistant cell lines compared to the commercially available drug [8a,d]. It is noteworthy that the methoxy-substituted compounds are more potent than the hydroxyl-substituted ones. The tetra-methoxy substituted phenantherone lactam has highly potent cytotoxic activity [11]. Although the cytotoxicity of aristolactams is well known, structure-activity relationships have not been explored mainly as a consequence of the synthetic difficulties associated with preparing a diverse array of aristolactam analogues. Therefore, it is important the continuous knowledge about the native Piperaceae species of Brazil since many of them have been used in folk medicine to treat many conditions. To the best of our knowledge this is the first report of aristolactams in the genus *Ottonia.*
References


Anti-angiogenic Activity Evaluation of Secondary Metabolites from *Calycolpus moritzianus* Leaves

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Angiogenesis is a crucial step in many pathological conditions like cancer, inflammation and metastasis formation; on these basis the search for antiangiogenic agents has widened. In order to identify new compounds able to interfere in the Vascular Endothelial Growth Factor Receptor-1 (VEGFR-1, also known as Flt-1) recognition by VEGFs family members, we screened *Calycolpus moritzianus* (O. Berg) Burret leaves extracts by a competitive ELISA-based assay. MeOH and CHCl₃ extracts and several their fractions demonstrated to be able to prevent VEGF or PlGF interaction with Flt-1, with an inhibition about 50% at concentration of 100 µg/mL. Phytochemical and pharmacological investigation of the active fractions led to the isolation of flavonoids, and terpenes.

**Keywords:** *Calycolpus moritzianus*, Angiogenesis, VEGFR1/Flt-1, VEGF and PlGF, bioassay-oriented study.

In the last decade the inhibition of angiogenesis and vascular targeting has been the focus of new treatment strategies against the cancer. Among the long list of growth factors involved in the angiogenic process, VEGF-A has been considered for years the most important mediator of tumor angiogenesis [1]. Consequently, several strategies have been developed to inhibit the release of this growth factor, or to interfere in its interaction with receptors, VEGF receptor 1 (Flt-1) and VEGF receptor 2 (Flk-1 in mouse, KDR in human) [2]. Recent data support the concept that tumor infiltration by bone marrow-derived myeloid cells confers resistance to current antiangiogenic drugs targeting primary VEGF-A and its receptors (VEGF(R)s) [3]. For this reason, novel targets out of VEGF-A have been studied to diversify antiangiogenic treatments and to overcome resistance [4]. Genetic and pharmacological studies have identified Flt-1 and Placental Growth Factor (PIGF) as possible therapeutic targets for anticancer therapy [5]. Furthermore, has been proven that a combination of lower amount of VEGF(R)s inhibitors and compounds able to block PIGF showed equal antitumor efficacy compared to the standard dose of VEGF(R)s [5].

These findings suggested that molecules able to inhibit the activity of both PIGF and VEGF-A driven angiogenesis may be an opportunity for patients with cancer who may suffer excessive or prohibitive adverse effects from VEGF(R)s inhibitors.

Accordingly to these data, the research of new natural compounds which may inhibit both PIGF and VEGF-A activity, has been the target of the present study. We carried out a screening of *Calycolpus moritzianus* (O. Berg) Burret leaves extracts by a competitive ELISA-based assay [6]. We aimed to identify natural molecules as inhibitors of PIGF and VEGF-A recognition by Flt-1.
Several extracts from *C. moritzianus* leaves have been tested at the doses of 0.5, 0.1, and 0.02 mg/mL.

Methanol and chloroform residues exhibited a good activity in the inhibition on both PI GF/Flt-1 and VEGF-A/Flt-1 interaction, with a binding reduction higher than 60% at 20 µg/mL (Figure 1). Therefore these extracts were submitted to a bioassay-oriented fractionation. *C. moritzianus* MeOH extract was fractioned by sephadex column chromatography giving 9 fractions (A-I), while CHCl₃ extract was separated using silica gel column chromatography giving 7 fractions (AA-GG). The effect of the obtained fractions was tested on both PI GF/Flt-1 and VEGF-A/Flt-1, leading to the results reported in Figure 2-4.

*C. moritzianus* MeOH extract fractions were assayed in dose-dependent experiments at concentration ranging between 500 and 20 µg/mL on PI GF/Flt-1; the active frs. D-F and H were then assayed on VEGF-A/Flt-1 at concentration of 100 and 20 µg/mL. Among the MeOH fractions, Fr. H revealed the highest dose-dependent activity for PI GF/Flt-1 inhibition, provoking a reduction of its Flt-1 binding to 20% at 500 µg/mL and to 60% at 100 µg/mL, while at dose 100 µg/mL a 25% reduction of VEGF-A/Flt-1 interaction was observed.

Also D, E, F, I fractions exhibited a moderate inhibition for PI GF/Flt-1 complex, even if only Fr. D revealed to be able to inhibit also hVEGF-A / Flt-1 complex, leading to a binding reduction of 60% at 100 µg/mL (Figure 2). The active fractions were studied in order to identify the compounds responsible for this inhibitory activity.

Chromatographic and spectroscopic analyses of active Frs. D-F indicated the presence of flavonoidic derivatives as main components, which were identified as quercetin 3-O-β-D-glucopyranoside 1 [7], kampferol-3-O-β-D-glucopyranoside 2 [7], quercetin-7-O-β-D-glucopyranoside 3 [8], kaempferol-3-O-β-D-rhamnopyranoside 4 [9], quercetin-3-O-β-D-rhamnopyranoside 5 [9], and quercetin 6 [10].

The main compound identified in Fr. H was quercetin (85% w/w abundance). All isolated compounds were identified by means of 1D- and 2D-NMR spectroscopy, ESI-MS analysis, and by comparison of their data with those reported in the literature.

Among the pure compounds assayed, only quercetin showed a moderate activity (60% inhibition of PI GF/Flt-1 interaction at 100 µg/mL), compounds 1, 3, 5 its glycosides were inactive (Figure 3). These data suggest that the glycosylation at C-3 and C-7 of quercetin core is fatal for the activity. To better investigate a structure-activity relationship we tested also kaempferol aglycon of compounds 2, 4. Kaempferol was inactive in our test. The structures of quercetin and kaempferol are very similar except for the substituent at C-3'; the different activity observed for these compounds indicated that the presence of OH group at C-3' influence the resultant activity.

Fractions obtained from *C. moritzianus* CHCl₃ extract were assayed on PI GF/Flt-1 and VEGF-A/Flt-1 interaction by a competitive ELISA screening at concentration of 100 and 20 µg/mL.

Data in Figure 4 showed that the most active fraction was Fr. AA which revealed a moderate activity for both the growth factors causing a reduction of their Flt-1 binding to 40% for PI GF and 60% for VEGF at dose 100 µg/mL.
Antiangiogenic metabolites from *Calycolpus moritzianus*

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Nonetheless BB showed a moderate inhibition for both PIGF / Flt-1 and VEGF / Flt-1 interaction with a binding reduction of about 40% at dose 100 µg/mL.

Chromatographic separation of AA and BB fractions allowed to obtain the pure components: rosifoliol 7 [11], platanic acid 8 [12], oleanolic acid 9 [13], (-)-4,10-di-epi-5β,11-dihydroxyeudesmane 10 [14], 4,5-dioxoseco-γ-eudesmol 11 [15], and ursolic acid 12 [13], all identified by means of 1D- and 2D-NMR spectroscopy, ESI-MS analysis, and a comparison of their data with those reported in the literature.

The isolated pure compounds were tested in dose dependence manner on both PIGF/Flt-1 and hVEGF/Flt-1 systems (Figure 5). Only compound 8 was moderately able to inhibit PIGF/Flt-1 recognition; anyway the inhibition activity showed by this compound cannot explain by itself the activity of the original fraction.

On the basis of our results, we could hypothesize that the inhibition activity of PIGF and VEGF interaction with Flt-1 receptor by the *C. moritzianus* CHCl3 extracts and fractions may be due to the presence of a combination of compounds acting synergistically or as vehicles enhancing the biological activity. However, we cannot rule out that the activity of the extracts and fractions could be due to a very minor compound not isolated.

**Experimental**

**General experimental procedures:** The instrumentation used in this work is described in our previous paper [13].

**Plant material:** The leaves of *C. moritzianus* were collected in Venezuela in 2008 and identified by Ing. Juan Carmona of Herbarium (MERF), Facultad de Farmacia y Bioanálisis - Universidad de Los Andes, Merida; where a voucher specimen n.761 is deposited.

**Extraction and isolation:** The air-dried powdered leaves of *C. moritzianus* (890 g) were defatted with n-hexane and extracted successively by exhaustive maceration (3 x 1 L, for 48 h) with CHCl3, CHCl3-MeOH (9:1), and MeOH. The MeOH extract (5 g) was chromatographed over a sephadex LH-20 column (100 x 5 cm) with MeOH as the eluent. A total of 110 fractions were collected (15 mL each) and combined according to TLC analysis [silica 60 F254 gel-coated glass sheets with n-BuOH-AcOH-H2O (60:15:25) and CHCl3-MeOH-H2O (40:9:1)] to give nine pooled fractions (A-I). Fraction D (106 mg) was purified by RP-HPLC with a C18 µ-Bondapak column (30 cm x 7.8 mm, flow rate 2 mL/min) using MeOH-H2O (35:65) to obtain compound 1 (4.0 mg, tR = 20 min), and 2 (15.0 mg, tR = 26 min).

Fraction E (95 mg) was purified by RP-HPLC with a C18 µ-Bondapak column (30 cm x 7.8 mm, flow rate 2 mL/min) using MeOH-H2O (35:65) to obtain compound 2 (5.0 mg, tR = 26 min). Fractions F (90 mg) was separately purified by RP-HPLC using MeOH-H2O (2:3) to give compounds 3 (16 mg, tR = 10 min), 4 (6 mg, tR = 16 min), and 5 (2 mg, tR = 20 min). Fraction H (20 mg) was identified as quercetin.

The CHCl3 extract (5.0 g) was submitted to silica gel flash column chromatography eluting with CHCl3 followed by increasing concentrations of MeOH (between 1% and 70%). The following volumes of solvents were used: 4.2 L of CHCl3, 1 L of CHCl3-MeOH (99:1), 4.3 L of CHCl3-MeOH (49:1), 1 L of CHCl3-MeOH (95:5), 0.5 L of CHCl3-MeOH (9:1), 0.5 L of CHCl3-MeOH (1:1), 0.5 L of CHCl3-MeOH (3:7), and 0.3 L of MeOH. Fractions of 30 mL were collected and analyzed by TLC on silica 60 F254 gel-coated glass sheets eluting with CHCl3 or mixtures CHCl3-MeOH, 99:1, 49:1, 95:5, 9:1, 4:1, and grouped into seven fractions (AA-GG). Fraction AA (95 mg) was subjected to RP-HPLC on a C18 µ-Bondapak column.

**Figure 4:** Inhibitory properties of chloroformic extract fractions were assayed on PIGF/Flt-1 interaction (A) and VEGF/Flt-1 interaction (B). The fractions were used at 100-20 µg/mL. As control a specific inhibiting peptide was used (CP). The white bar refers to ELISA experiment carried out without inhibitors. Each experiment was performed three times and average values ± SD were reported.

**Figure 5:** Inhibitory activity of compounds 7-12 assayed at concentration of 100-20 µg/mL on PIGF/Flt-1 (A) and VEGF/Flt-1 (B). As control a specific inhibiting peptide was used (CP). The white bar refers to ELISA experiment carried out without inhibitors. Each experiment was performed three times and average values ± SD were reported.
Plant extracts, fractions and compounds 1-13 dissolved in DMSO (Sigma) were properly diluted and added to the wells pre-mixed with ligand. For dose-dependent experiments, concentration ranging between 20 and 500 μg/mL were used.

Acknowledgements - Authors are grateful to Ing. Forestal Juan Carmona of Herbarium (MERF), Facultad de Farmacia y Bioanalisis - Universidad de Los Andes, Merida for the help in collecting the plant material.

References


(30 cm x 7.8 mm, flow rate 2.0 mL/min) with MeOH-H2O (73:27) to yield compounds 7 (4 mg, tR = 10 min), 8 (10 mg, tR = 16 min) and 9 (2 mg, tR = 34 min). Fractions BB (70 mg) and was purified by RP-HPLC with MeOH-H2O (37:13) to give compounds 10 (3.5 mg, tR = 16 min), 11 (8 mg, tR = 4 min), 12 (1.5 mg, tR = 26 min).

ELISA-based assays: The ELISA based assay for plant extract, fractions and pure compounds screening was performed as described elsewhere [6].
Chemical and Biological Activity of Leaf Extracts of *Chromolaena leivensis*

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The flavonoids 3,5-dihydroxy-7-methoxy-flavanone, 3,5-dihydroxy-7-methoxyflavone and 3,5,7-trihydroxy-6-methoxyflavone were isolated from the leaves of *C. leivensis*. Preliminary observations in K562 cells (human erythroleukemia) using the trypan blue test, showed a 90% viability at a concentration of 100 µg/mL; however, further testing of the flavonoids at concentrations of 25, 50 and 100 µg/mL showed toxicity affecting the morphology of human erythroleukemia cells (K562) and human melanoma cells (A375). Induction of apoptosis was produced by 3,5-dihydroxy-7-methoxyflavone at 72 hours after treatment with arrest in the G2/M phase of the cell cycle. The A375 cells treated with 50 µg/mL of 3,5-dihydroxy-7-methoxyflavone for 24, 48 and 72 hours, display effects on the behavior of the cell cycle. The flavonoid 3,5-dihydroxy-7-methoxyflavone has activity on the mitochondrial membrane at concentrations of 25, 50 and 100 µg/mL, at time intervals of 8 to 12 hours. The flavonoids 3,5-dihydroxy-7-methoxy-flavanone and 3,5-dihydroxy-7-methoxyflavone at a concentration of 25 µg/mL increased the expression of costimulatory molecules corresponding to the phenotype presented by mature dendritic cells with differentiation markers CD40, CD83, CD86 and HLA-DR. The two flavonoids at concentrations between 0.39 and 100 µg/mL slightly increased the proliferation of peripheral blood mononuclear cells in the presence and in the absence of phytohemagglutinin. These flavonoids at concentrations of 50 and 100 µg/mL slightly increased the proliferation of fibroblasts.

**Keywords:** *Chromolaena leivensis*, flavonoids, cytotoxicity, apoptosis.

Chromolaena species are invasive and cosmopolitan with morphological diversity given by adaptations to different environments and are considered weeds. This genus is constituted by 202 species of which only 13 have undergone chemical studies, and only a few studies have included biological activity. There are no known reports on the species *Chromolaena leivensis*, *C. pergleraba*, *C. tacotana*, *C. subscandens*, *C. opadoclina*, *C. odorata*, *C. arnottiana*, *C. morii*, *C. Collina*, *C. connivens*, *C. glaberrima*, *C. pseudoinsignis* and *C. chasleae*. Compounds such as sesquiterpenes, diterpenes, triterpenes, flavonoids, cyclic fatty acids, sesquiterpene lactones, germacranoles, have been identified from Chromolaenas. A sesquiterpene lactone was identified from the dichloromethane extract of *Chromolaena opadoclina* [1]. 5,3-dihydroxy-6, 7,4′-trimethoxyflavone, 5-hydroxy-6,7,3′,4′-tetramethoxyflavone, 5-hydroxy-6,7,3′,4′,5′-penta-methoxyflavone and a common derived 3,4-dihydroxy-acetophenone have been identified in *Chromolaena arnottiana* [2]. The heliangolide 4′-dihydrochromolaenid and a sesquiterpene lactone were found in *Chromolaena glaberrima* [3]. The acid 7α-acetoxy-trans-communinc was identified in *Chromolaena morii*, germacrane D, squalene, flavonols and a fatty acid type prostaglandin were found [5b].

Many studies on biological activity have been conducted in *Chromolaena odorata* and a few in *C. hirsuta*, *C. moritziana*, *C. pergleraba*, *C. bullata* and *C. tacotana*. Among the tested biological activities the following are highlighted: pesticide, insect repellent, antiprotozoal, insecticidal, trypanocidal, antibacterial, antifungal, cytotoxic, antioxidant, mutagenic, proliferative agent of human keratinocytes and fibroblasts. Taleb et al. [6] evaluated the antiprotozoal effect of total extracts and purified flavonoids from *Chromolaena hirsuta* and determined antiprotozoal activity against trypomastigotes of Trypanosoma cruzi and amastigotes of Leishmania amazonensis; the crude extracts significantly reduced parasite viability and the flavonoids showed an antiproliferative effect on these. Phan found that phenolic compounds of *Chromolaena odorata*, p-hydroxybenzoic acid and p-coumaric acid, flavones, flavanones and chalcones protect skin cells from oxidative damage and repair skin conditions [7]. Bouda observed the effect of essential oils from leaves of *Chromolaena odorata* on the mortality of Sitophilus Curculionidae (Coleoptera) with a LD50 of 6.78%[8]. Thang evaluated the antioxidant effect of extracts of *Chromolaena odorata* on human dermal fibroblasts by measuring the protectant effect against damage from hydrogen peroxide and hypoxanthine-xanthine oxidase [9]. Phan showed that Eupolin extract...
adhesion complex and fibronectin in human keratinocytes [10]. They found increased expression of integrin b1 and b4 induced by the extract at concentrations of 0.1 and 1 µg/mL, but the expression was reduced at higher doses of Eupolin (10 to 150 µg/mL). Other researchers [11] have studied the proliferation of fibroblasts and endothelial cells treated with hydroethanolic leaves extracts of Chromolaena odorata (Eupolin). The greatest growth of fibroblasts and endothelial cells was found at concentrations of 10 µg/mL and 100 µg/mL of Eupolin extract, but it was found to be toxic at concentrations exceeding 250 µg/mL.

Table 1: Effect of the flavonoids on cell viability at 24h of treatment.

<table>
<thead>
<tr>
<th>FLAVONOID -µg</th>
<th>Trypan blue</th>
<th>% TO 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,5-dihydroxy-7-methoxyflavanone-100</td>
<td>94.4</td>
<td></td>
</tr>
<tr>
<td>3,5-dihydroxy-7-methoxyflavanone-50</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>3,5-dihydroxy-7-methoxyflavanone-100</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>3,5-dihydroxy-7-methoxyflavone-50</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Concentrations that affect cell morphology.

<table>
<thead>
<tr>
<th>EFFECT ON MORPHOLOGY</th>
<th>CELL-K562 [µg/24 H]</th>
<th>CELL-K562 [µg/48 H]</th>
<th>CELL-A375 [µg/24H]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,5-dihydroxy-7-methoxy flavone</td>
<td>25</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>3,5-dihydroxy-7-methoxy flavone</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 3: Percentage of cells in each phase of the cell cycle.

<table>
<thead>
<tr>
<th>EFFECT ON CELL CYCLE, A375 CELLS</th>
<th>FLAVONOID - µg</th>
<th>G0/G1 - %</th>
<th>S-%</th>
<th>G2/M - %</th>
<th>SUB GI- %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,5-dihydroxy-7-methoxy flavone-50</td>
<td>75.9</td>
<td>19.39</td>
<td>4.62</td>
<td>5.99</td>
<td></td>
</tr>
<tr>
<td>CONTROL (+) DMSO</td>
<td>54.21</td>
<td>26.3</td>
<td>19.49</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>CONTROL (+) G2/M VINC</td>
<td>8.63</td>
<td>16.27</td>
<td>75.10</td>
<td>2.29</td>
<td></td>
</tr>
</tbody>
</table>

The species Chromolaena perglabra, C. tacotana, C. bullata, C. subscandens, C. leivensis and C. scabra are found in the Cundiboyacense region of Colombia, and C. barranquillensis is found in the Atlantic coast. These species have not been studied at depth with respect to their biological activities, specifically as antiparasitic agents against Chagas and Leishmania and their cytotoxic and antitumor potential.

In this investigation we studied the production of secondary metabolites in the leaves of C. leivensis and isolated the flavonoids 3,5-dihydroxy-7-methoxyflavanone, 3,5-dihydroxy-7-methoxyflavone and 3,5,7-trihydroxy-6-methoxyflavone, the first two compounds were tested for their activity on cancer cell lines. Using the trypan blue test, it was observed in K562 cells (erythroleukemia) viability percentages of 90% using a concentration of 100 µg/mL, which indicates that flavonoids at these concentrations are not cytotoxic. The flavonoids tested at concentrations of 25, 50 and 100 µg/mL showed toxicity affecting the morphology of human erythroleukemia cells (K562) and human melanoma cells (A375). Induction of apoptosis was produced by the flavonoid 3,5-dihydroxy-7-methoxyflavone at 72 hours of treatment with arrest in G2/ M. In A375 cells treated with 50 µg/mL of the flavonoids for 24,
48 and 72 hours, it was observed that the flavonoid 3,5-dihydroxy-7-methoxy-flavanone influences the behavior of the cell cycle. The flavonoid 3,5-dihydroxy-7-methoxy-flavone have activity on mitochondrial membrane at concentrations of 25, 50 and 100 μg/mL, at time intervals of 8 to 12 hours. It was also observed that the flavonoids 3,5-dihydroxy-7-methoxy-flavanone and 3,5-dihydroxy-7-methoxyflavone at a concentration of 25 μg/mL increased the expression of co-stimulatory molecules corresponding to the phenotype presented by mature dendritic cells with differentiation markers CD40, CD83, CD86 and HLA-DR. The two flavonoids at concentrations between 0.39 and 100 μg/mL slightly increased the proliferation of peripheral blood mononuclear cells in the presence and in the absence of phytohemagglutinin. It was also determined that fibroblast proliferation increased slightly at concentrations of 50 and 100 μg/mL of these flavonoids.

Experimental

Materials were collected in the outskirts of Bogota, Colombia; a control sample was sent to the National Herbarium of Colombia for identification and was determined to be Chromolaena leivensis (Hieron). King & H. Rob. with the number COL-535 219 Colombian National Herbarium. The extraction was carried out in Soxhlet with 95% ethanol (4L) with a yield of 31.04%. 200 g of extract was mixed with silica gel (1:2) and Soxhlet with 95% ethanol (4L) with a yield of 31.04%. The extraction was carried out in Soxhlet with 95% ethanol (4L) with a yield of 31.04%. The biological activity was determined to be 2.66% for the crude extract solid - liquid with petroleum ether (3L), toluene (200 g of extract was mixed with silica gel (100 g silica gel Merck Kieselgel), eluting with petroleum ether, toluene and methanol in various proportions. By fractional crystallization three flavonoids were isolated and identified as 3,5-dihydroxy-7-methoxyflavanone, 3,5-dihydroxy-7-methoxyflavone [13] and 3,5,7-trihydroxy-6-methoxyflavone.[14].

Cell viability: Cell viability was measured using trypan blue, a negatively charged chromophore that interacts with cell membranes whose integrity has been altered. Living cells exclude the dye while dead cells allow entry and are stained. The effect of flavonoids 3,5-dihydroxy-7-methoxy-flavanone and 3,5-dihydroxy-7-methoxyflavone on cell populations with concentrations less than or equal to 100 μg/mL were analysed at 24 and 48 hours. The data are listed in Table 1.

Evaluation of cell cycle distribution: To demonstrate the effect of flavonoids 3,5-dihydroxy-7-methoxy-flavanone and 3,5-dihydroxy-7-methoxyflavone on the cell cycle in the A375 tumor cell line, cells were synchronized in G1 phase by total withdrawal of fetal bovine serum for 3 days. Once synchronized, the cells were grown in twelve-well plates at a density of 400,000 cells/mL and incubated in the presence and absence of the test compounds. After the incubation period, samples were analyzed in a flow cytometer, using Cell Quest Pro program and subsequent analysis was performed by the Modfit program V. 2.0. (FACSCalibur, Beckton Dickinson). The calibration parameters were established, and the linearity of the laser was measured for further analysis with the program Modfit. The data are shown in Table 3.

Mitochondrial membrane depolarization: The iodide of 5,5',6,6',tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbonic amino (JC-1) is a lipophilic cationic compound sensitive to changes in mitochondrial membrane potential. In healthy cells the tagged mitochondria emits red fluorescence. The negative charge established by the intact mitochondrial membrane allows the JC-1, which has delocalized positive charge to enter the mitochondrial matrix where it accumulates. When it reaches a critical concentration it forms J-aggregates that emit red fluorescence. In apoptotic cells, the membrane potential of mitochondria declines, and the JC-1 cannot accumulate within the organelle and remains in the cytosol as a monomer emitting green fluorescence. The aggregate red form has a maximum absorption / emission 585/590 nm, while for the green monomer is 510/527 nm. The test was performed using the cationic lipophilic fluorochrome JC-1, at concentration of 10 μg/mL, from a diluted stock solution in DMSO and kept at 4°C. The K562 cells (1X106) were treated with flavonoids 3,5-dihydroxy-7-methoxy-flavanone and 3,5-dihydroxy-7-methoxyflavone in 24 well plates and read at 4, 8 and 12 hours. The cells were then incubated for 10 minutes at 37°C and read immediately using a flow cytometer, using the Cell Quest Pro program (FACSCalibur, Beckton Dickinson). The data obtained are shown in Table 4.

Dendritic cells analysis: Peripheral Blood mononuclear cells (PBMC) were obtained and washed in RPMI 1640 supplemented with 1% fetal bovine serum (FBS). The viability and cell numbers were assessed by trypan blue staining and Neubauer cell counting chamber, respectively. The CD14+ cells were incubated in RPMI 1640 in the presence of 35 μg/mL of interleukin (IL-4) and 50 μg/mL growth factor granulocyte and monocyte GM-CSF (R&D system) for 5 days of culture, verifying their morphology by light microscopy. At day 5 of culture, DCs were treated with different concentrations (12, 25 and 50 μg/mL) of flavonoids 3,5-dihydroxy-7-methoxy-flavanone and 3,5-dihydroxy-7-methoxyflavone, and 1 μg/mL of lipopolysaccharide (LPS) as a positive control for differentiation. The expression of surface markers was assessed by flow cytometry after 48 hours using antibodies against CD40, CD83, CD86 and HLA-DR. The data obtained showed proliferation of dendritic cells.

Evaluation of the effect of the flavonoids on the viability of PBMNC and fibroblasts: The effect of flavonoids 3,5-dihydroxy-7-methoxy-flavanone and 3,5-dihydroxy-7-methoxyflavone on cell viability of peripheral

Secondary metabolites in the leaves of C. leivensis
blood mononuclear cells (PBMNC) cells was analyzed by seeding the cells in 96-well plates at a density of 200,000 cells/well in 200 μL of RPMI 1640 without phenol red supplemented with 10% FBS and stimulated for 12 hours with the mitogen PHA (250 μL/100 mL) and then placed in contact with different concentrations of each flavonoid. Cells treated with DMSO (vehicle) were used as a control. Fibroblasts were analyzed in a similar manner. Briefly, cells were seeded at a density of 15,000 cells/well in 200 μL of medium in a 96-well plate and allowed to adhere for 24 hours; different concentrations of the flavonoids were added and incubated for 24 hours (37°C, 5% CO2, 95% humidity). After incubation with the flavonoids, 110 μL of medium was added to 10 μL of 12 mM MTT and incubated for 4 hours (37°C, 5% CO2, 95% humidity) protected from light. After this time, the crystals of formazan resulting from the metabolism of MTT by mitochondria of viable cells were dissolved with 100 μL of SDS-HCl 0.01M. The plates were incubated again under the same conditions for 4 hours. The color intensity was read by absorbance at 540 nm in an ELISA reader (Labsystems Multiskan MCC/3340).

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References

Fresh juice from bergamot (*Citrus bergamia* Risso) has been studied to evaluate the polyphenolic composition by HPLC-DAD analysis and total polyphenols content by UV method. The main constituent, Naringin, has been selected as analytical and biological marker of the juice. Juice has been loaded onto maltodextrin matrix by spray-drying. The produced maltodextrin/juice powder (BMP) showed neither significant change in total polyphenols content nor decrease in antioxidant properties with respect to fresh juice. Moreover, BMP displayed high *in vitro* dissolution rate of the bioactive constituents in water and in simulated biological fluids. BMP appears as promising functional raw material for food, nutraceutical and pharmaceutical products. With this aim, a formulation study to develop tablets (BMT) for oral administration has been also performed. The produced solid oral dosage form preserved high polyphenols content, showed complete disaggregation in few minutes and satisfying dissolution rate of the bioactive constituents in simulated biological fluids.

**Keywords:** *Citrus bergamia* Risso, fresh juice, polyphenols content, Naringin, maltodextrin/juice powder, tablets, *in vitro* disaggregation and dissolution tests.

Bergamot (*Citrus bergamia* Risso) is a natural hybrid fruit derived from bitter orange and lemon. The plant grows almost exclusively in the Reggio Calabria region (South Italy). Bergamot is used for production of essential oil obtained from the peel, and its fruit juice is considered a waste product of the industrial process [1a-1e]. Bergamot juice has drawn attention for its polyphenolic, mainly flavonoids content [2a-2c], being an attractive raw material for the food and nutraceutical industry. It is well known that the consumption of polyphenol-rich products, mainly due to their antioxidant properties, is beneficial for human health [3a,3b]. Flavonoids from citrus fruits have many health benefits including anticancer, antiviral, and anti-inflammatory activities, as well as effects on capillary fragility, and inhibition activity on human platelet aggregation [4a,4b]. In recent studies [5a,5b], bergamot juice has been shown to be effective in the prevention of diet-induced hyperlipidemia. Moreover, it is able to enhance the antioxidant values of others industrial juices, acting as synergistic compound for the synthetic additives normally used [6].

Despite these beneficial effects, the unprocessed fresh bergamot juice, showing penetrating smell and bitter taste, involves practical difficulties for an industrial use. Alterations of the functional and organoleptic properties of polyphenols can take place during the storage period due to constituents release and degradation/oxidative process [7,8]. A convenient way to increase the shelf-life and to improve the organoleptic characteristics of a plant derivative is to transform it into a stable dry powder form [9a,9b]. Spray-drying is a micro-encapsulation technique appropriate for sensitive components such as polyphenols, and commonly used in pharmaceutical and food industry [10]. Food ingredients and additives in spray-dried powder form have reduced bulk weight and size, long-lasting biological stability, and are suitable for transportation and handling. Common carriers for spray-drying process include carbohydrates, gums, semisynthetic cellulose derivatives and synthetic polymers [11]. Currently, maltodextrins, water soluble modified starch derivatives, are used alone or in combination with other materials in food and drug processing of plant extracts, aromatic additives, carotenoids and vitamins [12a-12c]. Maltodextrins have multifaceted functions including bulking, caking resistance, film formation, binding of flavour and fat as well as reduction of oxygen permeability of wall matrix. Moreover, as one of the administration problems of the bergamot juice is the bitter taste, maltodextrins are also able to sweeten the final product.

This paper reports on the evaluation of polyphenol components and antioxidant properties of fresh bergamot juice, as well as on the production and characterization of powders obtained loading the fresh juice onto maltodextrins as carrier (BMP) by spray-drying. Moreover, a
formulation study to develop tablets containing BMP for oral administration has been performed. Characteristics of the tablets (BMT) were evaluated in term of disintegration time and active compounds release in water and simulated biological fluids.

Hand-squeezed crude bergamot juice was analyzed by HPLC-DAD method. As shown in the chromatogram (Figure 1A) the eluted constituents were three flavanone neohesperidosides, Neoeriocitrin (1), Naringin (2), Neohesperidin (3), and a flavone neohesperidoside, Neodiosmin (4) (Figure 1B). Each compound was identified by comparison of retention time, MS and UV spectra with those of standards. In agreement with literature data [2a-2c], compounds (1-3), were found as the most abundant flavonoids in the bergamot juice (0.6 ± 0.01, 0.6 ± 0.01, and 0.4 ± 0.01 mg/mL, respectively). To produce the powder form, an aqueous liquid feed, containing both maltodextrins and bergamot juice, was prepared and processed by spray-drying technique, as described in the experimental section. The production yield of Bergamot-Maltodextrin Powder (BMP) was very high (90%). The presence of maltodextrin, having high water solubility, significantly reduced apparent viscosity of the feed dispersion favouring the atomization and drying of the liquid feed [12b]. Moreover, increasing temperature during the spray-drying process, maltodextrins are able to induce the rapid formation of a glassy surface which allows air expansion inside particles, favouring the increase of particles diameter [12b]. For this reason, smallest and lightest particles which are normally lost with the exhaust of the spray dryer are reduced, and the yield increases. On the other hand, a low viscosity liquid feed led to a low retention of core material because of the delay in the formation of a semi-permeable layer by the internal components during drying [12b,13].

Polyphenol content of both unprocessed juice (actual polyphenol content, APCB) and BMP (APC_{BMP}) was determined by UV method (5.49 and 3.57%, respectively). These values led to calculate the effectively loaded juice (actual juice content, AJC_{BMP} 32.7%) as described in the experimental section. AJC_{BMP} was reasonable with respect to the theoretical juice content (TJC 50.0%). Consequently, the loading efficiency (LE) value, calculated as the ratio of AJC to TJC, was 65.4%.

The functional stability of juice, before and after the spray-drying process, was evaluated as free-radical scavenging activity using the DPPH test [14]. The antioxidant activity of bergamot juice, expressed as EC_{50} (130 ± 5 and 140 ± 12 μg/mL, respectively) was at the same level and remained quite unaltered after the spray-drying process. The process conditions used did not determine significant loss of antioxidant activity. To evaluate the dissolution/release profile of juice from the powder, its solubility in each dissolution medium was previously detected as described in the experimental section. Solubility of BMP resulted 4.0 g/L in water, 3.4 g/L in simulated gastric fluid (GF) and 4.0 g/L in simulated intestinal fluid (IF), respectively. Sink conditions, which describe a dissolution system sufficiently dilute so that the dissolution process is not impeded by saturation of the solution, resulted 1.0 g/L. The in vitro dissolution/release profiles of active compounds from the BMP in each dissolution medium (water, GF and IF) are reported in Figure 2. After 5 minutes a high amount (about 90%) of juice was dissolved/released both in water and simulated intestinal fluid; and about 60% in GF, in agreement with solubility results previously reported. The complete dissolution (about 100%) was achieved after 30 minutes in both water and IF, and after 2 hours in GF (Figure 2). These results are very interesting, because the high water solubility of the powder, displaying high in vitro dissolution rate with complete release of the active compounds in all dissolution media. Formulation of BMP into tablets meets the challenge to retain the original properties of powder during compression. This was achieved by keeping low the compression pressure and using the direct compression procedure instead of wet granulation, thus avoiding lengthy granulation steps and exposure to solvents used in wet granulation [15]. BMP itself was used both as a directly compressible binder and as diluent. Bergamot juice is rich in sugars which can act as binder, and maltodextrins, used as carrier, which may also act as diluent in the tablets preparation [15]. Moreover, CMC was used as disintegrant and directly compressible filler, and magnesium stearate as a lubricant. The final formulation of bergamot-maltodextrin tablets (BMT) is reported in the experimental section. Each BMT resulted 8-mm tick (Figure 3) and their disintegration time (see experimental section) was in about 15 minutes. Figure 3 shows the dissolution profiles of BMT in three different dissolution media. In 5 minutes, about 20% and 30% of juice was dissolved in water and IF, respectively. In the same time only 5% was dissolved in GF. 90% of dissolution was obtained in 90 minutes in IF, and in 150 minutes, a total release was observed in all dissolution media. Release of juice from the BMT resulted slower than from the BMP, probably due to the enhancement of binding forces of sugars, contained in the juice, during the compression. Any how, this effect was balanced by the presence of CMC which promotes the disintegration of tablets and enhancement of the active compounds dissolution rate. In conclusion, the obtained water-soluble powder BMP is able to preserve the polyphenols content and antioxidant activity of unprocessed juice. Furthermore, the spray-dried powder is suitable for the production of oral dosage tablets (BMT) by directly compression as well as for manufacturing raw material functional food, and for pharmaceutical and food supplements products.

**Experimental**

**Chemicals:** HPLC grade methanol (MeOH), formic acid (HCOOH) and the reagents used for the extractions were purchased from Carlo Erba (Rodano, Italy). HPLC grade water (18mΩ) was prepared using a Millipore Milli-Q
purification system (Millipore Corp., Bedford, MA). Neoeriocitrin, Naringin, Neohesperidin, Neodiosmin, and Folin-Ciocalteau’s phenol reagent were provided from Sigma Chemical Co. (Milan, Italy). Maltodextrins D.E. 16, magnesium stearate and microcrystalline cellulose from Acef, Italy.

**Instruments:** HPLC analysis was carried out on an Agilent 1100 series system equipped with a Model G-1312 pump, and Rheodyne Model G-1322A loop (20 μl), and a DAD G-1315 A detector. Peaks area were calculated with an Agilent integrator. ESI-MS was performed on a Finnigan LC-Q Deca instrument s (Thermoquest, San Jose, CA) equipped with Xcalibur software. To produce the juice-maltodextrin powder a Mini Spray Dryer B-191 Büchi (Laboratoriums-Tecnik, Flawil, Switzerland) was used. Dissolution test was carried out by SOTAX AT Smart Apparatus (Basel, CH) on line with a spectrophotometer (UV/Vis spectrometer Lambda 25, Perkin Elmer Instruments, MA, USA). Balance Crystal 100 CAL – Gibertini (max 110 g d=0,1 mg; + 15°C/30°C). Mixer Galena Top (Ataena, Tecno-Pro srl, Italy). Alternative compression apparatus GP1, Costamac srl, Casatenovo (LC) Italy.

**Plant material:** *Citrus bergamia* fruits Risso were collected in February 2009 from plants growing in Reggio Calabria, Italy. Bergamot juice was prepared by hand squeezing fresh fruits, immediately after collection. It was filtered through steel sieves of 1 mm and stored at -20°C until required for our study.

**Sample preparation for HPLC and UV analyses:** Unprocessed bergamot juice and processed spray dried juice (BMP) were extracted according to Pernice et al. 2009 [6] with slight modifications. 2 mL of bergamot juice was extracted wht 10 mL methanol, agitated and sonicated for 10 min. Juice was centrifuged for 10 min at 4000 rpm; supernatant was collected, while the pellets was extracted a second time using the same procedure. Supernatants were combined and centrifuged for 10 min at 2000 rpm. The concentration of solid material was 20.4 mg/mL.

**HPLC-DAD analysis:** A part of supernatant was separated by HPLC using a 3.9 × 300 mm i.d. C18 μ-Bondapack column. The mobile phase consisted in water (solvent A) containing 0.1% formic acid, and methanol (solvent B). The elution gradient was as follows; 0→5 min, 15→30% B; 5→10 min, 30→35% B, 10→20 min, 35→50% B, 20→30 min, 50→75% B; 30→35 min, 75→95% B; 35→40 min, 100% B. The flow rate was 1.0 mL min⁻¹ with a DAD detector set at 284 nm. Elution yielded four major compounds (Figure 1): Neoeriocitrin (1, tR 12.6 min), Naringin (2, tR 15.1 min), Neohesperidin (3, tR 16.6 min), and Neodiosmin (4, tR 18.4 min), in according to data reported in literature [2c]. Identification of constituents was carried out by comparison of their retention times, UV and MS spectra data with those of standard compounds, and confirmed by co-injections.

**Bergamot-powder (BMP) production by Spray drying:** 200 g of maltodextrins (16 D.E.) were dissolved in 200 mL of fresh bergamot juice, with a 1:1 polymer/juice weight ratio. The liquid feed was spray dried under the following process conditions: inlet temperature 120°C; outlet temperature 69-71°C; spray flow feed rate 5 mL/min; nozzle diameter 0.5 mm; drying air flow 500 l/h, air pressure 6 atm, aspirator 100%. In order to keep
homogeneity, while feed was pumping into the spray dryer, the suspension was gently stirred using a magnetic stirring. Each preparation was carried out in triplicate. Spray-dried bergamot-maltodextrin powder (BMP) was collected and stored under vacuum for 48 h at room temperature until the characterization.

Quantitative HPLC analysis: HPLC equipment and conditions were the same used for the qualitative analysis. Unprocessed juice and BMP were subjected to extraction as reported in Sample preparation. Reference standard solutions of Neoeiocitrin, Naringin and Neohesperedin were prepared at three concentration levels in the range 0.25-1.0 mg/mL. Standard curves were analyzed using the linear least-squares regression equation derived from the peak area (R²>0.9999) corresponding to each compound. Results were expressed as mg/mL of compound ± standard deviation.

Total polyphenol content: Actual phenol content of bergamot unprocessed juice (APC_B) and BMP (APC_BMP), were determined by UV/Vis spectrometry at λ 284 nm. Each analysis was made in triplicate. APC was expressed in percentage as total Naringin (N) equivalents (mg N/100 mL juice).

Yield of the process and loading efficiency: Production yield was gravimetrically determined and expressed as the weight percentage of the final product compared to the total amount of the materials sprayed.

Theoretical juice content (TJC) was calculated as percentage of juice content compared to the initial total content of all feed components before spray-drying. Actual juice content (AJC), theoretical polyphenol content (TPC) and loading efficiency (LE) were calculated as reported in the following formula:

\[
\text{AJC}% = \frac{\text{APC}_{\text{BMP}}}{\text{APC}_B} \times 100 \\
\text{TPC}% = \frac{\text{APC}_B \times \text{loaded juice}}{\text{feed components}} \\
\text{LE}% = \frac{\text{AJC}}{\text{TJC}} \times 100
\]

BMP Solubility: Solubility of the powder was determined in distilled water and in simulated biological fluids (gastric fluid, pH 1.2, and intestinal fluid, pH 7.5 without enzymes) prepared according to USP 31 (2008) [16] at the conditions reported elsewhere [8]. Concentration of juice in the media was determined by UV/Vis spectrometry at λ 284 nm and expressed as Naringin equivalents. Each analysis was made in triplicate. Naringin calibration curves in the same solvents were previously worked out. Proportionality between absorbance and concentration was verified in the range 1 g/L to 5 g/L (R²>0.999).

Bleaching of the Free-radical 1,1-Diphenyl-2-picyrylhydrazyl (DPPH Test): Free radical scavenging activity of unprocessed juice and BMP was determined using the DPPH (1,1-diphenyl-2-picyrylhydrazyl) method [14]. Results were expressed as amount (µg/mL) of antioxidant necessary to decrease the DPPH initial concentration by 50% (EC50± standard deviation (SD)). α-Tocopherol (EC50 10.1±1.3 µg/mL) was used as a positive control in the test.

Tablets products: Ingredients and their relative amounts used for the formulation of Bergamot-maltodextrin-tablet (BMT) were: BMP 1000 mg, magnesium stearate 10 mg, and microcrystalline cellulose (CMC) 5 mg. The ingredients were mixed until uniformity. The resultant mixture was compressed using round double concave punches of 10 mm diameter.

In vitro dissolution test: In vitro dissolution/release tests were carried out according with the Farmacopea Ufficiale Italiana (F.U.I. XII, 2009) [17]. Release profile of BMP and BMT were determined in water, phosphate buffer at pH 6.8 (simulated intestinal fluid without enzymes) and hydrochloric acid buffer pH 1.2 (simulated gastric fluid without enzymes). Samples were analyzed spectrophotometrically at λmax 284 nm. Briefly, 1000 mg of BMP or one BMT were placed in six dissolution vessels containing 1000 mL of dissolution medium on a dissolution test apparatus n.2: paddle, 100 rpm at 37°C±0.5°C. All the dissolution tests were made in triplicate; only the mean values are reported (standard deviations < 5%). Amount of juice dissolved was measured as Naringin equivalents. Results are graphically expressed as the dissolution rate (in percentage) with respect to the time (in minutes).

In vitro disintegration time: Test was carried out according with the Farmacopea Ufficiale Italiana (F.U.I. XII, 2009) [18]. A modified dissolution apparatus (paddle type) was used. The disintegration fluid was HCl pH 1.0 (1000 mL) at the temperature of 37°C±0.5°C with a stirring of 100 rpm. Six tablets were placed individually in six sinkers and disintegration time was determined as the point at which the tablet disintegrated completely and passed through the screen of the sinker.

References

Polyphenol and antioxidant properties of fresh bergamot juice


A new phenolic derivative, 4-methoxyphenol 1-O-β-D-apiofuranosyl-(1→6)-O-β-D-glucopyranoside (1), has been identified together with uncommon 3,4-dimethoxyphenol 1-O-β-D-apiofuranosyl-(1→6)-O-β-D-glucopyranoside (2) and 3-hydroxy, 4-methoxyphenol 1-O-β-D-apiofuranosyl-(1→6)-O-β-D-glucopyranoside (3) from the leaves of Martinella obovata (Kunth) Bureau & K. Schum., an Honduran species used in folk medicine for the treatment of eyes diseases. Verbascoside, isoverbascoside, leucoceptoside A, vitexin, isovitexin, luteolin 8-C-β-D-glucopyranoside and spireoside were also found. All structures were elucidated on the basis of mass spectrometry and 2D NMR techniques.

**Keywords:** Bignoniaceae, Martinella obovata, Phenolic apiosides, 1D and 2D NMR.

The Bignoniaceae family includes about 120 genera and 800 species, growing mainly in Africa, Central and South America. Species of the Bignoniaceae are used for many purposes, such as horticulture, timber, dyes and medicine. The best-known medicinal use of the Bignoniaceae is the application of bark preparations of various species of Tabebuia as cancer cures [1]. However, members of the family have been sparsely chemically investigated [2].

Martinella (Bignoniaceae) is a tropical genus consisting of nine species. Root extracts of the Martinella iquitosensis vine, found in Amazonian lowland rainforests, are used by indigenous peoples to treat various eye ailments, including inflammation and conjunctivitis [3]. This medicinal use may be attributed, at least in part, to the presence guanidine alkaloids which have been demonstrated to be modest antibiotics and micromolar binders of several G-protein coupled receptors [4]. A predominance of information regarding M. obovata use comes from Amazon Indian tribes of Peru, where this liana, uniformly called “yuquilla”, is often cultivated as the preferred treatment for eye diseases. In general, the thick fleshy root bark, with the rough outside part scraped off, is pounded and the resultant juice strained through cloth. One or two drops of this juice placed into the eyes is said to have an immediate effect on inflammation. However, there are no data in the literature concerning the possible pharmacological effects and the chemical constituents; this is the first chemical investigation of M. obovata leading to the isolation of 10 phenolic compounds.

The ESIMS in negative mode of compound 1 exhibited a quasi-molecular ion peak at m/z 417 [M-H] and a high resolution measurement indicated the molecular formula, C_{18}H_{26}O_{11}, in accordance with 13C NMR data. Major fragments at m/z 285 and 123 were assigned to the loss of a pentose unit (132 amu) and the successive loss of an hexose unit (162 amu). The 1H NMR spectrum of 1 exhibited a set of AAXX coupling system at δ H 7.09 (2H, d, J=8.5, H-2, H-6), and δ H 7.01 (2H, d, J=8.5, H-3, H-5), a methoxy signal at δ H 3.85 (3H, s, OMe-3), and two anomeric proton signals at δ 5.01 (1H, d, J=2.5 Hz, H-1”) and 4.78 (1H,d, J=8.0 Hz, H-1`). The 13C-NMR spectrum of 1 revealed 19 carbon signals, including one benzene ring carbon signal, a set of hexose carbon signals, a set of pentose carbon signals, and a methoxy signal. NMR data were in agreement with a 1,4-disubstitution of benzene ring. The nature of the terminal sugar unit as β-D-apiofuranosyl was deduced by the following evidence: the 1H NMR spectrum indicated an anomic signal at δ 5.01 (H-1”, d, J = 2.0 Hz); in the 1D TOCSY experiment, selective excitation of the signal at δ 5.01 led
to the the enhancement only of H-2" (δ 4.03, d, J = 2.0 Hz); and the multiplicity of H-2" may be derived only from the presence of a quaternary carbon at C-3" characteristic of an apiofuranosyl structure. The 13C NMR spectrum gave 11 carbon signals for the sugar moiety, of which three methylenes were ascribable to C-4" (δ 75.4) and C-5" (δ 66.2) of an apiofuranosyl unit and to C-6' (δ 69.0) of a glucopyranosyl unit, respectively. Analysis of the correlated 13C NMR signals in the HSQC spectrum and of the resonances of the quaternary carbon signal (δ 80.5, C-3") matched well with a terminal β-D-apiofuranosyl linked to an inner β-D-glucopyranosyl. C-6' of the glucopyranosyl unit was shifted downfield (β-effect) demonstrating the (1→6) linkage between the apiosyl and glucosyl units. The interglycosidic linkage was also confirmed unambiguously to be at C-6' based on the HMBC cross-peak, between H-1" and C-6'. Correlations due to long-range HMBC couplings were also observed between H-1" and C-1. Therefore, the structure of 1 was determined as 4-methoxyphenol 1-O-β-D-apiofuranosyl-(1→6)-O-β-D-glucopyranosyl (1).

Comparison with the NMR spectral data that showed the NMR signals in 2 were similar to those of 1, except for the presence of an extra O-methyl, suggesting that 2 was a 1,3,4-trisubstituted benzene with two methoxyl groups and one apiofuranosyl(1→6)-glucopyranosyl group. The location of these groups was verified by HMBC and NOE spectra. Therefore, compound 2 was concluded to be 3,4-dimethoxyphenol 1-O-β-D-apiofuranosyl-(1→6)-O-β-D-glucopyranosyl reported previously only in two species Symlocos caudata (Symlocaceae) [5] and Tabebuia impetiginosa (Bignoniaceae) [6].

Compound 3 has the molecular formula C18H26O12, as deduced from ESIMS analysis. The 1H and 13C NMR spectra indicated the presence of 1,2,4-trisubstituted aromatic ring, with one methoxyl group as well as a β-D-apiofuranosyl-(1→6)-O-β-D-glucopyranosyl unit. The spectroscopic data of compound 3 suggested the same skeleton as compound 2, but lacking a methoxyl group. The complete assignment was established by the resonance of C-3 (δ 147.4) shifted upfield by 3.6 ppm and of C-4 (δ 144.7) and C-2 (δ 104.6) shifted downfield by ca. 2.4 and 1.6 ppm with respect to dimethoxylated model (2). The 3-hydroxy, 4-methoxy substitution was also confirmed by HMBC experiments, the correlation peaks of H-2/C-6, C-4; H-6/C-2, C-4, H-5/ C-1, C-3; CH3O−/ C-4; H-1'/C-1 and H-1''/C-6' indicated that benzene ring was substituted at C-1 with the apiofuranosyl-(1→6)-glucopyranosyl unit and the methoxyl group was located at the C-4. Therefore, the structure of 3 was determined as 3-hydroxy, 4-methoxyphenol 1-O-β-D-apiofuranosyl-(1→6)-O-β-D-glucopyranoside, reported previously only in the Indonesian medicinal plant Fagara rhesa (Rutaceae) [7].

The structures and molecular formulas of compounds 4-10 were determined from their ESIMS spectra, as well as from 1D and 2D 1H and 13C NMR data and by comparison of their NMR data with those in the literature. Compound 4 was identified as verbascoside by HPLC comparison with authentic standard and according to its 1H, 13C NMR, and ESI-MS data [8]. The structure of isoverbascoside (5) was confirmed using 1H and 13C NMR. The 1H NMR spectrum of isoverbascoside was similar to that of verbascoside, except for differences in the chemical shifts of H-4' (verbascoside, δ 4.81; isoverbascoside, δ 3.41) and 2H-6 (verbascoside, δ 3.63 and 3.84; isoverbascoside, δ 4.34 and 4.50) in their glucosyl moiety. The 13C NMR chemical shifts of isoverbascoside were close to those of verbascoside, but slight differences were observed in the shifts at C-3', C-4', and C-6' (verbascoside, δ 81.66, 70.69, 62.49; isoverbascoside, δ 84.45, 70.94, 65.20) [9]. Compound 6 showed ESI-MS and 1H and 13C NMR data superimposable with those reported in the literature for leucoceptoside A [10]. Compounds 7-9 were identified as the flavones vitexin, isovitexin and luteolin 8-O-β-D-glucopyranoside, compound 10 as the flavonol spireoside on the basis of their spectroscopic data and specifically by comparison of their NMR data with those in the literature [11].

**Experimental General**

**Experimental Procedure**: A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for 1H and at 150.86 MHz for 13C, was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ H 3.34 and δ C 49.0 for CD3OD; coupling constants, J, are in Hertz. DEPT, 13C, DQF-COSY, HSQC, HMBC and NOESY NMR experiments were carried out using the conventional pulse sequences as described in the literature. Electrospray
ionization mass spectrometry (ESIMS) was performed
using a Finnigan LCQ Deca instrument from Thermo Electron (San Jose, CA) equipped with Xcalibur software. Instrumental parameters were tuned for each investigated compound: capillary voltage was set at 3 V, the spray voltage at 5.10 kV and a capillary temperature of 220°C and the tube lens offset at -60 V was employed; specific collision energies were chosen at each fragmentation step for all the investigated compounds, and the value ranged from 15-33% of the instrument maximum. Data were acquired in the MS1 scanning mode (m/z 150-700). All compounds were dissolved in MeOH : H2O (1:1) and infused in the ESI source by using a syringe pump; the flow rate was 5 μL/min. Exact masses were measured by a Q-TOF premier (Waters, Manifold, MA, USA) instrument. Chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden) employing MeOH as solvent. Column chromatography was carried out employing Silica gel RP18 (0.040–0.063 mm; Carlo Erba) and MeOH:H2O gradients. HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a Kromasil C18 column (250 x 10 mm i.d., 10 μm, Phenomenex). HPLC-grade methanol was purchased from Sigma Aldrich (Milano, Italy). HPLC-grade water (18 Ω) was prepared by a Milli-Q50 purification system (Millipore Corp., Bedford, MA). TLC analysis was performed with Macheray-Nagel precoated silica gel 60 F254 plates.

**Plant Material:** The leaves of *M. obovata* (Kunth) Bureau & K. Schum. were collected in Pico Bonito, Francisco Morazan, Honduras, in August 2005. The plant was identified by Dr. Cirilo Nelson. A voucher specimen was deposited in the herbarium of the Botanical Department of the Universidad Nacional Autonoma de Honduras, Tegucigalpa, Honduras, (Voucher No. 314).

**Extraction and Isolation Procedure of Compounds 1-10:** Dried and powdered leaves (1 kg) of *M. obovata* were extracted for a week, three times, at room temperature using solvents of increasing polarity; namely, petroleum ether, chloroform, and methanol. Part (3 g) of MeOH extract was chromatographed on a Sephadex LH-20 column (100 cm x 5.0 cm) using CH3OH as mobile phase and a flow rate of 1 mL/min to furnish 6 fractions (I-VI). Fraction II and III (258.4 mg) were purified by RP-HPLC (40% CH3OH) to give 2 (9.5 mg) and 3 (4.9 mg) and 1 (9.2 mg). Fr. IV (184.1 mg) was purified by RP-HPLC (35% CH3OH) to give 4 (16.8 mg), 5 (6.4 mg) and 6 (3.8 mg). Finally Fr. V and VI containing flavones were purified with 70:30 MeOH-H2O to yield compounds 7 (15.1 mg), 8 (12.2 mg), 9 (6.1 mg) and 10 (7.2 mg).

**References**


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**Phenolic compounds from Martinella obovata**

4-methoxyphenol 1-O-β-d-apiofuranosyl-(1→6)-O-β-d-glucopyranoside (1)
White amorphous solid. 
[α]D: -58.9 (c 0.20, MeOH).

UV/Vis λmax (MeOH) nm (log ε): 202 (4.42), 223 (3.85), 279 (3.42).

1H and 13C NMR data were consistent with those previously reported [10]. ESI-MS m/z 433 [M-H]-, 447 [M-H]-, m/z 301 [M-132]- and m/z 139 [M-132-162]-

HREIMS m/z 448.2560 (calcld for C19H28O12, 448.4430).

3,4-dimethoxyphenol 1-O-β-d-apiofuranosyl-(1→6)-O-β-d-glucopyranoside (2)

1H and 13C NMR data were consistent with those previously reported [4].

ESI-MS m/z 447 [M-H]-, m/z 315 [M-132]- and m/z 153 [M-132-162]-

HREIMS m/z 448.2560 (calcld for C19H28O12, 448.4430).

3-hydroxy, 4-methoxyphenol 1-O-β-d-apiofuranosyl-(1-6)-O-β-d-glucopyranoside (3)

1H and 13C NMR data were consistent with those previously reported [7].

ESI-MS m/z 433 [M-H]-, m/z 301 [M-132]- and m/z 139 [M-132-162]-

HREIMS m/z 434.3177 (calcld for C18H26O12, 434.7100).

**Verbascoside (4)**

1H and 13C NMR data were consistent with those previously reported [8]. ESI-MS m/z 621 [M-H]-.

**Isoverbascoside (5)**

1H and 13C NMR data were consistent with those previously reported [9]. ESI-MS m/z 621 [M-H]-.

**Leucoceptoside A (6):** 1H and 13C NMR data were consistent with those previously reported [10]. ESI-MS m/z 635 [M-H].

**Vitexin (7)**

1H and 13C NMR data were consistent with those previously reported [11]. ESI-MS m/z 431 [M-H]-.

**Isovitexin (8)**

1H and 13C NMR data were consistent with those previously reported [11]. ESI-MS m/z 431 [M-H].

**Luteolin 8-C-β-d-glucopyranoside (9)**

1H and 13C NMR data were consistent with those previously reported [11]. ESI-MS m/z 447 [M-H].

**Spireoside (10)**

1H and 13C NMR data were consistent with those previously reported [11]. ESI-MS m/z 463 [M-H].


Phenolic Chemical Composition of *Petroselinum crispum* Extract and Its Effect on Haemostasis

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From the aqueous extract (Pc) of *Petroselinum crispum* (Mill) flat leaves specimens were isolated and identified the flavonoids apigenin (1), apigenin-7-O-glucoside or cosmosiin (2), apigenin-7-O-apisyl-(1→2)-O-glucoside or apin (3) and the coumarin 2''''3''''-dihydroxy-furanocoumarin or oxypeucedanin hydrate (4). The inhibitory activity toward clotting formation and platelet aggregation was assessed for Pc flavonoids (1) and (2), and the coumarin (4). Pc showed no inhibition on clotting activity when compared with the control. On the other hand, a strong antiplatelet aggregating activity was observed for Pc (IC\(_{50} = 1.81\) mg/mL), apigenin (IC\(_{50} = 0.036\) mg/mL) and cosmosiin (IC\(_{50} = 0.18\) mg/mL). In all cases ADP was used as inductor of platelet aggregation. Our results showed that Pc, apigenin and cosmosiin interfere on haemostasis inhibiting platelet aggregation. To the best of our knowledge this is the first report for the cosmosiin antiplatelet aggregation *in vitro* activity.

**Keywords:** *Petroselinum crispum*, parsley, flavonoids, cosmosiin, cardiovascular disease, haemostasis, platelet aggregation.

The species *Petroselinum crispum*, known as parsley, is an aromatic herb from Apiaceae family that has been employed in food, pharmaceutical, perfume and cosmetic industries [1]. Widespread in all continents, parsley may be one of the oldest herbs used as condiment in food. Previous studies on the chemical composition of parsley have revealed the presence of flavonoids [2-5], coumarins [6-8] and terpenes [9,10]. In popular medicine, parsley is used to treat various illnesses such as Alzheimer’s disease, thrombosis and strokes [11,12]. In Morocco and Brazil, parsley is widely employed against cardiovascular diseases [12-14].

The ethnopharmacological knowledge is useful to identify potential therapeutic targets from medicinal plants. Substances from vegetal kingdom, which have already contributed with several compounds in prophylaxis and treatment of a large variety of pathologies, have been investigated for their potential as antithrombotic agents [15,16].

Aspirin, an antiplatelet agent and warfarin, an oral anticoagulant were developed from secondary metabolites. However, aspirin consumption can increase the risk of gastrointestinal bleeding and other adverse effects [16]. Antiplatelet and anticoagulant drugs are used to treat cardiovascular diseases and strokes preventing or slowing down blood clots formation and enlargement of existing blood clots. [17].

According to World Health Organization (WHO), in the next 20 years there will be 24 million deaths from cardiovascular diseases [18]. The current spending with antithrombotic treatment is extremely high [19]. Many antithrombotic substances from synthetic or semi-synthetic origin or derived from natural products are currently under clinical evaluation (phases I, II, III or IV). About 450 substances are considered promising candidates as new antithrombotic drugs in the Stroke Trials Registry [20].
This study led to the isolation of known 5,7,4’-trihydroxy-flavone (apigenin), apigenin 7-O-glucoside (cosmosin), apigenin 7-O-apiosyl-(1→2)-O-glucoside (apiin) and 2”,3”-dihydroxyfuranocoumarin (oxypeucedanin hydrate) identified according to reported NMR data [21,22]. These flavones and the coumarin derivative were previously described for this species [4,6]. Two other flavones diosmetin apiosyl-glucoside and diosmetin apiosyl-glucoside isomer were identified with basis on HPLC-DAD chromatogram and comparison with literature data [4]. Apiin is the most abundant flavonoid in parsley, while apigenin is the minor component as reported previously in the literature [3].

In experiments assessing the intrinsic pathway (aPTT), and the extrinsic pathway (PT) of coagulation, Pc extract, apigenin, cosmosin and oxypeucedanin hydrate did not show any significant activity, since the clotting time was not significantly increased. Figures 2, 3 and 4 show the antiplatelet aggregation effect observed for Pc, apigenin and cosmosin, respectively. A strong antiplatelet aggregation activity was observed for Pc (IC50 = 1.81 mg/mL), apigenin (IC50 = 0.036 mg/mL) and cosmosin (IC50 = 0.18 mg/mL).

Our results confirm the already known antiplatelet activity reported for Pc and apigenin [12,23]. Cosmosin exhibited a significant antiplatelet activity, although less active than apigenin. We can deduce from our findings that the presence of glycosylation decreases the activity of cosmosin, since the apigenin skeleton is common to both structures [24].

Studies on the medicinal species P. crispum (Pc) showed that its aerial parts aqueous extract was able to inhibit platelet activity induced by ADP [13]. In these studies, a crude extract at 10 mg/mL inhibited the platelet aggregation by 78%. In our study we observed that Pc leaves extract significantly inhibited (IC50 = 1.81 mg/mL) the platelet aggregation induced by ADP in human platelet–rich plasma. Moreover, at concentrations 2.6 times lower (3.80 mg/mL) than that used by Mekhfi et al. (2004) we obtained a higher platelet aggregation inhibition (94.4%) [13].

A possible explanation for the difference in these results should be due to the different extraction procedures employed in both studies. We prepared a decoction from fresh leaves at 10% (w/v), while Mekhfi et al. (2004) prepared an infusion at 5.5% (w/v) without mention if fresh or dried aerial parts were used. The variety of P. crispum was not mentioned by those authors, while we used the flat leaf specimens. Furthermore, other factors such as cultivation conditions, sunlight exposure and season may lead to differences in the production of bioactive secondary metabolites [25].
platelet aggregation induced by ADP [12,13]. Our results showed that Pc extract, apigenin and cosmosiin interfere on haemostasis inhibiting platelet aggregation. To the best of our knowledge this is the first report for the cosmosiin in vitro-antiplatelet aggregation activity. The study of apiin and the coumarin oxypeucedanin hydrate effect on the coagulation process is undergoing in our laboratories.

Experimental

Chemical

General: Melting points were determined using a Koppler melting point apparatus. Optical rotations were measured on a Jasco P-2000 digital polarimeter. All 1D and 2D experiments were performed on a Varian 400 MHz spectrometer. The NMR spectra were recorded in DMSO-d$_6$. ESI-MS spectra were recorded on a tandem – triple quadrupole m/z 30-3000. HPLC separation was performed using a Shimadzu liquid chromatograph LC-10AD equipped with an UV SPD-10A wavelength detector. The reversed-phase column used was Merck C18 (5 µm, 250 mm, 2.5 mm) with mobile phase consisted of water containing phosphoric acid 0.01% (eluent A) and methanol (eluent B). The samples were run for 44 minutes at 1 mL/min and absorbance was monitored between 200 – 500 nm. The gradient used was 0 – 5 min (100 – 65% A), 5 – 15 min (65 – 55% A), 15 – 25 min (55 – 52% A), 25 – 35 min (52 – 45% A), 35 – 40 min (45 – 20% A), 40 – 42 min (20 – 0% A) and 42 – 44 min (0 – 100% A). Thin layer chromatography (TLC) was performed on silica gel 60 F$_{254}$ (Merck) eluted with n-butanol/acetic acid/water (BAW) 8:1:1, visualized under UV light (254 and 365 nm) and developed with ceric sulfate solution for flavonoids. Coumarin was detected using 5% potassium hydroxide solution in ethanol.

Plant material: Leaves from Petroselinum crispum (Mill.) Nym.ex A.W. Hill (flat leaf specimens) were collected out of blooming season from specimens grown in an experimental garden at Severino Sombra University (Vassouras, RJ, Brazil). A voucher specimen (RFA – 31241) was classified by Dr. Ricardo C. Vieira and deposited in the herbarium of the Institute of Biology, UFRJ, Brazil.

Extraction and isolation: Fresh leaves (160 g) were triturated using a food processor and extracted with distilled water (10% w/v) by decoction (10 minutes). After the extract filtration, a spontaneous precipitation at room temperature yielded a solid that was separated by centrifugation. This precipitate (1.6 g) was re-suspended in methanol and chromatographed over Sephadex LH-20 (30 x 1.5 cm; MeOH) yielding 2 fractions: a minor methanol-soluble fraction (396.2 mg) and a methanol-insoluble one (1.2 g). Each fraction was purified over Sephadex LH-20 (23 x 0.7 cm; MeOH/H$_2$O 1:1) affording cosmosiin (2), for the soluble fraction, as a yellow powder (23.4 mg) and apiin (3), for the insoluble fraction as a white powder (676.8 mg). After the precipitate separation the extract was filtered (Whatman filter paper Nr.1), frozen at -20°C and lyophilized (1.8 g). After its re-suspension in water, the resulting solution was partitioned successively with ethyl acetate (3 x 300 mL) and n-butanol (3 x 300 mL). The ethyl acetate fraction (301.0 mg) was chromatographed over Sephadex LH-20 (23 x 0.7 cm; ethanol) affording three fractions. The second fraction (64.8 mg) showed a yellow crystalline solid (22.3 mg) that was separated by centrifugation and identified as apigenin (1). The third fraction was purified on an RP-2 column (30 x 1.2 cm; H$_2$O/EtOH), followed by a Sephadex LH-20 chromatography (23 x 0.7 cm; MeOH/H$_2$O 1:1), affording oxypeucedanin hydrate (4) as a brown powder (15.4 mg).

Biological

In vitro determination of activated partial thromboplastin time (aPTT) and prothrombin time (PT): Blood samples were centrifuged (2000 x g, 10 min), and the platelet-poor plasma was stored at -20°C until use. aPTT and PT were measured on an Amelung KC4A coagulometer as follows. For aPTT tests, cephalin plus kaolin (aPTT reagent, BioMérieaux, RJ, Brazil) were incubated for 1 min with 50 µL of pre-warmed plasma (37°C) and P. crispum (Pc), apigenin, cosmosiin and oxypeucedanin hydrate at various concentrations (suspension in PBS buffer). The reaction was started by addition of 100 µL of pre-warmed CaCl$_2$ (25 mM).

For PT tests, 50 µL of pre-warmed plasma was incubated with Pc extract, apigenin, cosmosiin and oxypeucedanin hydrate at various concentrations (suspension in PBS buffer) for 2 min (37°C) and reaction was started by addition of 100 µL of pre-warmed thromboplastin with calcium (PT reagent, BioMérieaux, RJ, Brazil). Apiin was not evaluated since it is insoluble in water.

Platelet aggregation assays: Human blood was collected in EDTA 0.2 M (9:1 v/v). Platelet-rich plasma (PRP) was prepared by centrifugation (500 x g, 10 min) at room temperature. The platelet-poor plasma (PPP) was prepared by centrifugation of the PRP (2000 x g, 10 min) at room temperature). In some cases, experiments were performed using washed platelets as described [28]. Platelet aggregation was monitored by the turbidimetric method on a Chrono-Log aggregometer. PRP (400 µL) was incubated (37°C, 1 min) with continuous stirring at 900 rpm. Platelet aggregation was induced by ADP (2-10 mM). P. crispum extract (Pc), apigenin, cosmosiin and oxypeucedanin hydrate at various concentrations (suspension in PBS buffer) or vehicle (0.5% DMSO v/v) was added to PRP samples 1 min before addition of the agonist.

Supplementary data: Compounds from Petroselinum crispum (Mill.) Nym.ex A.W. Hill, an aromatic herb popularly known as parsley.

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References


Bioactivities of *Chuquiraga straminea* Sandwith

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Methanolic extracts of *Chuquiraga straminea* Sandwith, subfamily Barnadesioideae (Asteraceae) showed the presence of quercetin-3-O-glucoside, quercetin-3-O-rutinoside, kaempferol, kaempferol-3-O-glucoside and kaempferol-3-O-rutinoside. Antioxidant and antimicrobial activity was determined. The total extracts showed antioxidant activity by DPPH and ABTS method (SC\(50\) 14.5 to 34.9 \(\mu\)g/mL). A significantly positive correlation was observed between the antioxidant activity and the total phenolics (R\(^2\)>0.93). The extracts were active against ten methicillin resistant and sensitive *Staphylococcus aureus* strains isolated from nosocomial infection (MIC values between 200 to 800 \(\mu\)g/mL). These preliminary studies are highly interesting as they open new ways for further applications in the treatment of infections by methicillin resistant *S. aureus*.

**Keywords:** Flavonoids, *Chuquiraga straminea*, Antioxidant activity, Antimicrobial activity.

From an estimated 250,000 higher plants in the world only 5-15% have been studied for a potential therapeutic value. The Argentinian flora offers great possibilities for the discovery of new compounds with medicinal uses. The genus *Chuquiraga* is represented in Argentina by 15 species distributed in arid regions between the Andes and Patagonia. Previous reports indicated that the flavonoids identified in the species of *Chuquiraga* genus are identical, so the compounds are useful as phylogenetic micromolecular markers in the genus [1]. *Chuquiraga straminea* is a medium xerophytic shrub. Its distribution is in southern Argentina, from northwestern Chubut province to eastern Neuquen province. It inhabits in Patagonian phytogeographic province between 600 and 1000 meters above sea level (masl). This species has been employed as traditional medicine by native people, either as building and crafts material and forage [2].

It is reported that phenolic compounds from herbs are active against many human pathogenic bacteria and fungi and have antioxidant activity [3-5]. Recently, there has been considerable interest in the use of such antioxidants and antimicrobial compounds from natural sources, not only in pharmaceutical industry but also for the preservation of foods and improving the shelf life of food products, for increasing the stability of fats and oils and to control the plant diseases of microbial origin. *Staphylococcus aureus* is a common pathogen associated with serious community and hospital acquired diseases and has long been considered a major problem of Public Health. The aim of this study was to investigate antioxidant and antimicrobial activities of *C. straminea* extracts obtained from aerial parts and flowers.

Extracts were analyzed for their contents of phenolic compounds (total phenols, flavonoids). A relationship between antimicrobial activity, antioxidant activities and the content of phenolic compounds was evaluated.

The content of total phenols and flavonoids of flower and aerial parts extracts from *C. straminea* are given in Table 1. The amount of total phenolic compounds extracted ranged from 1.56 to 2.74 mg gallic acid equivalent (GAE)/mL extract. The *C. straminea* aerial parts extracts contained the highest amounts of phenolic compounds.

<table>
<thead>
<tr>
<th>Alcoholic Extracts</th>
<th>Phenolic compounds (mg GAE/mL)</th>
<th>Flavonoids (mg QE/mL)</th>
<th>Compounds isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flowers</td>
<td>1.56±0.04</td>
<td>0.12±0.01</td>
<td>quercetin-3-O-glucoside, quercetin-3-O-rutinoside, kaempferol, kaempferol-3-O-glucoside and kaempferol-3-O-rutinoside</td>
</tr>
<tr>
<td>Aerial Parts</td>
<td>2.74±0.08</td>
<td>0.14±0.01</td>
<td>kaempferol-3-O-rutinoside</td>
</tr>
</tbody>
</table>

Kaempferol, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, kaempferol-3-O-glucoside and kaempferol-3-O-rutinoside were identified in the extracts. The flavonoid
glycosides are comparable with those previously isolated, by us from other argentine species belonging to *Chuquiraga* genus, [1,6].

**Antioxidant activity:** Free radical species play a critical role in cardiovascular and inflammatory diseases as well as in neurodegenerative disorders, cancer and aging. The *C. straminea* extracts were effective as ABTS [2,2’-azinobis (3-ethylbenzothiazoline-6 sulfonic acid) diammonium salt] and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavengers. The results obtained are represented in Figure 1 (SC50 values denote the sample concentration required to scavenge 50% ABTS or DPPH free radicals). Aerial parts extracts were the most effective radical-scavengers with SC50 of 18.0±0.7 and 14.5±0.79 µg GAE/mL for DPPH and ABTS respectively, flowers extracts result also active with SC50 of 34.5±1.2 and 31.0±1.1 µg GAE/mL for DPPH and ABTS radicals, respectively.

A significantly positive correlation was observed between the antioxidant potential determined by ABTS and DPPH assays, and the total phenolics (R2>0.93).Contact autography, indicated that more than one compounds with antioxidant activity in all crude extracts. The flavonoids could be the more active metabolites in this plant specie.

**Antimicrobial activity:** The antibiotic resistant clinical *S. aureus* strains assayed in this work were isolated from human infections from a local hospital.

By means of bioautographic assay was qualitatively demonstrated that several phenolic compounds in the methanolic extracts were active against methicillin resistant and sensitive *S. aureus* strains. Table 2 show the antimicrobial activity of *C. straminea* methanolic extracts against ten *S. aureus* antibiotic resistant and sensitive strains.

The two methanolic extracts of *C. straminea* were active against all methicillin resistant and sensitive *S. aureus* strains (MRSA and MSSA, respectively) and the observed differences between minimal inhibitory concentration (MIC) values with respect to the control strain were not significant. Similar behavior was observed for methicillin sensitive and resistant *Staphylococcus coagulase* negative, MSSC: methicillin sensitive *Staphylococcus coagulase* negative, ATCC: American Type Culture Collection, r and s, resistance or susceptibility to antibiotics. Met, methicillin; Ox, oxacillin; Gen, gentamicin; Van, vancomycin. MIC was defined as the lowest concentration of extract that had restricted growth to a level <0.05 at 550nm. MBC (minimal bactericidal concentration) was defined as the lowest extract concentration at which 99.9% of the bacteria have been killed.

Differences between minimal inhibitory concentration (MIC) values with respect to the control strain were not significant. Similar behavior was observed for methicillin sensitive and resistant *Staphylococcus coagulase* negative, MSSC: methicillin sensitive *Staphylococcus coagulase* negative, ATCC: American Type Culture Collection, r and s, resistance or susceptibility to antibiotics. Met, methicillin; Ox, oxacillin; Gen, gentamicin; Van, vancomycin. MIC was defined as the lowest concentration of extract that had restricted growth to a level <0.05 at 550nm. MBC (minimal bactericidal concentration) was defined as the lowest extract concentration at which 99.9% of the bacteria have been killed.

The reported data in the present work for *S. aureus* was similar to those obtained for other *Chuquiraga* species that grow in arid regions of Argentine [5].

Up to the present, there are limited reports about the bioactivities of *C. straminea*. The ethnobotanical data indicated that this species was used by mapuches and tehuelches as the main therapeutic tool in traditional
medicine as antinflammatory and antiseptic on skin infection [2]. According with our results the C. straminea extracts could serve as good candidates for the development of new antimicrobial and antioxidant agents and/or standardized phytomedicines.

**Experimental**

**Plant material:** The plant was collected in the province of Neuquén, Department Collan Cura. Grow in patches on the slope of Piñon Cerrito, on rocky ground. A voucher specimen is deposited at the Fundación Miguel Lillo Herbarium (LIL 605812).

**Preparation of plant extracts:** The aerial parts (flowers and leaves) of C. straminea were dried and extracted with methanol 80%. The solvent was evaporated at reduced pressure and then, resuspended in DMSO (dimethylsulfoxide) for avoid the extraction solvent interference in the biological screening.

**Compounds identification:** The extracts were chromatographed bidimensionally according to Mabry [9] using TBA (tert-butanol-acetic acid-water 3:1:1) and 15% AcOH as development solvents. Eluted spots were analyzed by paper and thin layer chromatography in different solvent systems. The plates were observed under ultraviolet light, in the absence and presence of ammonia and natural product reagent. Spectral data with shift reagents, NaOMe (sodium methoxide), AlCl3 (aluminum trichloride), HCl (hydrochloric acid), NaOAc (sodium acetate), H3BO3 (boric acid) were used.

**Free radical scavenging activity**

**The DPPH method:** The reduction capability of extracts was measured by DPPH method according to Zampini et al [10]. DPPH solution (1.5 mL of 300 μM in 96% ethanol) was incubated with the samples (5-50 μg GAE). The reaction mixture was shaken and incubated during 20 min. at room temperature. Then, absorbance was measured at 515 nm.

The percentage (%) of radical scavenging activity (RSA) was calculated using the following equation:

\[ RSA \% = \left[ \frac{(A_0 - A_t)}{A_0} \right] \times 100 \]

Where \( A_0 \) is the absorbance of the control and \( A_t \) is the absorbance of the samples at 515 nm. SC\(_{50} \) values denoted the sample concentration required to scavenge 50% DPPH free radicals.

**Antimicrobial activity**

**Bioautographic assays:** Extracts (25 μg) were seeded on TLC plates and the components were separated using chloroform-ethyl acetate (80:20; v/v) as development solvents. Then, the plates were dried overnight and covered with 3 mL of soft medium (agar 1% containing 1 mL ABTS\(^+\) (7 mM ABTS and 2.45 mM potassium persulfate) or DPPH (1mg/mL)). Plates were incubated at room temperature during 1 minute in the dark. Active samples appeared as light spots against a green-blue or purple background for ABTS or DPPH assay, respectively [12].

**Microorganism:** The microorganisms used in this study consisted of ten Staphylococcus aureus strains recovered from clinical samples obtained from the Hospital Nicolás Avellaneda, San Miguel de Tucumán, Tucumán, Argentina: methicillin resistant S. aureus (MRSA) (n=3), methicillin sensitive S. aureus (MSSA) (n=3), methicillin resistant S. coagulase negative (MRSCN) (n=2) and methicillin sensitive S. coagulase negative (MSSCN) (n=2). A reference strain was included in the study: S. aureus ATCC 29213.

**Antibiotic susceptibility assay**

**Broth microdilution susceptibility assay:** This assay was performed in sterile 96-well microplates. The extracts were transferred to each microplate well in order to obtain two-fold serial dilutions of the original extract (25 to 800 μg/mL). The inoculum (100 μL) containing 5×10\(^7\) CFU...
was added to each well. A number of wells were reserved in each plate for sterility control (no inocula added), inocula viability (no extract added), and solvent effect (DMSO) [14].

Plates were aerobically incubated at 35°C. After incubation for 16-20 h, bacterial growth was indicated by the presence of turbidity and a pellet on the well bottom. A cytotoxicity assay was also carried out. After the broth microdilution susceptibility assay, 20 µL of methylthiazolyltetrazolium chloride solution (MTT) (12 mg/mL in PBS) was added to the wells and incubated for 1 h. Cellular viability was determined by absorbance at 550 nm.

MIC was defined as the lowest concentration of extract that had restricted growth to a level <0.05 at 550nm (no macroscopically visible growth).

To confirm MIC and to establish MBC, 10 µL of each culture medium was removed from each well with no visible growth and inoculated in Müller Hinton Agar plates. After 16-20 h of aerobic incubation at 35°C, the number of surviving organisms was determined.

MBC was defined as the lowest extract concentration at which 99.9% of the bacteria have been killed.

MIC values were also determined for different commercial antibiotics. Resistance was defined for each case: methicillin (Met, MIC > 16 µg/mL), oxacillin (Oxa, MIC > 16 µg/mL), gentamycin (Gen, MIC > 100 µg/mL) and vancomycin (Van, MIC > 6 µg/mL) for S aureus strains. All experiments were carried out in triplicate.

Statistical analysis: Data are represented as mean ± standard deviation. The statistical tests were carried out by analysis of variance (one-way ANOVA) and the post-test of Turkey, using a probability level of less than 5% (p < 0.05).

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References


Free Radical Scavenging Activity, Determination of Phenolic Compounds and HPLC-DAD/ESI-MS Profile of Campomanesia adamantium Leaves

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Numerous diseases are induced by free radicals via lipid peroxidation, protein peroxidation and DNA damage. It has been known that a variety of plant extracts have antioxidant activity to scavenge free radicals. \(\text{Campomanesia adamantium}\ (\text{Myrtaceae})\) is a small tree with edible fruit, commonly known as “guavira” or “guabiroba-branca” that has been used in popular medicine as depurative anti-diarrhoeic, anti-inflammatory, anti-rheumatic and to liver diseases. In this study, the antiradical activities of ethanol crude extract of the leaves from \(\text{C. adamantium}\) and the ethyl acetate and butanol fractions obtained by partition, were determined using DPPH (2,2-Diphenyl-1-picrylhydrazyl radical) and ORAC-FL (Oxygen Radical Absorbance Capacity) assays. The total phenol content in the samples was estimated by Folin Ciocalteau method (FCR). In an initial evaluation the ethanolic extract and the fractions ethyl acetate and butanol have shown levels of phenolic compounds between 15- 74 mg GAE/g in FCR assay, showed DPPH free-radical scavenging activity with SC50 in the range of 7.77-13.35 µg/mL and demonstrated antioxidant capacity between 2648-3502 µmol TE/g of extract and fractions in the ORAC-FL assay. HPLC-DAD and ESI-MS analysis revealed that the extract of the leaves of \(\text{C. adamantium}\) studied appears to contain flavonoids as major constituents, including isoquercetrin and quercetin that exhibit proven antioxidant activity.

**Keywords:** radical scavenger, DPPH, Myrtaceae, \(\text{Campomanesia adamantium}\), HPLC-DAD/ESI-MS.

The organic and inorganic molecules and atoms that contain one or more unpaired electrons, with independent existence, can be classified as free radicals [1]. Free radicals are responsible for lipid peroxidation occurred during production and storage of nutrients [2], and are directly involved in some cancers, cardiovascular disorders, diabetes [3], Alzheimer’s disease, atherosclerosis and others human pathologies [4]. The radicals \(\text{O}_2\)\(^*\) and their reduction products, \(\text{H}_2\text{O}_2\), and especially the radical \(\text{OH}\)\(^*\), are some of those responsible for cell damage by promoting lipid peroxidation, with damage to mitochondria, lysosomes and cell membrane itself, leading to cell death. In animal, different biochemical routes involve free radicals formation, but in these cases defense mechanisms against the oxidative process propagation are also involved. These mechanisms do not show a constant efficacy [3]. However, exogenous antioxidant compounds act as an auxiliary function in this defense processes. Antioxidants block the free radicals formation through different ways and establish important control function in some oxidative stress diseases [4] and in food conservation [5]. Thus, new natural antioxidants, mainly those isolated from medicinal plants, acquire great pharmacological importance and the research on these classes of compounds has been increased in the last years [6-8].

The genus \(\text{Campomanesia}\) (Myrtaceae) comprises around 30 species of shrubs or small trees, aromatic, distributed mainly in tropical and subtropical South America [9]. Most species produce edible fruits that are widely used to make liqueur, juices and jellies [10]. Several species are considered medicinal and have been used in folk medicine mainly against digestive problems and diarrhea [11]. \(\text{Campomanesia adamantium}\) Camb. is a small tree, known as “guavira” or “guabiroba-branca”, largely spread in Brazil. It can be found growing wild in the Midwest, Southeast and South regions of Brazil, and frequently is cultivated in home gardens for its fruits [12]. Its leaves and fruits have been used against rheumatism, liver and urinary diseases [13]. Previous phytochemical studies in \(\text{Campomanesia}\) have reported the identification of quercetin, myricetin and rutin in \(\text{C. xanthocarpa}\) [14] and \(\beta\)-triketone type compounds, named champanones in \(\text{C. lineatifolia}\) [15]. Recently it was reported the isolation of...
flavonoids in *C. adamantium* [16,17] as well as the antioxidant activity of extracts and fractions [17,18]. However, these studies were carried on specimens growing in Midwest region that can have a chemical profile different from those growing in other regions of country. These facts prompted us to investigate the antioxidant capacity of the ethanolic extract and fractions of *C. adamantium* from South region of Brazil and characterize the major constituents responsible for antioxidant activity.

The samples analyzed in the present study showed a total phenol content in the range of 15.78 – 74.83 mg GAE/g extract (Table 1). Phenolic compounds are recognized as one of most important class responsible for antioxidant capacity in plants [19].

Ethanol extract, ethyl acetate and butanol fractions exhibited antioxidant activity concentration-dependent in DPPH assays, with SC50 varying from 7.77 to 13.35 µg/mL. The highest antioxidant activity was exhibited by butanol fraction. In ORAC-FL kinetic assay, based on hydrogen transfer mechanism, the extracts showed antioxidant capacity between 2648 and 3502 µM of TROLOX equivalent per gram of extract (Table 1). In comparison with previous studies [16,18], our extracts and fractions showed higher antioxidant activity and there were chemical differences between *C. adamantium* leaves analyzed in this study of South region of Brazil and *C. adamantium* growing in Midwest region of Brazil [16-18]. The chalcones were previously reported in *C. adamantium* growing in Midwest region of Brazil [16], while the flavonols isoquercitrin, myricetin, quercitrin and quercetin are being reported for the first time in this plant. Myricetin had already been identified in *C. xanthocarpa* [14].

To confirm the presence of the flavonoid isoquercitrin, we made the HPLC-UV/DAD analysis of standard isoquercitrin and of the crude extract. We noted the formation of a peak with a retention time coincident with the same pattern and absorption peaks. Furthermore, the identity of major constituent isoquercitrin was also confirmed through co-elution with authentic standard sample. These results are enough to confirm that one of the major constituents and the responsible for antioxidant activity is the flavonoid isoquercitrin, since that the same mass was also found in the analysis of TLC spot yellow sample that was revealed with solution of DPPH.

Thus, the results of the present study suggest that the antioxidant capacity of *C. adamantium* is correlated to the content of flavonoids, including isoquercitrin, which is present in the crude ethanolic extract and TLC spot yellow sample. Moreover, this activity presents a positive correlation with the total phenolic soluble content measured by FCR assay. However, further investigations are necessary to confirm if this plant and its constituents represent a source of powerful antioxidant products useful *in vivo*.

### Experimental

**Plant Material:** The leaves of *Campomanesia adamantium* were collected from wild specimen growing in Curitiba, Paraná State, Brazil (25°25'48” S, 49°16’15” W) at 934 m of altitude. The plant was identified by Dr. Armando Carlos Cervi, which deposited a voucher specimen at the herbarium of UFPR (UPCB 60503).

**Extracts preparation:** The powder was subjected to the process of maceration with ethanol at a ratio of powder / solvent of 1:5 (weight / volume). The ethanolic crude extract was suspended in methanol/water (9:1, v/v) and fractionated by liquid-liquid extraction with hexane and ethyl acetate. The hydroalcoholic phase remaining was partitioned with n-butanol and water to afford an n-butanol-soluble portion. This procedure yielded the fractions of hexane (Hex), ethyl acetate (EtOAc) and butanol (BuOH).

### Table 1: Total phenol content and antioxidant capacity by the DPPH and ORAC assays of ethanol extract of *Campomanesia adamantium* leaves and its fractions ethyl acetate (EtOAc) and butanol (BuOH).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Phenol contenta (mg of GAE/g)</th>
<th>DPPH assayb SC50 (µg/mL)c</th>
<th>ORACd TE/gd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol extract</td>
<td>35.04 (5.48)</td>
<td>13.00 (5.03)</td>
<td>2648 (1.77)d</td>
</tr>
<tr>
<td>EtOAc fraction</td>
<td>74.83 (10.77)</td>
<td>13.35 (16.85)</td>
<td>3502 (5.71)d</td>
</tr>
<tr>
<td>BuOH fraction</td>
<td>15.78 (15.29)</td>
<td>7.77 (5.00)</td>
<td>3150 (6.66)d</td>
</tr>
<tr>
<td>Quercetin*</td>
<td>-</td>
<td>12.80 (2.00)</td>
<td>5.62 (0.89)e</td>
</tr>
<tr>
<td>Isoquercitrin*</td>
<td>-</td>
<td>-</td>
<td>5.21 (1.60)e</td>
</tr>
<tr>
<td>Trolox*</td>
<td>-</td>
<td>2.55 (1.40)</td>
<td>-</td>
</tr>
</tbody>
</table>

*Experimental positive controls.

- not evaluated.

*aMean (%RSD, relative standard deviation) of triplicate assays.

*bTotal phenolics data expressed as milligrams of gallic acid equivalents per gram (mg of GAE/g) of extract or fractions.

*cDPPH assay data expressed as SC 50 (concentration that inhibited 50% of the DPPH radical) in micrograms per milliliters (µg/mL).

*dORAC data expressed as relative Trolox equivalent, mean (%RSD, relative standard deviation) of triplicate assays.
The antioxidant activity was expressed as the calculated Trolox equivalent for pure compounds. The analyses were performed in triplicate.

**Evaluation of antioxidant capacity by ORAC assay:** The antioxidant capacity of the ethanolic extract and its fractions, dissolved in ethanol, were analyzed for their total soluble phenolic content according to the Folin-Ciocalteau colorimetric method [24]. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract or fraction (mg of GAE/g). The analyses were performed in triplicate.

**HPLC-UV/DAD/ESI-MS profile:** The TLC yellow spot sample, crude extracts and fractions of *C. adamantium* leaves were diluted in a solution containing 50% (v/v) methanol (chromatographic grade), 50% (v/v) deionized water and, 0.5% of ammonium hydroxide (Merck, Darmstadt, Germany). In the fingerprinting ESI-MS analysis, the general conditions were: source temperature of 100 °C, capillary voltage of 3.0 kV and cone voltage of 30 V. For measurements were performed according to the described in the literature [25]. The yellow spot was scraped, dissolved in methanol, filtered and this was called TLC yellow spot sample.

**Radical scavenging activity by DPPH assay:** The antiradical activity of extract and fractions of EtOAc and BuOH were determined using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) [22]. Fifty microliters of a 250 μM DPPH solution in ethanol was added to a range of solutions of different concentrations (seven serial 3-fold dilutions to give a final range of 100 to 1.6 μg mL⁻¹) of extracts to be tested in ethanol (10 μL). Absorbance at 517 nm was determined 20 min after the addition of each of the compounds tested, and the percentage of activity was calculated. Quercetin and Trolox were used as positive compounds tested, and the percentage of activity was expressed as the percentage of activity of extract and fractions tested in ethanol (10 μL). All samples were tested in triplicate.

**Separation and analysis of antioxidants on thin layer chromatography:** The sample was applied on TLC and eluted with BAW (butanol: acetic acid: water), after that we sprayed with a solution 500 µg/mL of DPPH in ethanol. After solvent evaporation (about 5 minutes), the potential anti-free radical was verified by the appearance of yellow spots on violet background, according to the described in the literature [25]. The yellow spot was scraped, dissolved in methanol, filtered and this was called TLC yellow spot sample.

**Quantitative determination of total soluble phenols:** The dried ethanolic extract and its EtOAc and BuOH fractions, dissolved in ethanol, were analyzed for their total soluble phenolic content according to the Folin-Ciocalteau colorimetric method [24]. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract or fraction (mg of GAE/g). The analyses were performed in triplicate.

**HPLC-UV/DAD/ESI-MS profile:** The TLC yellow spot sample, crude extracts and fractions of *C. adamantium* leaves were diluted in a solution containing 50% (v/v) methanol (chromatographic grade), 50% (v/v) deionized water and, 0.5% of ammonium hydroxide (Merck, Darmstadt, Germany). In the fingerprinting ESI-MS analysis, the general conditions were: source temperature of 100 °C, capillary voltage of 3.0 kV and cone voltage of 30 V. For measurements were performed according to the described in the literature [20,21]. Structural analysis of single ions in the mass spectra from extract and fractions of interest was selected and submitted to 25 eV collisions with argon in the collision quadrupole. The compounds were identified by comparison of their ESI-MS/MS fragmentation spectra.
Statistical analysis: Data are reported as mean (%RSD, relative standard deviation) of triplicate determinations. The statistical analyses were carried out using the Microsoft Excel 2002 software package (Microsoft Corp., Redmond, WA)

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References
Activity of Cuban Propolis Extracts on *Leishmania amazonensis* and *Trichomonas vaginalis*


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In this paper we analyzed the antiprotozoal effects of eighteen Cuban propolis extracts (brown, red and yellow type) collected in different geographic areas, using *Leishmania amazonensis* (as a model of intracellular protozoa) and *Trichomonas vaginalis* (as a model of extracellular protozoa). All evaluated propolis extracts caused inhibitory effect on intracellular amastigotes of *L. amazonensis*. However, cytotoxicity on peritoneal macrophages from BALB/c mice was observed. Only five samples decreased the viability of *T. vaginalis* trophozoites at concentrations lower than 10 µg/mL. No correlation between the type of propolis and antiprotozoal activity was found. Cuban propolis extracts demonstrated activity against both intracellular and extracellular protozoa model, as well as the potentialities of propolis as a natural source to obtain new antiprotozoal agents.

**Keywords:** Propolis, antiprotozoal, *Leishmania amazonensis*, *Trichomonas vaginalis*.

Propolis is a resinous hive product that honey bees produce employing parts of plants as buds, leaves, exudates or resins, and beeswax. Its chemical composition is highly variable and depends greatly according to the plants found around the hive [1a]. Cuban propolis has shown significant differences in its chemical composition with respect to propolis from temperate zone. They also vary significantly among themselves and can be clearly divided into three groups: brown Cuban propolis type (BCP type) has shown to be rich in polyisoprenylated benzophenones, red type (RCP type), containing isoflavonoids as the main constituents, and yellow type (YCP type) with a variety of triterpenoids as the major chemical components [1b].

Diverse pharmacological activities of propolis have been explored, such as: anti-inflammatory and anti-tumoral effect [2a]. Up to now, antimicrobial properties have been widely investigated; including antibacterial [2b], antiviral [2c] and antifungal activity [2d]. Antiparasitic activities have also been reported against *Trypanosoma cruzi* [2e] and *Giardia duodenalis* [2f]. However, only a few studies have been carried out for the antileishmanial [3a-3c] and antitrichomonacidal activity [4a]. In Cuba, propolis has displayed therapeutic potentialities as antipsoriatic, anti-inflammatory, analgesic [4b], antibacterial [4c] and antitumoral [4d]. Scarce reports can be found about its antiparasitic activity. Thus, its biological potentiality has not been explored totally.

In the present work, we analyzed the antiprotozoal effects of brown, red and yellow Cuban propolis extracts collected in different geographic areas, using *Leishmania amazonensis* (as a model of intracellular protozoa) and *Trichomonas vaginalis* (as a model of extracellular protozoa). *Leishmania* are protozoa that cause leishmaniasis [5a]. The disease is endemic in 88 countries throughout Latin America, Africa, Asia and Southern Europe. Approximately 350 million people are thought to be at risk with a worldwide prevalence of 12 million and annual incidence of 2 million new cases [5b].

All propolis samples caused inhibition growth against *L. amazonensis*, with IC$_{50}$ < 27 µg/mL; although a high toxicity on peritoneal macrophage from BALB/c mice was observed (Table 1). The better selectivity was showed by BCP-1, a propolis sample that exhibited a high content of nemorosone [1b]. However, other BCP samples containing also high content of this compound exhibited higher values of IC$_{50}$. BCP-16 and RCP-29 showed unspecific action. In general, RCP was the most active (IC$_{50}$ average of 13.9 µg/mL) and the most cytotoxic (CC$_{50}$ average of 37.2 µg/mL). There are a number of studies documenting the antiprotozoal activity of flavonoids [6a-6e]. These compounds have shown activity against *Plasmodium falciparum, Leishmania spp., Trypanosoma cruzi* or *Giardia intestinalis*. Four flavonoids detected in RCP including biochanin A, 3,8-dihydroxy-9-methoxy-pterocarpan, formononetin, and liquiritigenin have shown
inhibitory activity against parasites mentioned above. These observations correlate well with the in vitro studies obtained herein due to these flavonoids are among the major components of RCP.

Previously, Ayres et al. reported the activity of Brazilian propolis on L. amazonensis promastigotes and amastigote form [3a]. In this sense, Machado et al. compared the antileishmanial activity of Brazilian and Bulgarian propolis against four different species of Leishmania (L. amazonensis, L. braziliensis and L. chagasi from New World and L. major from Old World). They also observed significant differences in the leishmanicidal activities with IC50 values that ranged from 2.8 to 229.3 µg/mL [3b]. Duran et al., demonstrated that propolis samples from Turkey also reduced the proliferation of T. vaginalis [4a]. Thus, it is very interesting to note that all these results were obtained employing propolis samples from different geographical origins containing very dissimilar chemical constituents.

On the other hand, T. vaginalis is a flagellated protozoa that causes trichomoniasis, a common but overlooked sexually transmitted human infection, with approximately 170 million cases occurring annually worldwide [7]. Only five propolis extracts showed activity against T. vaginalis; while thirteen were inactive (Table 2). A high contrast was observed since only some samples belonging to each propolis type caused an important inhibition growth of trophozoites. In this sense, YCP samples were the most active, which should be corroborated using in vivo models of infection. A previous report demonstrated that propolis possesses in vitro anti-trichomonas activity [4a].

In both protozoa, the reference drug caused better activity, which is logical since the pentamidine and metronidazol are pure compounds and propolis extracts are complex mixture of substances. Chemical studies revealed that BCP has shown nemorosone as the main component and contained also other prenylated benzophenone derivatives such as garcinielliptone I, hyperibone B and propolone B, C and D [8a]. RCP is composed by isoliquiritigenin, liquiritigenin, biocanin A, formononetin, vestitol, neovestitol, isosativan, medicarpin, homopterocarpin and vesticarpan, mainly [8b]; while YCP samples are rich in triterpenoids including lanosterol, α- and β-amyrin, β-amyrin acetate, β-amyroside, germacranol and germacranol acetate, lupeol and lupeol acetate, cycloartenol, lanosterol acetate and 24-methylene-9,19-cyclopanstan-3β-ol [8c].

Although Cuban propolis has been characterized and grouped into three different types according its main chemical components, we could not correlate its antiparasitic activity against both protozoa with the chemical composition. Promissory results were found in some samples of each type of propolis, but this activity has not been shown by all samples belonging to a same group. This apparent contradiction can indicate that the antiparasitic activity can be associated to compounds that are present in a minor percentage in the sample or due to interaction of different compounds that act as synergistic and enhance the effects or interact as antagonist, which provoke the lost of activity in some samples of the same type. This unresolved situation is very common in products that exist in the nature as complex mixture of substances, which can interact and change the expected pharmacological activity. Further experiments with the compounds obtained from all types of propolis can be developed in order to elucidate the responsible of the high antiparasitic activity showed in some cases.

In conclusion, some Cuban propolis extracts exhibited activity against both intracellular and extracellular protozoa model. Our results corroborated the high potentiality of propolis as a natural source of new antiprotozoal agents with a wide spectrum of activity. This study also confirms that both the chemical composition of propolis and its biological potentiality should be evaluated during the quality control process of this natural product.

### Table 1: Antileishmanial activity and cytotoxicity of Cuban propolis extracts.

<table>
<thead>
<tr>
<th>Propolis</th>
<th>IC50 (µg/mL) ± SD</th>
<th>CC50 (µg/mL) ± SD</th>
<th>SI†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCP-15</td>
<td>7.3 ± 0.1</td>
<td>51.8 ± 4.5</td>
<td>7.3</td>
</tr>
<tr>
<td>BCP-3</td>
<td>9.1 ± 0.8</td>
<td>50.7 ± 1.5</td>
<td>5.5</td>
</tr>
<tr>
<td>BCP-4</td>
<td>10.1 ± 0.9</td>
<td>51.8 ± 3.2</td>
<td>5.1</td>
</tr>
<tr>
<td>YCP-1</td>
<td>11.8 ± 1.8</td>
<td>51.2 ± 0.1</td>
<td>4.4</td>
</tr>
<tr>
<td>YCP-2</td>
<td>12.0 ± 1.1</td>
<td>51.2 ± 0.1</td>
<td>4.2</td>
</tr>
</tbody>
</table>

†: IC50: Concentration of drug that caused 50% of inhibition growth. †: SD: Standard deviation. §: CC50: Concentration of drug that caused 50% of mortality. SI: Selectivity index = CC50/IC50

### Table 2: Activity of Cuban propolis extracts on T. vaginalis.

<table>
<thead>
<tr>
<th>Propolis</th>
<th>IC50 (µg/mL) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCP-1</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td>BCP-4</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>BCP-5</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>BCP-6</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>BCP-9</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>BCP-10</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>BCP-11</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>BCP-12</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>BCP-13</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>BCP-14</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>BCP-15</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>BCP-16</td>
<td>&gt; 200</td>
</tr>
</tbody>
</table>

†: IC50: Concentration of drug that caused 50% of inhibition growth. †: SD: Standard deviation.
**Experimental**

**Propolis samples:** Eighteen samples of Cuban propolis were provided by “La Estación Experimental Apícola”, Havana, Cuba, between October 2003 and December 2004. Samples were collected in nine provinces of Cuba including Eastern, Central and Western regions (Table 3). Propolis samples were extracted by maceration with methanol (10 mL, 3 times) for 1 hour at room temperature employing agitation. Extracts were filtered on paper filters and the solvent was evaporated at 40°C under reduced pressure to obtain dry extracts. Each sample was characterized and classified using a combination of NMR, HPLC-PDA and HPLC-ESI/MS techniques as previously described [1b]. Cuban propolis samples used in this study, their origin and classification are reported in table 3. The extracts were dissolved in dimethylsulphoxide (DMSO, BDH, Poole, England) at 20 mg/mL and stored at 4°C.

**Activity of propolis samples on L. amazonensis amastigotes:** Peritoneal macrophages were harvested from normal BALB/c mice in RPMI medium (Sigma) and plated at 10³/mL in Lab-Tek 16 chamber slides (Costar®, Naperville, US) and incubated at 37°C and 5% CO2 for 2 hours. Non-adherent cells were removed and stationary-phase L. amazonensis promastigotes were added at a 4:1 parasite/macrophase ratio. The cultures were incubated for 4 hours and washed to remove free parasites. Propolis was added at a concentration ranging from 100 to 12.5 µg/mL for 48 hours. The cultures were then fixed with absolute methanol, stained with Giemsa, and examined under light microscopy [9b]. The number of intracellular amastigotes was determined by counting the amastigotes residents on 100 macrophage per each sample, and the results were expressed as percent of reduction of the infection rate (%IR) in comparison to that of the controls [%IR = 100 – (infection rate of the treated culture/infection rate of the untreated culture x 100)]. The infection rates were obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophages [9c].

**Cytotoxicity of propolis samples on macrophase:** Peritoneal macrophages were collected from normal BALB/c mice in RPMI medium supplemented with antibiotics, and seeded at 30000 cell/well. The cells were incubated for 2 hours at 37°C in 5% CO2. Non-adherent cells were removed and dilutions of propolis in 1 µL DMSO were added to 200 µL medium at 10% HFBS and antibiotics. The macrophages were treated by eight concentrations of the product ranging from 200 to 1.7 µg/mL for 48 hours. The viability was determined using the colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (SIGMA, St. Louis, MO, USA). MTT solutions were prepared at 2.5 mg/mL for 4 hours. The cultures were then fixed with absolute methanol, stained with Giemsa, and examined under light microscopy [9b]. The number of intracellular amastigotes was determined by counting the amastigotes residents on 100 macrophage per each sample, and the results were expressed as percent of reduction of the infection rate (%IR) in comparison to that of the controls [%IR = 100 – (infection rate of the treated culture/infection rate of the untreated culture x 100)]. The infection rates were obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophages [9c].

**Parasites culture:** The strain of L. amazonensis (MHOM/77BR/LTB0016) was kindly provided by the Department of Immunology, Oswaldo Cruz Foundation, Brazil. The parasites were routinely isolated from mouse lesions and maintained as promastigotes at 26°C in Schneider’s medium (Sigma Chem Co, St. Louis, Mo, US), containing 10% heat-inactivated foetal bovine serum (Sigma), 200 U penicillin/mL and 200 µg streptomycin/mL (Sigma). The parasites were not used after the fifth passage. For examining the effect of these propolis samples on T. vaginalis, the isolate C173, axenized from a symptomatic woman suffering of trichonomiasis, was used [9a]. Parasites were cultured in TYI-S-33 supplemented with heat-inactivated bovine serum at final concentration of 10%, under anaerobic conditions, at 37°C.
800 x g), and the supernatant medium was aspirated from the wells, as completely as possible, without disturbing the formazan crystals or the cells on the plastic surface. DMSO (100 μL) was added to each well to dissolve the formazan crystals, the plates were shaken for 1 min and immediately the optical density was determined at 560 nm, using 630 nm as the reference wavelength.

Statistical analysis: The 50 % inhibition concentration \((IC_{50})\) value was determined from the linear concentration-response curves in each parasite evaluation. The 50 % cytotoxic concentration \((CC_{50})\) was obtained from dose-response curves fit to data by means of the equation for the sigmoidal \(E_{max}\) model \([9e]\). Selectivity indices \((SI)\) were then calculated through of division the \(CC_{50}\) for host cells by the \(IC_{50}\) for \(L. amazonensis\) \([9f]\).

References


Antioxidant compounds can be useful to prevent several degenerative diseases or as preservative in food and toiletries. Species of the Myrtaceae family are able to accumulate phenolic substances and those are closely related to the antioxidant activity due to their capacity to scavenge free radicals, protect against lipid peroxidation and quench reactive oxygen species. These facts prompted us to investigate the antioxidant capacity of the ethanolic extracts of the leaves of four Myrtaceae plants collected of the south of Brazil. Eugenia chlorophylla O. Berg., Eugenia pyriformis Cambess, Myrcia laruotteana Cambess and Myrcia obtecta (Berg) Kiacrsk. The antioxidant potential was performed using the DPPH (a single electron transfer reaction based assay) and ORAC (Oxygen Radical Absorbance Capacity, a hydrogen atom transfer reaction based assay) assays. Moreover, the total soluble phenolic content was also measured using the Folin-Ciocalteu reagent. A preliminary evaluation of the Ethanolic extracts of these Myrtaceae plants revealed high levels of phenolic compounds (343.7-429.3 mg GAE) as well as high antioxidant activity according to both methods (1338 ± 3785 µmol of TE/g of extract in ORAC and SC50 in the range of 1.70 and 33.7 µg/mL in the DPPH). The highest antioxidant activity obtained by DPPH assay was exhibited by ethanol extract of the leaves of E. pyriformis (1.70 µg/mL), followed by extracts of M. laruotteana (3.38 µg/mL) and M. obtecta (6.66 µg/mL). In comparison with controls, in the DPPH assay, the extract of E. pyriformis was more active than trolox (SC50 = 2.55 µg/mL), while the extracts of M. laruotteana and M. obtecta were more active than quercetin (SC50 = 7.80 µg/mL). In the ORAC assay, all species also show good antioxidant capacity (>1000 µmol of TE/g). Initial HPLC-UV/DAD and ESI-MS confirmed the presence of phenolic acids constituents in the ethanol extracts. The results indicate the presence of compounds possessing promising antioxidant/free-radical scavenging activity in the analyzed extracts of Myrcia and Eugenia plants of the south of Brazil.

**Keywords:** Myrtaceae, Myrcia, Eugenia, radical scavenger, DPPH, HPLC-UV/DAD, ESI-MS.

Antioxidant compounds can be useful to prevent several degenerative diseases or as preservative in food and toiletries products. Currently there is an increasing interest in searching natural antioxidants to replace synthetic compounds, which can be dangerous for human health. Thus many plants have been screened for a possible source of non-toxic and effective antioxidants [1-3]. Compounds with antioxidant activity are generally phenolics and the free radical-scavenging is a suitable method for preliminary search of them in plants [4]. Myrtaceae is an important family in Brazil, with more than 1000 species over the country. Myrcia and Eugenia, with around 400 and 350 species, respectively, are the major genera [5]. Many species produce edible fruits and, some have an essential ecological role in the conservation of Brazilian biodiversity [6]. Screening for antioxidant activity of Myrtaceae has been focused on its edible fruits [7-9], with only one report on leaves [10]. The aim of this work was evaluate the antioxidant capacity of leaves of Eugenia chlorophylla O. Berg., Eugenia pyriformis Cambess, Myrcia laruotteana and Myrcia obtecta (Berg) Kiacrsk. The selected plants are trees, growing in Southern region of Brazil [11]. Previous studies have reported the essential oil composition of these plants [12-17], identification of the flavonoids myricitrin, rutin and quercitrin in the leaves of E. pyriformis [18] and the antimicrobial and antioxidant activities of the fruits of E. pyriformis [7,19-20]. No phytochemical reports were found on E. chlorophylla, M. laruotteana and M obtecta.

In this study, the phytochemical screening showed the presence of triterpenes/sterols, phenolic compounds, tannins and saponins in all samples. Theses results are in agreement with previous reports of flavonoids, triterpenes and phenolic compounds in Eugenia and Myrcia [21-26]. The ethanol extracts showed a total phenol content in the range of 343.7-429.3 mg GAE/ g extract (Table 1). Phenolic compounds (including simple phenols, tannins and flavonoids) are recognized as one of most important class responsible for antioxidant capacity in plants [27].
All samples exhibited antioxidant activity concentration-dependent in DPPH assays, with SC50 varying from 1.70 to 33.72 μg/mL. In DPPH assays the highest antioxidant activity was exhibited by ethanol extract of the leaves of *E. pyriformis* (1.70 μg/mL), followed by extracts of *M. laruoetteana* (3.38 μg/mL), *M. obtecta* (6.66 μg/mL) and *E. chlorophylla* (33.72 μg/mL). In comparison with controls, the extract of *E. pyriformis* was more active than trolox (SC50 = 2.55 μg/mL), while the extracts of *M. laruoetteana* and *M. obtecta* were more active than quercetin (SC50 = 7.80 μg/mL) (Table 1). Moreover, in ORAC-FL kinetic assay the extracts showed a good antioxidant capacity with value between 1338.58 and 3785.70 μM of Trolox equivalent per gram of extract (μM of TE/g). In accordance to literature data, samples with good antioxidant capacity in this assay [28,29]. In ORAC-FL assays, highest antioxidant activity was exhibited by ethanol extract of the leaves of *M. laruoetteana*, followed by extracts of *E. chlorophylla*, *E. pyriformis* and *M. obtecta* (Table 1).

### Table 1: Total phenol content and antioxidant capacity by the DPPH and ORAC assays of ethanol extracts of Myrtaceae plants.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phenol content¹ (mg of GAE/g of extract or fraction)</th>
<th>DPPH assay, SC⁵₀</th>
<th>ORAC assay² (µmol of TE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eugenia chlorophylla</em></td>
<td>429.3 (5.2)</td>
<td>33.72 (2.25)</td>
<td>2197.20 (1.12)</td>
</tr>
<tr>
<td><em>Eugenia pyriformis</em></td>
<td>396.2 (4.5)</td>
<td>1.70 (1.19)</td>
<td>1456.50 (4.92)</td>
</tr>
<tr>
<td><em>Myrica laruoetteana</em></td>
<td>401.4 (2.5)</td>
<td>3.38 (3.38)</td>
<td>3785.70 (2.67)</td>
</tr>
<tr>
<td><em>Myrica obtecta</em></td>
<td>343.7 (4.9)</td>
<td>6.66 (5.30)</td>
<td>1338.58 (4.85)</td>
</tr>
<tr>
<td>Quercetin*</td>
<td>-</td>
<td>7.80 (2.00)</td>
<td>5.62 (0.89)</td>
</tr>
<tr>
<td>Caffeic acid*</td>
<td>-</td>
<td>10.80 (2.60)</td>
<td>2.86 (2.02)</td>
</tr>
<tr>
<td>Chlorogenic acid*</td>
<td>-</td>
<td>12.15 (1.80)</td>
<td>2.65 (1.50)</td>
</tr>
<tr>
<td>Trolox*</td>
<td>-</td>
<td>2.55 (1.40)</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Mean value (%RSD, relative standard deviation) of triplicate assays.
²Total phenolics data expressed as milligrams of gallic acid equivalents per gram (mg of GAE/g).
³DPPH assay data expressed as SC⁵₀ (concentration that inhibited 50% of the DPPH radical) in micrograms per milliliters (µg/mL).
⁴ORAC data expressed as micromol of Trolox equivalents per gram (µmol of TE/g).
⁵ORAC data expressed as relative Trolox equivalent, mean (%RSD, relative standard deviation) of triplicate assays.

The differences in best antioxidant capacity of the species studied among the two assays happens due to differences of sensibility and on the basis of the chemical reactions involved in each test: ORAC is a hydrogen atom transfer reaction based assay (HAT) and DPPH is a single electron transfer reaction based assay (ET). It is apparent that the hydrogen atom transfer reaction is a key step in the radical chain reaction. Therefore, the HAT based method is more relevant to the radical chain-breaking antioxidant capacity. Overall, there are a multitude of ET-based assays for measuring the reducing capacity of antioxidants. The assays are carried out at acidic (FRAP and DPPH), neutral (TEAC and ORAC), or basic (total phenols assay by Folin-Ciocalteau reagent, FCR assay) conditions. The pH values have an important effect on the reducing capacity of antioxidants. At acidic conditions, the reducing capacity may be suppressed due to protonation on antioxidant compounds, whereas in basic conditions, proton dissociation of phenolic compounds would enhance a sample’s reducing capacity [28,29].

Thus, the results documented in this study demonstrated that all extracts analyzed showed antioxidant capacity (measured by DPPH and ORAC assays) and this activity present a positive correlation with the total phenolic content (measured by FCR assay). Moreover the caffeic and chlorogenic acids presented considerable antioxidant (Table 1) capacity and were detected in some of the extracts studied (Table 2).

Phenolic compounds has been presented as important substances in combating free radical production mainly due to its chemical structure and redox capacity, allowing them to act as reducing agents, hydrogen donating, neutralizing free radicals [30], chelating of transition metals and inhibiting lipid peroxidation [31]. In biological systems this capacity confers pharmacological properties to this compounds that act preventively against diseases related to oxidative stress.

The results for *E. pyriformis* corroborate previous work that reported the inhibition of xanthine oxidase, an enzyme responsible by super-oxide anion production [32] and antioxidant activity of fruits [7,19].

The ESI-MS fingerprints technique with direct infusion [33-35] was used to characterize the presence of compounds with potent free-radical scavenging activity in this work. The extracts were analyzed by direct insertion both in the negative and positive ion modes. However, ESI(+-)MS fingerprints produce by far the most characteristic mass spectra; hence only the ESI(-)-MS data will be presented and discussed. This method in the negative ion mode provides a sensitive and selective method for the identification of polar organic compounds with acidic sites, such as the phenolic organic acids. Deprotonated forms of the compounds of interest were then selected and dissociated and their ESI-MS/MS were compared to those of standards.

Chlorogenic acids are esters of *trans*-cinnamic acids (coumaric, caffeic, ferulic, and 3,4-dimethoxycinnamic) with quinic acid. The *trans*-cinnamic acids can be esterified at one or more of the hydroxyls at positions 1, 3, 4, and 5 of quinic acid, originating series of positional isomers. In HPLC-UV/DAD and ESI-MS analysis the *Myrica* samples presented *m/z* 353 as base peaks (bp) in negative ionization mode mass spectra and UV spectra characteristics of caffeoylquinic derivatives (UV max: ≈298 and 325 nm) which, when taken together, suggest positional isomers of a quinic acid (QA) esterified with a single caffeoyl (CAF) unit. The product ion spectra obtained by negative ion MS/MS for precursor ions *m/z* 353 were different from each other, and comparison with

![Image](https://via.placeholder.com/150)
The caffeoylquinic acids (CQA) identification keys [36,37] led to the individualization of three CQA positional isomers. The product ion spectrum for m/z 353 showed m/z 191 (bp) and m/z 179 at 4% ri. The greater relative intensity of m/z 179 in the product ion spectrum, led to the identification of peak as 5-O-(E)-caffeoylquinic acid (5-CQA). Furthermore, the identity of this compound was also confirmed through co-elution with a 5-CQA authentic standard. In the ESI-MS fingerprints of the samples of Myrtaceae extracts (Figure 1, Table 2) the following components were identified in their deprotonated forms: caffeic acid (m/z 179), quinic acid (m/z 191), ferulic acid (m/z 193), and chlorogenic acid 5-CQA (m/z 353), figure 2. The content of phenolic compounds in the extracts, possibly could explain the high antioxidant activity verified for these extracts of Myrtaceae.

The investigation by direct infusion electrospray ionization mass spectrometry (ESI-MS) provided important information about bioactive components present in the Myrtaceae extracts, that are widely reported as potent antioxidants, probably explaining the antioxidant activity of the studied extracts [35, 38-40].
Experimental

Plant Material: The leaves of Eugenia chlorophylla O. Berg. (I), Eugenia pyriformis Cambess (II), Myrcia laruoatiana Cambess (III) and Myrcia obtecta (Berg) Kiacrsk. (IV) were collected in Curitiba, Paraná State, Brazil. Voucher specimens were deposited at the herbarium of Universidade Federal do Paraná (UPCB 53304, 16741, 53303 and 60504, respectively).

Extracts preparation and chemical analysis: Dried and powdered leaves of each plant (50 g) were extracted with ethanol (3 X 300 mL) at room temperature. The solvent was removed under reduced pressure to give the crude extracts (E. chlorophylla 5.4%, E. pyriformis 5.7%, M. laruoatana 14.1% and M. obtecta 21.4%), which were used in DPPH assays.

Phytochemical Analysis: Phytochemical tests for sterols/triterpenes, phenolic compounds, tannins, saponins and alkaloids were carried on according usual methodology [41].

Quantitative determination of total soluble phenols: The extracts, dissolved in methanol, were analyzed for their total soluble phenolic content according to the Folin-Ciocalteau colorimetric method [42-43], using gallic acid as reference. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract or fraction (mg of GAE/g). The analyses were performed in triplicate.

Radical scavenging activity using the DPPH method: The antiradical activity of extracts was determined using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) [44]. The test was performed in 96-well microplates. Fifty microliters of a 250 μM DPPH solution in MeOH was added to a range of solutions of different concentrations (seven serial 3-fold dilutions to give a final range of 100 to 1.6 μg mL⁻¹) of extracts to be tested in MeOH (10μL). Absorbance at 517 nm was determined 30 min after the addition of each of the compounds tested, and the percentage of activity was calculated. Quercetin and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were used as positive controls. All samples were tested in triplicate. The antioxidant activity of each sample was expressed as the SC₅₀ value, which is the concentration in μg mL⁻¹ of each extract that scavenged 50% of the DPPH radicals. All of the results are expressed as mean of three different trials.

Evaluation of antioxidant capacity by ORAC assay: The antioxidant capacity of the ethanolic extract was assessed through the oxygen radical absorbance capacity (ORAC) assay. This assay measures antioxidant scavenging activity against peroxyl radicals using fluorescein as the fluorescent probe. ORAC assays were carried out on a Synergy HT multi-detection microplate reader system. The temperature of the incubator was set at 37°C. The procedure was carried out according to the method established by Ou and co-workers [29] with modifications [45]. The data were expressed as micromoles of Trolox equivalents (TE) per gram of extract on dry basis (μmol of TE/g) and as relative Trolox equivalent for pure compounds. Quercetin and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were used as positive controls. The analyses were performed in triplicate.

HPLC analysis: HPLC analyses were conducted using a RP-18 column (Lichrospher, 5 μm, 225x4.6 mm, Merck). The mobile phase consisted of a linear gradient combining solvent A (acetonitrile) and solvent B (water/acetic acid, 99:1, v/v, pH 2.88) as follows: 15% A (15 min), 15-40% A (5 min), 40-60% A (5 min), 60-100% A (5 min), 100-15% A (5 min), 15% A (5 min). The analyses were carried out in triplicate at a flow rate of 0.8 mL/min and an injection volume of 20 μL. UV-DAD detector was set to record between 200 and 600 nm, and the UV chromatograms were measured at 254 and 330 nm. The samples were analyzed at 1 mg/mL. The standard sample of quinic acid and chlorogenic acid (5-O-(E) caffeoylquinic acid) were also analyzed and then used for co-elution with authentic standard.

Electrospray ionization mass spectrometry fingerprinting: Crude extracts of Myrtaceae plants were diluted in a solution containing 50% (v/v) chromatographic grade methanol and 50% (v/v) deionized water and 0.5% of ammonium hydroxide (Merck, Darmstadt, Germany). In the fingerprinting ESI-MS analysis, the general conditions were: source temperature of 100 °C, capillary voltage of 3.0 kV and cone voltage of 30 V. For measurements in the negative ion mode, ESI(-)-MS, 10.0 μL of concentrated NH₄OH were added to the sample mixture having a total volume of 1000 μL yielding 0.1% as final concentration. For measurements in the positive ion mode ESI(+)-MS, 10.0 μL of concentrated formic acid were added giving a final concentration of 0.1%. ESI-MS was preformed by direct infusion with a flow rate of 10 μL min mL⁻¹ using a syringe pump (Harvard Apparatus). Structural analysis of single ions in the mass spectra from extract was performed by ESI-MS/MS. The ion with the m/z of interest was selected and submitted to 15–45 eV collisions with argon in the collision quadrupole. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation. The compounds were identified by comparison of their ESI-MS/MS fragmentation spectra with literature data [33-35].

Statistical analysis: Data are reported as mean (%RSD, relative standard deviation) of triplicate determinations. The statistical analyses were carried out using the Microsoft Excel 2002 software package (Microsoft Corp., Redmond, WA)
Antioxidant capacity of four Myrtaceae plants

Acknowledgments - The authors are grateful to Dr. Armando C. Cervi, from Departamento de Botânica, Universidade Federal do Paraná, for plant identification and to FAPESP, CNPq and FAEPEX-UNICAMP for financial support.

References


Cytotoxicity of Active Ingredients Extracted from Plants of the Brazilian “Cerrado”

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Cytotoxicity assays are needed for the screening of natural products with potential anti-inflammatory. The purpose of this study was to compare the basal cytotoxicity of active ingredients extracted from plants of the Brazilian “cerrado”. The viability was assayed with the neutral red uptake assay in Mac Coy cells after 24h of exposition. The dose evaluated was 50 µg/µL. The test substances were: cinnamic acid, p-coumaric acid, chlorogenic acid, syringic acid, vannilic acid, homogentisic acid, scandenin, palustic acid, diosgenin, cabraleone.

Studies of cytotoxicity demonstrated that all active compounds evaluated have low toxicity in vitro. The substances showed cell viability above 60% for the concentration used. However, the cinnamic acid, scandenin and palustic acid showed highest toxicity with a 50% reduction in cell viability for the dose of 50 µg/µL. Cytotoxic screening results are useful to estimate the best concentrations of those compounds with potential anti-inflammatory without their cause cell death.

Keywords: Natural products, Cytotoxicity, Brazilian Cerrado.

The Cerrado is one of the world's threatened biodiversity hotspots [1a]. About 60% of its vegetation has already been removed [1b] and the remaining areas are isolated in forest fragments [2a]. Due to the devastation many natural compounds with potential biological activities were lost.

Screenings of natural compounds using cytotoxicity assays offer the advantage of evaluate several compounds simultaneously. The comparative sensitivity of cells to toxicity tests that evaluate direct-acting and indirect-acting cytotoxicants may be important to explaining their mode of action [2b]. Natural products with low cytotoxicity activities constitute an excellent alternative search for complementary treatments for inflammatory disease.

The values obtained from the cytotoxicity test performed on cells MacCoy can be used as parameters for doses used in trials of anti-inflammatory activity. The purpose of this study was to compare the basal cytotoxicity of active compounds extracted from plants of the Brazilian “cerrado” and find those whose toxicity is low to further anti-inflammatory assays. Due to this purpose dose of 50 µg was used. The percentage of cell viability was established for each of the compounds evaluated and the results are presented in Table 1.

<table>
<thead>
<tr>
<th>Natural Products (50 µg/mL)</th>
<th>nm (±SD)*</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.519 ± 0.010</td>
<td>100%</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>0.155 ± 0.015</td>
<td>30%</td>
</tr>
<tr>
<td>Scandenin</td>
<td>0.207 ± 0.012</td>
<td>40%</td>
</tr>
<tr>
<td>Palustic acid</td>
<td>0.249 ± 0.016</td>
<td>48%</td>
</tr>
<tr>
<td>Homogentisic acid</td>
<td>0.259 ± 0.013</td>
<td>50%</td>
</tr>
<tr>
<td>Diosgenin</td>
<td>0.404 ± 0.014</td>
<td>78%</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>0.415 ± 0.013</td>
<td>30%</td>
</tr>
<tr>
<td>Cabraleone</td>
<td>0.337 ± 0.012</td>
<td>65%</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>0.337 ± 0.015</td>
<td>65%</td>
</tr>
<tr>
<td>Vannilic acid</td>
<td>0.363 ± 0.016</td>
<td>70%</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.415 ± 0.013</td>
<td>80%</td>
</tr>
</tbody>
</table>

* Values presented with standard deviation.

The compounds showed cell viability above 60% for the concentration used. However, the cinnamic acid, scadenin and palustic acid showed highest toxicity with a 50% reduction in cell viability for the dose of 50 µg.

The natural products obtained from the Brazilian cerrado show large potential for pharmaceutical product development. Some researches have shown the use of these compounds as an alternative for the treatment of cancer [3]. In addition, the potential of these products might be exploited to treat other inflammatory disorders such as physiological dysfunctions. Several xanthones,
coumarins, terpenoids and phenolic compounds were isolated from plants Brazilian cerrado and with remarkable activities including antiHIV [4], antibacterial [5], trypanocidal [6], and anticancer against the cell lines KM12 (colon adenocarcinoma), U251 (glioma), PC3 (prostate), and K562 and HL60 (leukemia) [7-9a]. Our results agree with those previously reported. According to [9] and K562 and HL 60 (leukemia) [7-9a]. Our results agree with those previously reported. According to (prostate), and K562 and HL 60 (leukemia) [7-9a]. Our results agree with those previously reported. According to

The differences between the chemical structures of the substances evaluated, was the determining factor for difference of cytotoxicity. Complex structures (diosgenin, cabraleone) showed less toxic, possibly because these compounds are not able to go into de cells. Structures with coumarin ring were the most toxic (cinnamic acid, p-coumaric acid) Glycoside radical (acid chlorogenic) usually shows low toxicity due to the presence of hydroxyls that make difficult the transport of the compounds to into the cells. Natural compounds with low toxicity and potential as anti-inflammatory drugs have been a source of research, once the search for new drugs with fewer side effects has increased significantly.

**Experimental**

**Chemical and culture media:** Neutral red was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Cultiab (Campinas, SP, Brazil). Dulbecco’s Modified Eagle Medium (DMEM) was obtained from Instituto Adolfo Lutz (São Paulo, SP Brazil).

**Cell Line:** Mac Coy mouse fibroblast cell line (CCL1; American Type Culture Collection, USA, from Cell Culture Section of the Adolfo Lutz Institute, São Paulo, Brazil) was maintained in Eagle medium with 7.5% fetal bovine serum at 37°C.

**Cytotoxicity assay:** After trypsinization, 0.2 mL aliquots of Eagle, containing approximately 10^4-10^5 cells/mL were transferred to 96-well microtiter tissue-culture plates and incubated at 37°C. After 24 hr, the medium was removed and the cells were covered in unmodified medium (control) or in medium modified with various concentrations of the test compound. After incubating for another 24 hr, the medium was removed and the plates were prepared for the neutral red (NR) assay [9b]. After brief agitation, the plates were transferred to a microplate reader (Spectra (Shell) & Rainbow (Shell) Reader, Tecan Austria GMBH) and the optical density of each well at 620 nm was measured, using a 540 nm filter. All experiments were performed at least four times, using three wells for each concentration of chemical tested. The cytotoxicity data was standardized by determining absorbance and calculating the corresponding chemical concentrations [9c].

**Compounds:** The test substances were obtained after maceration of different plants species from Brazilian cerrado, resulted in hexane, dichloromethane, ethanol and hydro-ethanol extracts, depending on extraction procedures. The substances, cinnamic acid, p-coumaric acid, chlorogenic acid, syringic acid, vanillic acid, homogentisic acid, scandenin, palustacic acid, diosgenin, cabraleone were obtained using methods described by dos Santos [9d].

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**References**

Propagation and Conservation of Native Forest Genetic Resources of Medicinal Use by Means of *in vitro* and *ex vitro* Techniques

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In Argentina, there are numerous native species which are an important source of natural products and which are traditionally used in medicinal applications. Some of these species are going through an intense extraction process in their natural habitat which may affect their genetic diversity. The aim of this study was to establish vegetative propagation systems for three native forestal species of medicinal interest. This will allow the rapid obtainment of plants to preserve the germplasm. This study included the following species which are widely used in folk medicine and its applications: *Erythrina crista-galli* or “seibo” (astringent, used for its cicatrizant properties and for bronchiolitic problems); *Acacia caven* or “espinillo” (antirheumatic, digestive, diuretic and with cicatrizant properties) and *Salix humboldtiana* or “sauce criollo” (antipyretic, sedative, antispasmodic, astringent). The methodology included the micropropagation of seibo, macro and micropropagation of *Salix humboldtiana* and the somatic embryogenesis of *Acacia caven*. The protocol for seibo regeneration was adjusted from nodal sections of seedlings which were obtained from seeds germinated *in vitro*. The macropropagation through rooted cuttings of “sauce criollo” was achieved and complete plants of this same species were obtained through both direct and indirect organogenesis using *in vitro* cultures. The somatic embryogenesis for *Acacia caven* was optimized and this led to obtain a high percentage of embryos in different stages of development. We are able to support the conservation of native forest resources of medicinal use by means of vegetative propagation techniques.

**Keywords:** Micropropagation, native forest species, macropropagation, somatic embryogenesis.

Medicinal plants have been used since ancient times as new therapeutic agents and their uses have been transmitted from generation to generation, either in oral or written forms, up to the present, and this is known as the “traditional therapeutic practice”, the use of extracts or active principles of plants, which has been essential to take care of people’s health in the first level of attention.

The developed countries as well as the developing ones have increased the use of medicinal plants or their products [1]. Medicinal plants have played a vital role in societies including Argentina for centuries. Most of these plants are wild plants which were available in some forest ecosystems. With the intensity of development and clearing of land many of these wild plants used for medicinal purposes are no longer available in their natural habitat. Now we can address this environmental situation by *ex situ* conservation and propagation of medicinal plants for its sustainable utilization using new and traditional techniques.

In Buenos Aires province (Argentina), there are different native forest species of medicinal interest and some of them are *Salix humboldtiana* or “sauce criollo”, *Erythrina crista-galli* or “seibo” and *Acacia caven* or “espinillo”.

*Salix humboldtiana* is distributed in river banks or islands, sometimes in sandy places, too. The different parts of the plant contain salicin. The bark is used in folk medicine as a quinine substitute (it contains glucosides). The decoction of this bark is used “against intermittent fever” (rubber). The bark is bitter and has febrifugal, tonic, sedative and spasmodic properties. It is also astringent [2]. *Erythrina crista-galli* has several important pharmacological uses. It is an astringent and a sedative to heal wounds (3% of bark decoction). It is antihemorrhoidal and used for vaginal lavage in candidiasis cases (bark). It is disinfectant and deodorant, it has cicatrizant properties and it is ahemostat, emollient for colds, coughs, catarrh, bronchitis and asthmatic pains (the leaves are smoked in a pipe or rolled up like a cigar). It has narcotic, sedative and hypnotic properties: this is attributed to the most inner part...
of the bark when it is used in an infusion (it contains several alkaloids). This plant is also used for muscular and rheumatic pains (a balm prepared with its bark and flowers in 70% of alcohol). The leaves are used as antihemorrhoidal for external use and they are antiseptic and astringent [2]. *Acacia caven* or “espinillo” is a medicinal plant that lives in South America and it is not tropical. It grows in the arid highlands in the centre and north of the country as well as on the islands in the Paraná River, in very humid areas. The parts of the plant which are used for medicinal purposes are its leaves, stems and seeds. The leaves of this medicinal plant have cicatrizant properties, and the seeds are used as a digestive. The leaves and stems have antiphlogistic and sedative properties (http://www.tusplantasmedicinales.com/).

Vegetative reproduction keeps the parental genotype and its characteristics are preserved in its off springs. Thus, the genotypes of selected trees which are propagated vegetatively reproduce identically and form clones. Grafts, cuttings and layering are the traditional methods of vegetative propagation [3].

In the last few years the use of *in vitro* culture techniques in trees has facilitated the clonation of select phenotypes, the preservation and the manipulation of vegetal material. These techniques allow the multiplication of clones in a short period of time, in any season of the year and in a limited space [4]. By using these techniques, the long time it takes a plant to reach maturity, the low viability of seeds, and the difficulties some species have to propagate by traditional methods can be reduced [5]. Besides, hundreds of clones from the same species can be reproduced *in vitro*. Later, they are taken to a plant nursery and then, they are cultivated in fields where they will develop and finally become a product with a specific economic interest (http://www.biologia.org/).

The aim of this study was to establish vegetative propagation systems for three native trees of medicinal interest: *Salix humboldtiana* (sauce criollo), *Erythrina crista-galli* (seibo) and *Acacia caven* (espinillo). This will help to obtain plants to conserve germplasm in a rapid way.

**Macropropagation of *Salix humboldtiana***: The substrate with the mixture of soil, perlite and vermiculite (6:3, 5:0, 5) was adequate to place the *Salix humboldtiana* cuttings. The cuttings rooted 15 days after they were placed in water. 90% of the cuttings which were treated with IBA rooted, and 75% of them originated complete plants (Figure 1).

**In vitro culture of *Salix humboldtiana***: The culture media used for callus, shoot and root induction were adequate. Callus with *de novo* shoots (indirect organogenesis) were obtained approximately 35 days after the leaves were cultured. The shoots formed roots 25 days later. In this way, *in vitro* plants were obtained and they became acclimatized successfully.

**Micropropagation of *Erythrina crista-galli***: Growth of the preformed shoots was induced in MS with 1 mg.L\(^{-1}\) of BAP and 0.5 mg.L\(^{-1}\) of NAA. These shoots were subcultured in a WPM medium with 0.1 mg.L\(^{-1}\) of IBA where they elongated. Whole plants were obtained in a WPM rooting medium with 0.1 mg.L\(^{-1}\) of NAA. These plants were acclimatized under controlled light and temperature conditions (16 hours of light and 8 hours of darkness, T 21°C +/-2°C) (Figure 3).

**Somatic embryogenesis of *Acacia caven***: Direct and indirect somatic embryogenesis were obtained on the
Vegetative propagation systems for three medicinal plants

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Figure 3a-d: Micropropagation of Erythrina crista-galli. a. In vitro seedlings. b. Shoot elongation from stem section. c. Induction of roots. d. Acclimatization of whole plants.

Figure 4a-e: Somatic embryogenesis of Acacia caven. a. Mature fruits of Acacia caven. b y c Seeds in chemical scarification. d. Somatic embryos in a globular and heart stage formed from cotyledons. e. Embryos in torpedo and cotyledonar stage.

culture medium and the PGR concentration (1 mg L⁻¹ of 2,4 D and 0.1 mg L⁻¹ of BAP) used. The somatic embryos occur directly over the cotyledons, on their adaxial side, or indirectly from the formation of the callus after 6 months of culture. Somatic embryos were observed in different stages of development, and this showed there is no synchronicity in their maturation (Figure 4). The somatic embryos in a globular stage were subcultured on MS medium free from PGR, and they germinated there. 30% of them were converted into complete plants.

Conclusion: When the vegetative propagation techniques of the species mentioned before are adjusted, they contribute to the preservation of the forest genetic resources. Excellent opportunities for scientific research are generated by installing germplasm banks with a wide range of genetic material. This becomes very important if we consider that these species have a great importance in the medicinal use and among others.

These studies pretend to be preliminary stages to achieve the production of native plants of medicinal interest, without the degradation of the genetic base of this resource, which is vitally important for the preservation of the forest resources.

Experimental

This study has been developed at the Centro Experimental de Propagación Vegetativa (C.E.Pro.Ve.) in the Facultad de Ciencias Agrarias y Forestales at Universidad Nacional de La Plata, Buenos Aires, Argentina. In order to reach the objectives, we used vegetative propagation strategies for each species.

For Salix humboldtiana or “sauce criollo”, we used micro and macropropagation systems. Macropropagation was done through the rooting of cuttings with the exogenous application of growth regulators and the optimization of their nutritional requirements. The micropropagation was done through the use of in vitro plant tissue culture techniques, following the direct and indirect organogenic pathways.

For Erythrina crista-galli or “seibo” and Acacia caven or “espinillo” we used micropropagation following the pathway of organogenesis and somatic embryogenesis. Mother plants which were in good sanitary conditions, optimal growth, and adaptability to the particularities of the local site were chosen as a donating source for the different explants. The methodology included:

Macropropagation of Salix humboldtiana: Between 100 and 150 plant stem cuttings with single node each were cut of approximately 30 cm long and from 0.8 to 1.5 cm in diameter. They were treated superficially with 1000 mg L⁻¹ of Bénomyl fungicide for 3 hours in order to avoid the presence of fungi. After that, the bases of the cuttings were dipped in 50 mg L⁻¹ of indole-3-butyric acid (IBA) for 24 hours to induce rooting. The control was not treated with IBA. Then, they were placed in running water for 15 days and they were planted in plastic flowerpots N° 14 (1500 cm³) with a mixture of soil, perlite and vermiculite (6:3.5:0.5) as substrate [6]. The pots were arranged in randomized block design and replicated three times.

In vitro culture of Salix humboldtiana: Explants were nodal sections with internodes (microcuttings) from adult plants and leaves from in vitro seedlings obtained from
immature embryos. The embryos were surface sterilized with 10% of commercial sodium hypochlorite (55% active chlorine) for 5 minutes in order to place them in vitro, and later obtain the seedlings. The leaves from the seedlings were placed on Murashige & Skoog (MS) [7] basal medium at full strength supplemented with 1 mg.L⁻¹ of benzyl amino purine (BAP) and 0.5 mg.L⁻¹ of naftalen acetic acid (NAA), 2% sucrose and 7.5 g. L⁻¹ agar. Calli were subcultivated on MS medium at full strength with 1mg/L⁻¹ of BAP and 1 mg.L⁻¹ of NAA. The nodal sections or microcuttings of adult "sausage" plant were washed with running water for 5 minutes, and they were surface disinfected with 2000 mg/ L⁻¹ of Bennomyl fungicide for 3 hours and 50% of commercial sodium hypochlorite (55% active chlorine) for 25 and 45 minutes. For shoot induction, the following culture media were tested: Woody Plant Medium (WPM) without growth regulators and WPM supplemented with 0.1; 0.5; 1; 1.5; and 2 mg.L⁻¹ of the 6- benzilaminopurine (BAP). The cultures were maintained in the culture room under a regime of 16 h photoperiod (intensity - 40 µEcm- 2/min/sec) at 21°C +/-2°C. All experiments were conducted at least three times with 15 replicates each.

Shoots from both explants were placed in a rooting medium with the macro and micronutrients of WPM [8] supplemented with 0.1 mg.L⁻¹ of Indolebutyric acid (IBA) (Table 1).

<table>
<thead>
<tr>
<th>Basal media</th>
<th>Growth regulator</th>
<th>mg.L⁻¹</th>
<th>Type of explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS complete</td>
<td>BAP/ANA</td>
<td>1:0.5</td>
<td>Leaves</td>
</tr>
<tr>
<td>MS complete</td>
<td>BAP/ANA</td>
<td>1:1</td>
<td>Leaves</td>
</tr>
<tr>
<td>WPM</td>
<td>IBA</td>
<td>0,1</td>
<td>Shoots</td>
</tr>
<tr>
<td>WPM</td>
<td>BAP</td>
<td>0.1; 0.5; 1; 1.5 y 2</td>
<td>Nodal sections</td>
</tr>
<tr>
<td>WPM</td>
<td>-</td>
<td>-</td>
<td>Nodal sections</td>
</tr>
</tbody>
</table>

**Table 1**: Culture media used to the different explants (leaves and nodal sections) of Salix humboldtiana.

**Micropropagation of Erythrina cristagalli**: In order to induce the shoot proliferation nodal sections from in vitro germinated seedlings were used as source of explants. Different culture media with different growth regulators were tested. The explants were cultured in MS medium in a complete, half, and a quarter concentrations, with the addition of different growth regulators: 1 and 2 mg.L⁻¹ of BAP, 0.5 mg.L⁻¹ of NAA, 1mg.L⁻¹ of IBA, 2% sucrose and 7.5 g.L⁻¹ of agar, alone or combined. Cultures were incubated at 21°C +/-2°C with a 16 hours photoperiod.

**Somatic embryogenesis of Acacia caven**: In this case, cotyledons from mature seeds were used as explants. These seeds were treated with 98% of sulfuric acid in order to scarification for two hours and they were washed with running water for 10 minutes. Then, they were disinfected with 70% of ethanol during 5 minutes and 20% of sodium hypochlorite (55% active chlorine) for 30 minutes. After that, they were washed 3 times with distilled water under a laminar flow hood and they were put in sterile water during 7 days in order to soften the seed coat and obtain the cotyledons. The latter were sowed in a Murashige & Skoog (MS) basal medium, at half concentration of macro and micronutrients, supplemented with 1 or 2 mg.L⁻¹ of 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.1 mg.L⁻¹ of BAP, 3% of sucrose and 7.5 g. L⁻¹ agar (Table 2). The cotyledons were placed with their abaxial side in contact with the culture medium and were maintained in the culture room under complete darkness at 21°C +/-2°C.

**Table 2**: Culture media used in the somatic embryogenesis of Acacia caven.

<table>
<thead>
<tr>
<th>Explants</th>
<th>Media</th>
<th>PGR</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotyledon (Control)</td>
<td>MS/2</td>
<td>0</td>
<td>25 +/- 2°C in the darkness.</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>MS/2</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>MS/2</td>
<td>2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**References**


Genotoxic Evaluation of a Methanolic Extract of *Verbascum thapsus* using Micronucleus Test in Mouse Bone Marrow

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*Verbascum thapsus* L. is a medicinal plant and has been used to treat numerous pulmonary diseases, asthma, inflammatory disease, spasmodic coughs and migraine headaches. Several studies have demonstrated that different extracts of *V. thapsus* present antimicrobial activity. Thus, the goal of this study was to evaluate the genotoxic and cytotoxic activities of a methanolic extract of *Verbascum thapsus*, using micronucleus test in mouse bone marrow. No toxicity in bone marrow was detected in the extract-treated groups. The methanolic extract of *V. thapsus* at doses of 100, 300 and 500 mg / kg, did not produce a significant increase in the frequency of MN-PCE in bone marrow and neither altered the relationship PCE / NCE respect to negative control. These cytogenotoxic findings contribute to the preclinical knowledge of methanolic extract of *V. thapsus* and provide security in its use as herbal medicine.

**Keywords:** *Verbascum thapsus* L., methanolic extract, micronucleus, bone marrow, genotoxicity.

The use of medicinal plants in therapy or as dietary supplements remounts centuries ago, but it has increased substantially in the last decades [1a,1b]. The popularity of herbal medicines is related to their easy access, therapeutic efficacy, relatively low cost, and assumed absence of toxic effects.

Widespread public opinion is that being a natural product, herbal medicines are harmless and free from adverse effects. However, the safety of their use has recently been questioned due to the reports of illness and fatalities [2a-2c]. Considering the complexity of herbas in general and their inherent biological variation, it is now necessary to evaluate their safety, efficacy and quality [1b]. Thus, an assessment of their mutagenic and cytotoxic potential is necessary to ensure the relatively safe use of plant-derived medicines.

Scrophulariaceae is an important family of plants comprising over 200 genera and about 2500 species. It includes *Mimulus*, *Penstemon*, *Digitalis*, *Veronica* and *Verbascum* [3a]. Different members have been valued for their curative properties and are widely employed in domestic and regular medicine. At least 250 species of *Verbascum* are known. Among the species traditionally used in medicine, the most important is *Verbascum thapsus* L., commonly known as mullein, common mullein, great mullein [3b]. *V. thapsus* is distributed worldwide. In Argentina this species is abundant however; it is considered an exotic plant. Many studies carried out with plants collected in other countries, have shown that different extracts have antimicrobial, antitumor and cytotoxic activities [4a-4c]. Therefore, given the abundance of the species in Córdoba province, Argentina, cytotoxic and antiviral properties of methanolic extract of *V. thapsus* were investigated. The results of these previous studies have indicated that the extract markedly inhibits *Herpes suis* virus type 1 at non cytotoxic concentrations [5a,5b]. Since this information is hopeful, it is necessary to define the cytogenotoxic potential to ensure the use of the extract at safe levels.

The aim of this study was to determine the genotoxic and cytotoxic activities of a methanolic extract of *Verbascum thapsus*, using micronucleus test in mouse bone marrow. Evaluation of micronucleus induction is the primary in vivo test in a battery of genotoxicity tests and is recommended by the regulatory worldwide agencies to be conducted as part of product safety assessment.

The results of micronucleus (MN) test in BALB/c mice treated with different doses of the extract are summarized in Table 1. In all cases these results are expressed as mean (± standard deviation).
### Table 1: Mean of polychromatic erythrocytes with micronuclei (MNPCE) observed in bone marrow cell of female (F) and male (M) BALB/c mice treated with a *Verbascum thapsus* methanolic extract, and respective controls.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose mg/kg</th>
<th>Number of MNPCE per animal</th>
<th>MNPCE (mean ± SD)</th>
<th>PCE/NCE (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F1</td>
<td>F2</td>
<td>F3</td>
</tr>
<tr>
<td>Negative control (saline)</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>V. thapsus</em> methanolic extract</td>
<td>100</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td><em>V. thapsus</em> methanolic extract</td>
<td>300</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><em>V. thapsus</em> methanolic extract</td>
<td>500</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Positive control (cyclophosphamide)</td>
<td>20</td>
<td>11</td>
<td>14</td>
<td>11</td>
</tr>
</tbody>
</table>

Thousand cells were analyzed per animal, for a total of 6000 cells per group. SD = Standard deviation. * p< 0.001, statistically significant difference from saline group (ANOVA. Tukey’s test).

**Figure 1:** A photomicrograph of mouse whole bone-marrow smear showing nucleated as well as enucleated cells (PCEs and NCEs). One the polychromatic erythrocyte also contains micronuclei.

Examples of polychromatic and normochromatic erythrocytes unaltered, normal, and the presence of micronucleated polychromatic erythrocytes are shown in Figure 1.

The percentage frequency of MN in the groups treated with 100, 300 and 500 mg / kg of methanolic extract, which were 2.17 (±1.60), 2.00 (±0.89), 2.00 (±1.55) respectively, showed no significant differences from the saline-treated group: 2.5 (±0.55). However, there was a significant increase in the frequency of micronucleus in PCE from the positive control group treated with cyclophosphamide (Figure 2).

No citotoxicity in bone marrow was detected in the extract-treated groups. Statistical analysis of the proportion PCE / NCE revealed no differences in any study group. There were no sex-dependent changes in any treatment.

*V. thapsus* methanolic extract contains iridoid glycosides (laterioside, harpagoside, ajugol, picroside IV), three iridoid ((+)-genipin, α-gardiol and β-gardiol), one phenylethyl glycoside (verbacoside), two sesquiterpenes (buddlindeterpene A and buddlindeterpene B), one diterpene (buddlindeterpene C), and one biflavonoid (amentoflavone) [6].

Therefore, the results obtained in the present study allow concluding that the methanolic extract of *Verbascum thapsus* does not contain genotoxic and cytotoxic compounds since its administration in mice at doses of 100, 300 and 500 mg/kg, showed no evidence of genotoxicity or cytotoxicity in vivo. The extract did not produce a significant increase in the frequency of MNPCE in bone marrow and neither altered the relationship PCE / NCE respect to negative control.

These cytogenotoxic findings contribute the preclinical knowledge of methanolic extract of *V. thapsus* and provide security in its use as herbal medicine.

**Experimental**

**Plant material and extraction:** Aerial parts of *Verbascum thapsus* L. were collected in San Luis province, Argentina. The plant material was identified by Ing. Luis A. del Vitto. A voucher specimen (N° #514) was preserved and deposited in herbal library of the “Herbario de la Universidad Nacional de San Luis, Argentina”. The leaves were dried and chopped finely using a blender. Eight hundred grams of dried material were successively extracted with 3.5 L of the following solvents: n-hexane, chloroform and methanol at room temperature for 48 h. The evaporation of the extracts in vacuum at 40°C yielded...
the hexane, chloroform and methanol extracts. The methanolic extract was dissolved in saline solution and subsequently diluted to appropriate working concentrations.

**Animal’s treatments:** Two months old male/female BALBc mice weighing ca. 20 g were intraperitoneally injected with a single dose of *V. thapsus* methanolic extract (volume 0.2 ml). Three doses were selected (100, 300 and 500 mg/kg) considering previous citotoxicity data obtained with Vero cells. Cyclophosphamide (Sigma) at 20 mg/kg and saline solution were used as positive and negative controls respectively.

**Mouse bone marrow micronuclei assay:** Six mice per dose were sacrificed at 24 h post-injection and femurs were removed. Femurs were prepared for the boned-marrow micronucleus test as previously described [7a]. Slides were stained with May-Grünwald and Giemsa solutions [7b] which maximized the differentiation between the polychromatic (PCE) and normochromatic (NCE) erythrocytes. To determine index of genotoxicity the number of micronucleated polychromatic erythrocytes (MNPCE) was obtained at an average of 1000 PCE, counted per animal per dose. In order to evaluate any cytotoxic effect of extract, the ratio of PCE/NCE was determined in the same sample. Statistical significance was determined by analysis of variance (ANOVA), applying software GraphPad Prism 5.0.

**Acknowledgments** - The authors are grateful to CONICET, MinCyT of Córdoba, Universidad Nacional de Río Cuarto and PICTOR program, BID 1728 /OC-AR for financial support. We also would like to thank Ing. Luis A. del Vitto for taxonomic determination of the plant specimen.

**References**


Study of Antiviral and Virucidal Activities of Aqueous Extract of *Baccharis articulata* against *Herpes suis* virus

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*Baccharis articulata* is native of América and traditionally used for the treatment of digestive disorders and urinary infections. Cytotoxicity of aqueous extracts of *B. articulata* was investigated in Vero cells. As the maximal non cytotoxic concentration has been established, this concentration has been used to evaluate antiviral and virucidal activities against *Herpes suis virus type 1*, member of the same subfamily of *Herpes simplex virus*. Aqueous extracts of *B. articulata* exhibited more than 95% of virucidal activity. These findings support their potential application as a disinfectant or antiseptic with low toxicity and provide a valuable knowledge to ethnopharmacology properties of *Baccharis articulata*.

**Keywords:** cytotoxicity, virucidal, antiviral activity, aqueous extract, *Baccharis articulata*, *Herpes suis* virus.

*Baccharis articulata*, commonly known as carqueja, frequently found in the hills region of Córdoba province of Argentina, exhibit antioxidant, antibacterial, anti-HIV and antifungal abilities, [1a-1c]. In treatment of herpetic infections one or more drugs currently available were used, but both continuous use and self-medication promote the development of resistance and tolerance of these viruses [2]. This background encouraged the study of cytotoxic, antiviral and virucidal activities of aqueous extracts of *B. articulata* against *Herpes suis type 1*, virus closely related to *Herpes simplex types 1* and *2*. The aim of the present study was to determine concentration of aqueous extracts that do not affect monolayer cell and to be used in later assays. Therefore cytotoxic effect on Vero cells of aqueous extracts was evaluated by daily microscopic observation of treated cells to determine the MNCC (Maximal Non Cytotoxic Concentration). The MNCC values were 1000 µg/mL and 600 µg/mL for cold aqueous extract (CAE) and hot aqueous extract (HAE), respectively. The cultures exposed to extract concentrations lower than MNCC exhibited morphology similar to control cultures. The cytotoxic effect was characterized by retraction cell and disruption of cell monolayer. Virucidal and antiviral assays performed at different stages of virus replication revealed percentages of inhibition shown in Table 1. The CAE inhibited 25 and 33% viral replication when the extract was added at 1000 µg/mL (MNCC) during the viral adsorption and later that step, respectively. The HAE, at 600 µg/mL, demonstrated to exert the inhibitory activity (54%) during the stage of viral adsorption and penetration. This value showed that *Herpes suis* virus were more sensitive at HAE than CAE of *B. articulata*.

Only the pre-treatment of Vero cells with HAE slightly interfered *Herpes suis type 1* adsorption to cellular receptor. Therefore, both extracts would not induce antiviral state in the cells and neither would interfere to the mechanism of endocytosis used in the entry of virus into the cell. The CAE and HAE demonstrated to exert strong extracellular virus inactivation mostly when the assays were carried out with extracts at double concentration of MNCC (97.8 and 96% respectively). Results obtained in this work allow concluding that the aqueous extracts of *B. articulata* exert slight antiviral activities against *Herpes suis virus type 1*.

It is known that plant aqueous extracts, among other components, contain anthocyanins, saponins, polypeptides and terpenes [3a]. Studies on the chemical composition of *B. articulata* have reported presence of several terpenes such as articulina, germacrene and α-pinene [3b]. These compounds in other plant species (*Glyptopetalum sclerocarpum, Thymus vulgaris*) have exhibited antiviral...
activity [3a,3c]. As a consequence they could be responsible of the antiviral activity demonstrate in this work. Furthermore, additional studies are needed in order to identify which compounds could be responsible for this effect and how they exert antiviral action.

**Experimental**

**Plant material:** *Baccharis articulata* was collected in the hills of Cordoba, Argentina. Taxonomic identification was performed by Prof. Margarita Grosso of the Universidad Nacional de Río Cuarto. A specimen of the plant was deposited (Nº RCV 1810) in the Herbarium. Dried aerial parts (branch and leaf) (15 g) were submitted to extraction with 700 mL of cold water at 4°C for 2 days (cold aqueous extract, CAE) and at 70°C for 2 days (hot aqueous extract, HAE). The extracts were filtered and lyophilized.

**Viruses and cells:** Vero cells (African green monkey kidney) were grown in Eagle’s minimum essential medium (MEM) supplemented with 8% FCS, 1% gentamicin and 1% L-glutamine and maintained at 37°C in 5% CO₂ atmosphere. *Herpes suis* virus type 1 strain RC/79 was isolated in Río Cuarto in 1979 [4a].

**Cytotoxicity assays:** Confluent cell monolayers cultivated in 96-well culture plates were treated with different concentrations of extracts and incubated at 37°C for 72 h. At this time, maximal non-cytotoxic concentration (MNCC) was determined by microscopic observation.

**Antiviral assays:** The antiviral activity of tested extracts was evaluated at different stages of viral replication by plaque reduction method. Virus titres were calculated by plaque forming units per mL (PFU/mL) [4b]. The percentage of inhibition was calculated as the ratio between virus titres in treated cells and in untreated cells.

**During adsorption and viral penetration:** Monolayer cells grown in 24-well culture plates were incubated for 90 min with *Herpes suis* virus type 1 (10⁵ PFU/mL) in combination or not with extract at MNCC. Then, residual virus was discarded. The cells were overlaid with an overlay medium containing 1% of methylcellulose. The plates were further incubated at 37°C for 72 h. Later cell monolayer was fixed with 10% formalin. The virus plaques formed on Vero cells were stained with 1% crystal violet. Percentage of viral inhibition was determined.

**Post-adsorption and penetration of virus:** Confluent monolayer of Vero cells grown in 24-well culture plates were infected with *Herpes suis* type 1 (10⁵ PFU/mL) and incubated at 37°C for 90 min. After residual virus was removed, the cells were covered with the overlay medium containing 1% of methylcellulose and extract at MNCC, and incubated at 37°C for 72 h. Percentage of viral inhibition was determined.

**Pretreatment:** Monolayer cells grown in 24-well culture plates were incubated for 2 h with extract at MNCC. After the extract was discarded, culture cells were inoculated with *Herpes suis* type 1 (10⁶ PFU/mL) and incubated at 37°C for 90 min. The remainder virus was discarded and the cells were incubated with the overlay medium containing 1% of methylcellulose at 37°C for 72 h. Percentage of viral inhibition was determined.

**Virucidal activity assay:** Viral suspensions (10⁵ PFU/mL) were incubated with extract at MNCC and at double concentration. After incubation of virus at 37°C for 2 h, monolayer cells grown in 24-well culture plates were infected with treated virus. The infected cells were incubated at 37°C for 90 min. The remainder virus was discarded and the cells were incubated with overlay medium containing 1% of methylcellulose at 37°C for 72 h. Percentage of viral inactivation was determined.

**Acknowledgments** - The authors thank Universidad Nacional de Río Cuarto and PICTOR program, BID 1728 /OC-AR for financial support. We are grateful to Prof. Margarita Grosso for help in identification of the plant specimen.

**References**


Evaluation of Cytogenotoxic Effects of Cold Aqueous Extract from Achyrocline satureioides by Allium cepa L test

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Achyrocline satureioides ("marcela del campo") is native to America. Numerous investigations have reported several bioactive properties such as anti-inflammatory, hepatoprotective, immunomodulatory, antimicrobial and antiviral. Nowadays, few medicinal plants have been scientifically evaluated to test its safety, efficacy and potential benefits, despite the great public interest in these herbs.

The aim of this work was to evaluate the cytotoxic and genotoxic activities of cold aqueous extract obtained from A. satureioides using Allium cepa L test. The results demonstrated the absence of genotoxicity of the extract. Only higher concentrations induced cytotoxicity but interestingly this effect was reversible and was not associated with mutagenicity. The contribution of this research provides assurance of safety in the application of Achyrocline satureioides in treatment of microbial diseases and other pathologies helping to define selective toxicity.

Keywords: Achyrocline satureioides (Lam.) DC, Allium cepa L test, cytogenotoxicity, cold aqueous extract.

Asteraceae (Compositae) family includes some of the oldest and most valued plants for medicinal purposes [1a]. It is known that certain genus of this family contain toxic compounds such as tannic, cyanide, formic and malic acid [1b]. Achyrocline satureioides (Lam.) DC. is a relevant species that belongs to the family Asteraceae. This plant, commonly known as “marcela del campo”, is native to America and extends throughout the continent, as well as Europe and Africa. In our country it is often found in the hills of Córdoba, San Luis and Buenos Aires [2]. Numerous investigations have reported several bioactive properties, such as anti-inflammatory [3a], sedative [3b], hepatoprotective [3c], antioxidant [3d,3e], immunomodulatory and antimicrobial [3f], antitumor [3g], antiviral [3h,3i,3j] and photoprotective [3k].

Nowadays, few medicinal plants have been scientifically evaluated to test its safety, efficacy and potential benefits, despite the great public interest in these herbs [4]. Regulatory worldwide authorities require information on the genotoxic potential of new drugs as part of the safety evaluation process. Allium cepa L test allows assessment of toxicity of substances in terms of macroscopic parameters such as growth and form of roots and, evaluation of genotoxicity from microscopic parameters such as types and frequencies of chromosomal aberrations and abnormal cell divisions.

For all previously described, it is of paramount importance to know the cytotoxicity and genotoxicity of extracts from A. satureioides. In order to evaluate the cytotoxic and genotoxic activities of cold aqueous extract (CAE) obtained from Achyrocline satureioides experiments were performed using Allium cepa L test with modifications.

Table 1 summarizes the results of the effects of CAE of A. satureioides on root of Allium cepa. Taking into account the number of bulbs roots, the different concentrations of CAE induced normal development, similar to control.

The analysis of length of roots treated with CAE for 5 days showed statistically significant differences (p<0.05) among all treatments vs. negative control and absence of dose-response relationship. The inhibition of roots length was ≥ 50% (p<0.05) for all tested concentrations. Considering the bulbs treated for 2 days (with reversion), there were no significant differences between treatments of 0.5 and 2 mg/mL vs. negative control. By the contrary, there were significant differences for the remaining concentrations of CAE compared to negative control (Figure 1). The average root length for treatment of 2 days with CAE (with reversion) was always higher than of 5 days, although there was no statistically significant difference within the same concentration considered. The concentration of 0.5 mg/mL was the exception because it
Table 1: Macroscopic parameters analyzed in roots of *Allium cepa* L after treatment with different concentrations of CAE of *A. satureioides* for 5 and 2 days.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>TREATMENTS (n = 4 bulbs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C (-) C (+) 0.5 1 2 3 4 C (-) C (+) 0.5 1 2 3 4</td>
</tr>
<tr>
<td>Mean root number</td>
<td>54 37 67 41 46.5 60.5 56 48.5 34.5 43.5 62.5 39 38 39.5</td>
</tr>
<tr>
<td>Mean root length (mm) ± SEM</td>
<td>20.41 ±0.65 7.20 ±0.72 6.61 ±0.45 25.91 ±0.94 18.98 ±0.64 11.51 ±1.03 7.91 ±0.66 10.95</td>
</tr>
<tr>
<td>Abnormalities</td>
<td>Ha 8 32 11 0 2 10 3 10 20 7 5 10 6 4</td>
</tr>
<tr>
<td></td>
<td>Geb 0 0 0 10 14 16 25 0 0 0 15 6 15 37</td>
</tr>
<tr>
<td></td>
<td>Nec 0 0 16 17 6 4 0 0 0 12 17 1 0 18</td>
</tr>
<tr>
<td></td>
<td>Tud 10 5 2 0 0 0 1 9 5 0 1 3 0 0</td>
</tr>
</tbody>
</table>

*H*: hook; *Geb*: gelling; *Nec*: necrosis; *Tud*: tumor.

**Figure 1**: Roots length of bulbs of *Allium cepa* L treated with different concentrations of CAE of *A. satureioides* for 5 days vs. 2 days (with reversion).

**Figure 2**: Comparison of mitotic indices 1, 2 and 3 of the roots of the *Allium cepa* bulbs treated with different concentrations of CAE of *Achyrocline satureioides*.

... showed a statistical difference (p<0.001), indicating the recovery of the roots by mineral water action, after treatment with the extract. This result suggests that bulbs would have the ability to recover from damage induced by this concentration of extract.

Respect to macroscopic abnormality, the study revealed the presence of hooks and tumours in negative control with low frequency. These spontaneous changes are normal and concordant with other authors [5a,5b]. Positive control, paracetamol (acetaminophen 0.3 mg/mL), showed a high incidence of root hooks and also induced the appearance of tumours. None of the roots treated with CAE induced the development of hooks. The results of all treatments with the extract were markedly different to the effect caused by paracetamol.

Gelling and necrosis were present with varying frequency, having the first one the highest incidence. Roots treated with extract did not show significant values in the number of tumours. Pigmentation was observed in most of roots treated due to intense colour of extract.

Statistical analysis of mitotic index of bulbs treated with CAE did not show significant difference between negative control and treatments with 0.5 and 1 mg/mL, for 2 days (MI 1) and 5 days (MI 2). So, these concentrations did not exert toxicity. In contrast, there were significant differences between negative control and treatments of 2, 3 and 4 mg/mL. These concentrations would exert an inhibition of cell division. For the MI 2, the trend of the curve revealed a drastic reduction for MI% at the concentrations 2, 3 and 4 mg/mL. This behaviour could be due to these concentrations are extremely toxic when they are used for 5 days. The values of MI 3 (treatment with reversion) did not show statistically significant differences between treatments, neither between these treatments and negative control, indicating the ability of roots to recovery of the toxic action of CAE. The comparative analysis of MI 1 vs. MI 3, for each concentration, showed significant difference (p<0.5), indicating that the damage was reversed (Figure 2).

Analysis of phases index for bulbs treated for 2 and 5 days showed that cell division in roots treated with 0.5 and 1 mg/mL was similar to negative control. By the contrary, cell cycle stages were modified in the bulbs treated with 2, 3 and 4 mg/mL. Prophase was the most observed phase, demonstrating an arrest of cell division at this stage. Application of CAE at these concentrations would be affecting the formation of chromosomes and avoiding their placement in equatorial plane of the cell.

Statistical analysis of the results obtained in treatment with reversion did not show significant differences in phase index of treated roots vs. negative control, indicating the reversion of the changes induced by CAE of *A. satureioides*. These results point out that mitosis was normally developed as it confirms by the value of MI 3, (Figure 3).
Cytogenotoxic evaluation of the extract of *Achyrocline satureioides*

**Figure 3**: Phase index of cell division in roots treated with CAE of *Achyrocline satureioides* (A) MI 1, (B) MI 2, and (C) MI 3.

Microscopic evaluation of cells showed physiological and clastogenic aberrations as previously described [6]. The physiological aberrations observed were c-mitosis and, sticky and delayed chromosomes, while the other ones presented chromosomal bridges. These alterations were found more often in roots with normal cell division. Similar figures to apoptotic bodies were observed in interphase cells treated for 2 and 5 days with CAE, (Figure 4).

This study demonstrated the absence of genotoxicity of CAE from *A. satureioides*. High concentrations of the extract induced citotoxicity but this effect was reversible and was not associated with mutagenicity. The contribution of this research provides assurance of safety in the application of *Achyrocline satureioides* in treatment of microbial diseases and other pathologies helping to define selective toxicity. Nowadays, studies referred to chemical characterization of CAE from *A. satureioides* are in progress.

**Experimental**

**Plant material**: Healthy plants of *A. satureioides* species were collected manually from Villa Jorcore, southern Córdoba hills in 2009. The plant material was identified by Dr. Luis Del Vitto, Facultad de Farmacia y Bioquímica, Universidad de San Luis, San Luis, Argentina. A voucher specimen was deposited in the Herbarium of the University of San Luis (Nº 6362).

**Obtention of cold aqueous extract**: Dried aerial vegetal parts (15 g) were submitted to extraction with 700 mL of cold water at 4°C for 2 days. The mixture was filtered and lyophilized. This extract was identified as cold aqueous extract (CAE).

**Determination of genotoxic activity of cold aqueous extract from *A. satureioides* by the *Allium cepa* test**: *Allium* test as described [7a,7b,7c] was developed with some modifications. Qualitative and quantitative changes, macro and microscopic, induced by treatment with CAE in plant cells were assessed.

Onion root tips of *Allium cepa* L grown in mineral water, in darkness, with aeration and constant temperature of 25 ± 0.5 °C were employed. CAE was assayed at 0.5, 1, 2, 3 and 4 mg/mL of mineral water. Positive (paracetamol 0.3 mg/mL) and negative (mineral water) controls were included in the system. Extract concentrations were applied for different times: 2 and 5 days, and 2 days followed by 3 days with water (reversion). At the end of each treatment, 2-3 root tips from these bulbs were cut and fixed in a mixture of absolute alcohol:glacial acetic acid (3:1, v/v). These roots were hydrolyzed in 1N HCL for 5 minutes after which they were washed in distilled water. Two root tips were then squashed on each slide, stained with acetocarmine for 10 min and cover slips carefully

**Figure 4**: Microphotographs of meristematic cells of root tips of *Allium cepa*. Squash preparations, stained in acetocarmine and observed in light microscope. (A) A typical view of different size, shape and basophilia of nuclei and interphase and mitotic phases; (B) cells with sticky chromosomes; (C) cells with delayed chromosomes; (D) cells with chromosomal bridges; (E) physiological aberrations: c-mitosis; (F) figures similar to apoptotic bodies in interphase cells.
lowered on to exclude air bubble. The cover slips were sealed on the slides with clear fingernail polish as suggested by [8]. These roots were reserved for evaluating the cytogenetic aberrations (microscopic). Six slides were prepared for each concentration and controls (at 1000 cells per slide) were analyzed at ×1000 magnification for induction of chromosomal aberration. The mitotic index was calculated as the ratio between the number of cells in division and 1000 observed cells. In addition, phases indices were also calculated [7a,7c]. The percentage cell divisions in root tips of root number, length and presence of abnormalities, were evaluated.

Statistical significance was determined by analysis of variance (ANOVA) using GraphPad Prism 5.0 software.

Acknowledgments - The authors thank to CONICET, MinCyT of Córdoba, Universidad Nacional de Río Cuarto and the PICTOR programme, BID 1728 /OC-AR, for providing financial support. The authors are also grateful to Dr Luis Del Vitto for taxonomic classification of the plant specimen.

References


Previously, macroscopic changes of the roots in terms of shape and size were recorded. Macroscopic changes of the roots in terms of root number, length and presence of abnormalities, were evaluated.
Toxic Plants Used in Ethnoveterinary Medicine in Italy

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This study was conducted to document the use of toxic or potentially toxic plants for the treatment of ailments in livestock and pets in ethnoveterinary practice in Italy. More than 250 of the entities used (81% for curative purposes) can be toxic unless dosed appropriately. Many (55%) are dietary supplements. The list included 186 species (45%) for internal and 175 (55%) for external use, many used in places where animals are kept. The species belong to 71 families, among which the Fabaceae predominate. The purpose of the study was to provide information that can be validated and associated with correct determination, permitting even potentially dangerous plants to be used in veterinary practice.

Keywords: Toxic plants, ethnoveterinary medicine, Italy.

The number of plants used in Italy to treat domestic animals was previously reported to be 260 [1] and is now more than 500 [2a-c]. These plants include fungi, ferns, gymnosperms and angiosperms. Most are dietary supplements, chosen for their positive effect on growth and ease of administration. Many are used for prevention, but more than 60% of all uses are curative. Some plants are valued against parasites and as repellents, others for their toxic effects on fish. Other plants are considered to have magic properties. In this study we analyze use of toxic and potentially toxic plants in ethnoveterinary medicine in Italy.

Bibliographic and unpublished data in our database were examined and screened for toxic and potentially toxic plants. Shepherds and farmers generally avoid administering such plants, though many were used traditionally and considered relatively safe. More than 250 toxic or potentially toxic plants were identified, about 50% of all species used in ethnoveterinary medicine. Most (81%) are used for curative purposes and can be toxic if not appropriately dosed. A good number (55%) are dietary supplements. 186 species (45%) are used internally and 175 externally (55%) (Table 1). The active ingredients, largely glycosides and alkaloids, are listed in Table 2. The types of animals treated were cattle (23.93%), sheep (10.73%), poultry (9.5%), horses (7.83%), pigs (6.38%), goats (5%), dogs and cats (3.19%), rabbits (2.18%) and animals in general (27%). Many plants were used in places where animals are kept, such as stables, chicken houses, drinking troughs (3.92%) (e.g. Alnus glutinosa, Artemisia absinthium, Datura stramonium, Nerium oleander, Sambucus ebulus, S. nigra).

Toxic plants belong to 71 families, 62 of which are Angiosperms, one Gymnosperm, seven Pteridophytes, one fungus (Amanitaceae). The most common families were Fabaceae, followed by Asteraceae, Ranunculaceae, Labiatae, Euphorbiaceae, Apiaceae and Liliaceae. This differs slightly from the general statistics for ethnoveterinary medicine, which indicate species of the family Asteraceae to be the most numerous [3a,3b], as found in the Mediterranean area in general [4].

The toxic or potentially toxic species identified had largely curative uses. The main methods of administration were such, decoction, crushed and macerated. The main active ingredients were glycosides and alkaloids, with saponins and triterpenoids accounting for more than 13%, followed by tannins, volatile oils, terpenoids and resins [5a-5d]. In many species, the toxic substances are not distributed throughout the plant: many are concentrated in certain organs while the rest of the plant is innocuous; sometimes substances are influenced by the vegetative period or age of the plant. Toxic substances are often more abundant in certain phases of the life cycle, usually in seeds and juvenile plants, and in certain phases of the vegetative cycle, usually spring. Sometimes plants can be toxic if not appropriately dosed, if infected by fungi, if they accumulate harmful substances or if combined with conventional remedies [6a-6c].

Toxic plants were certainly used with caution in ethnoveterinary traditions, because loss of an animal was a serious event. Their use cannot be encouraged. The aim of the present study was to provide information that can be validated and associated with correct determination, permitting even potentially dangerous plants to be used in...
veterinary practice. The study is part of a series concerned with the enormous heritage of empirical experience and knowledge still traceable in Italy. Such documentation is useful to save animal lives, for phytochemical-pharmacological research and for the conservation of native flora. Benefits may range from local to European community level.

Table 1: Uses and examples of toxic or potentially toxic plants used in ethnoveterinary medicine in Italy.

<table>
<thead>
<tr>
<th>Internal use</th>
<th>Vermicides</th>
<th>Ichthyotoxic</th>
<th>Flea, mouse and mule repellents</th>
<th>Pesticides and repellents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrostemma githago</td>
<td>Allium sativum</td>
<td>Achiimia</td>
<td>Aconitum napellus</td>
<td>Colchicum autumnale</td>
</tr>
<tr>
<td>Amanita muscaria</td>
<td>Artemisia absinthium</td>
<td>Anthirinum mari</td>
<td>Atus glutinos</td>
<td>Anemone hortensis</td>
</tr>
<tr>
<td>Anemone hortensis</td>
<td>A. vulgaris</td>
<td>Conium</td>
<td>Artimisia absinthium</td>
<td>Colchicum autumnale</td>
</tr>
<tr>
<td>Crocus neapolitanus</td>
<td>Calamintha nepeta</td>
<td>Cyclamen</td>
<td>Calamintha nepeta</td>
<td>Cestrum parvum</td>
</tr>
<tr>
<td>Daphne mezereum</td>
<td>Cucurbita pepo</td>
<td>Daphne</td>
<td>Conium maculatum</td>
<td>Daphne laureola</td>
</tr>
<tr>
<td>Dryopteris filix-mas</td>
<td>Dryopteris filix-mas</td>
<td>Euonymus</td>
<td>Delphinium consolida</td>
<td>Delphinium consolida</td>
</tr>
<tr>
<td>Euphorbia dendroides</td>
<td>Fraxinus ornus</td>
<td>Euphorbia characias,</td>
<td>D. staphysagria</td>
<td></td>
</tr>
<tr>
<td>E. laethris</td>
<td>Gledchona hederacea</td>
<td>E. dendroides, E. helioscopia,</td>
<td>Delphiniun consolida</td>
<td></td>
</tr>
<tr>
<td>Eucalyptus resinifer</td>
<td>Juglans regia</td>
<td>E. laethris, E. paralias,</td>
<td>Laburnum alpinum</td>
<td></td>
</tr>
<tr>
<td>Helleborus sp.pl.</td>
<td>Mercurialis annua</td>
<td>E. pinea, E. pityusa,</td>
<td>L. anagyroides</td>
<td></td>
</tr>
<tr>
<td>Ilx aquilolumn</td>
<td>Polypondium australe</td>
<td>Juglans</td>
<td>Lupinus albus</td>
<td></td>
</tr>
<tr>
<td>Ligustrum vulgaris</td>
<td>Ruta angustifolia,</td>
<td>Marrubium</td>
<td>Ruscus aculeatus</td>
<td></td>
</tr>
<tr>
<td>Glechoma hederacea</td>
<td>R. chalepenis</td>
<td>Oenanthe</td>
<td>Ruta graveolens</td>
<td></td>
</tr>
<tr>
<td>Mercurialis annua</td>
<td>R. graveolens</td>
<td>Pistacia</td>
<td>Tanacetum vulgar</td>
<td></td>
</tr>
<tr>
<td>Papaver rheas,</td>
<td>Santolina insularis</td>
<td>Plumbago</td>
<td>Veratum album</td>
<td></td>
</tr>
<tr>
<td>P. somniferum</td>
<td>Sempervivum tectorum</td>
<td>Sambucus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polypondium australe</td>
<td>Verbascum thapus</td>
<td>Solarium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. vulgar</td>
<td>Teucrum chamaedrys</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polystichum setiferum</td>
<td>Thapsia gargarica</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bhannus sp.pl.</td>
<td>Urginea maritima</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>Verbascum pulvaulentum,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veratum alburnum</td>
<td>V. sinuatum, V. thapus</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Active principles of toxic or potentially toxic plants used in ethnoveterinary medicine in Italy.

<table>
<thead>
<tr>
<th>% no. of species</th>
<th>Notes (active compounds and the no. of species that contain them)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosides</td>
<td>25.82 110 coumarin and furfurocoumarin (11); saponic glycossides (13)</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>19.48 83</td>
</tr>
<tr>
<td>Saponins and triterpenoids</td>
<td>13.38 57 saponins (non saponic glycossides) (51)</td>
</tr>
<tr>
<td>Tannins</td>
<td>8.92 38</td>
</tr>
<tr>
<td>Volatile oils, terpenoids, resins</td>
<td>8.22 35 resins (5)</td>
</tr>
<tr>
<td>Organic acids</td>
<td>6.10 26 oxalates (13)</td>
</tr>
<tr>
<td>Pigments (flavonoids)</td>
<td>4.23 18</td>
</tr>
<tr>
<td>Diterpenoids</td>
<td>3.29 14</td>
</tr>
<tr>
<td>Phytosterogens</td>
<td>3.05 13 phytoestrogens, phytostersols</td>
</tr>
<tr>
<td>Nitrates and nitrates</td>
<td>2.11 9</td>
</tr>
<tr>
<td>Lignans and lignins</td>
<td>2.11 9</td>
</tr>
<tr>
<td>Toxic if infected with fungus or in cases of accumulation</td>
<td>1.88 8</td>
</tr>
<tr>
<td>Enzymes</td>
<td>1.41 6 thiaminase, urease, ficin</td>
</tr>
</tbody>
</table>

References

Diagnosis of Public Programs focused on Herbal Medicines in Brazil

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The present study is aimed to diagnose the current public programs focused on herbal medicines in Brazil by means of in loco visits to 10 programs selected by means of questionnaires sent to 124 municipalities that count on herbal medicine services. The main purpose of the implementation of program programs is related to the development of medicinal herbs. 70\% of them are intended for the production of herbal medicines and 50\% are aimed to ensure the access of the population to medicinal plants and or herbal medicines. The initiative of the implementation of these programs was related to the managers (60\%). The difficulties in this implementation were due to the lack of funding (100\%) of the programs. In 60\% of the programs, the physicians did not adhere to herbal medicine services due to the lack of knowledge of the subject. Training courses were proposed (80\%) to increase the adhesion of prescribers to the system. Some municipalities use information obtained from patients to assess the therapeutic efficiency of medicinal plants and herbal medicines. Of the programs underway, cultivation of medicinal plants was observed in 90\% and 78\% of them adopt quality control. In most programs, this control is not performed in accordance with the legal requirements. The programs focused on medicinal plants and herbal medicines implemented in Brazil face some chronic problems of infrastructure, management, operational capacity and self-sustainability, which can be directly related to the absence of a national policy on medicinal plants and herbal medicines.

Keywords: Medicinal plants, herbal medicines, public health.

The use of medicinal plants dates back thousands of years. However, the traditional allopathic medicine faces increased competition from alternative treatments due to the production of herbal medicines in accordance to the standards recommended by the legislation these days [1a]. The development of quality assurance within the pharmaceutical industry involves the concern with the production of seeds, planting, harvesting, drying, extraction, production practices and storage of drugs, and all these processes must be carried out according to a strict quality control, pre-clinical and clinical trials and data record. Quality assurance has enabled the health professionals to prescribe safely herbal medicines that the population has been taking for quite a long time [1b].

The use of medicinal plants for therapeutic purposes (both the traditional and popular usage of these plants and their use based on scientific evidence) is a common practice nowadays. The use of plants to mitigate symptoms or cure diseases has been a very common practice for long, especially because the resources used in the production of drugs are not available according to the needs of the population, or else, many of these drugs are currently being developed. These historical aspects of the use of medicinal plants clarify the importance of the interaction between local communities and their natural environment to the entire society in the present and in the future [1c]. At the same time, there is the urgent need to investigate the abundant biodiversity and, particularly, the medicinal flora, its proper and rational use, in order to find out how medicinal plants can be used by the population for medicinal purposes in an efficient and safe way [2a].

Data generated from the questionnaires completed has demonstrated the existence of well-structured programs, and the activities carried out within these programs involved pharmaceutical care, corroborating the expansion and effectiveness of the National Policy of Integrative and Complementary Practices at the SUS (Brazilian Public Health System) [2b]. Thus, visits to 10 programs focused on herbal medicines were scheduled and had excellent results, as demonstrated in the previously described research. [2c]. The visits were conducted from December 2008 to December 2009 and were aimed to collect information on the activities developed within the scope of the programs, as well as information on their
implementation, difficulties faced and solutions encountered to ensure the maintenance of the referred programs. The programs selected for visit were carried out in all the Brazilian regions as follows: 1 in the Southern region, 5 in the Southeastern region, 2 in the Center-West region, 1 in the Northeastern region and 1 in the Northern region. The selection was based on the data generated from the questionnaires sent to 124 Brazilian municipalities. It was found that in all programs visited (10) the main purpose of their implementation was the development of medicinal plants. In 70% of them the purpose was the production of herbal medicines, 50% were aimed to ensure the access of the population to these medicinal drugs and only one program (10%) was aimed to decrease the healthcare costs incurred by the population. 60% of the programs stemmed from the initiative of municipal governments, 50% were suggested by experts, 20% by users and 30% had different origins. The same results were obtained when the initiatives were carried out by more than one group. Regarding the inclusion of herbal medicine in pharmaceutical care programs in the municipalities, such inclusion was found to occur in 70% of them and in 30% of them it did not occur. These findings may have a direct influence on the maintenance of the programs, since funding issues can compromise the survival of those programs not included in the pharmaceutical care programs of the municipalities.

The difficulties in the implementation of these programs included: lack of funding (100%), poor adhesion by prescribers (60%), lack of space (70%) lack of qualified professionals (40%) and 1 (10%) mentioned the lack of interest of the population. Still regarding the difficulties encountered, it was found that 90% of the programs were funded by the municipality and 40% were funded by the state, but only 1 (10%) was exclusively funded by the state. Regarding the cultivation of medicinal plants, only 1 (10%) did not count on a garden and raw material for herbal medicines was obtained from suppliers. The selection of the medicinal species cultivated was based on literature research (100%), in an 80% of them it was also based on popular knowledge and in 40% the local species were used. Only 1 (10%) program did not count on a herbal medicine workshop, being focused on the distribution of medicinal plants. In all the programs involved herbal medicine handling a pharmacist was the technical responsible person, according to the country’s legislation. 7 out of these professionals (80%) are registered with their respective regional board of pharmacy and only 2 (20%) were not registered with their regulatory bodies. The pharmaceutical forms handled in the programs are distributed according to the number of quotations in the herbal medicine workshops: 67% of the programs deliver solutions, 22% suspensions, 55% capsules, 78% dyes, 11% elixirs, 89% syrups, 89% creams, 55% ointments and 44% liquid soaps. It has also been found that 78% of the programs that handle herbal medicines performed quality control.

The distribution of medicinal plants and herbal medicines is performed by the pharmacist in all the cases, and in some of them, it is also performed by physicians (50%), nurses (50%), technicians (40%), other professionals, such as dentists, biologists and nutritionists (20%). Therefore, in 80% of the total programs visited there was some kind of follow-up of patients taking medicinal plants or herbal medicines, 20% did not have any kind of follow-up service. However, in some of the programs that reported follow-up services, the evaluation was not recorded. Regarding efficiency assessment, 80% of the programs performed this assessment and 20% of them did not perform any efficiency assessment.

80% of the programs provide training courses to the professionals involved in herbal medicines and 20% of them do not count on any kind of training courses or activities. However, it was found that all the programs visited had educational activities targeted at the local community. One issue that has been greatly discussed with the coordinators of the programs visited was the partnership with universities or other institutions, both public and private, Only 3 (30%) of them have established partnerships, mostly with universities, and 70% have no kind of partnership. Nevertheless, most programs had tried unsuccessfully to establish partnerships, and the main reason for this was the lack of funding.

Finally, the results obtained in this study conclude that herbal medicine is getting considerable attention and has become a valuable asset that ensures the access of the population to basic health care.

References


The presence of a sildenafil derivative, the thiosildenafil, in an herbal product has been evidenced first by HPTLC and later determined by isolation and analysis of spectroscopic data. The analyzed product is nowadays marketed as dietary supplement containing herbal extracts and claimed for male and female sexual improvement. This report is noteworthy since it is clear that adulterated materials can cause serious health problems if they are consumed as herbal “natural” products, generally considered deprived of toxicity by the consumers. The use of a simple and reliable method, based on HPTLC, to determine synthetic adulterations is reported in this paper.

**Keywords:** thiosildenafil, sildenafil, dietary supplement, HPTLC, NMR.

The presence of synthetic drugs in the formulation of herbal products in order to improve the efficacy has been reported in several cases. The case seems to be very important in adulteration of herbal products marked as “natural” in cases of erectile dysfunction, since an abuse can be very dangerous for patients who unwittingly consume a synthetic potent drug instead of a botanical.

In the recent years, in particular, several cases have been reported about such herbal products produced in China [1-3]. The reported monitoring aspects were essentially based on the analytical analyses concerning the detection of the adulterants that in these cases are synthetic selective inhibitors of cyclic guanosine monophosphodiesterase-5 (PDE-5). Such active principles are the well known registered drugs, but also a plethora of analogues obtained by minor modifications to the basic structure of PDE-5 inhibitors was found. The reason of this proliferation is mainly due to the intention to escape analytical controls. Therefore, several efforts were focused on the best analytical tool to catch the adulterant, since first HPLC determination [4], followed by NMR [5], LC/MS and LC/MS/MS [6], until the recent 2D and 3D DOSY 1H NMR [7]. In this paper we report a further case of adulteration and propose the use of HPTLC as simple, direct and low cost method to detect such other adulterants, also in case of their presence in complex herbal mixtures.

Whereas sildenafil citrate (Viagra®, manufactured by Pfizer), vardenafil hydrochloride (manufactured by Levitra) and tadalafil (Cialis®, manufactured by Lilly) are well known compounds approved by the U.S. Food and Drug Administration for the treatment of erectile dysfunction, the analogues usually are not subjected to any control. In any case whereas registered products are prescription drugs and must be used under medical supervision, herbal products are self administered and generally regarded as being harmless because of their natural origin. Confusion between the two categories is therefore very dangerous.

Recently, a survey of the analysis of the presence of synthetic PDE-5 inhibitors in dietary supplements has been reported [3]. Among the seventeen considered commercial formulations of herbal drugs or dietary supplements marketed for sexual dysfunction, eight resulted adulterated, containing sildenafil, tadalafil, vardenafil, hydroxysildenafil and/or thiomethisosildenafil.

We were able to examine the content of an herbal supplement heavily marketed in internet sites as Sensual Tea or Jinshenkang, commercialized as able to rapidly solve any sexual problem of females and males. The product, also marketed in Italy, came from Spain, where now the product has been removed from the market. First, a HPTLC in dichloromethane: methanol (9:1, v/v) in a horizontal chamber (Camag 20X10) after saturation with the same mobile phase was performed. The plate showed a great spot very strong at UV lamp at 250 nm; its position and its intensity, in comparison with the other spots due to components of the herbal extracts, were an evident clue of the adulteration. Direct HPTLC comparison with sildenafil, vardenafil and tadalafil excluded the identity with these substances. Also densitometry analysis confirmed the differences.

Easily, extraction with ethyl acetate afforded a complete remove of the substance from the product and NMR spectrum of the extract resulted in a highly pure compound identified as thiosildenafil, by comparison with reported data and analysis of 2D spectra. In particular, making a $^1$H-NMR comparison with sildenafil [3], it is diagnostic the upfield shift ($\delta H = + 0.12$) of the N-Me, due the influence of the thiocarbonyl. The LC-MS analysis confirmed the presence of thiosildenafil, as well as a little quantity of sildenafil, probably as remaining product of the conversion into the thioderivative. Quantitative determination was obtained by the NMR method proposed by Balyssac [1] and gave for each package a quantity equal to that of a tablet of Viagra. Independently, HPLC analysis was performed confirming the non identity with previous compounds and affording similar quantitative results.

Herbal products spiked with synthetic drugs are dangerous to consumers and noxious for future correct developing of use of natural products. Controls must be based on simple, viable and low cost analyses. Therefore, HPTLC is a strong candidate to be used in the detection of anomalous constituents in botanicals to obtain easy clues of the adulteration.

**Experimental**

HPTLC in silica gel 60 in dichloromethane: methanol (9:1, v/v) developed in a horizontal chamber (Camag 20X10). Deposition with CAMAG Linomat IV, TLC scanner 3 WINCATS software. For the densitometric analysis a Camag TLC scanner 3 linked to winCATS software was used after multi-wavelength scanning between 250 and 400 nm. NMR by BRUKER AM400 at 400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR. Spectra were recorded in CDCl$_3$ using CHCl$_3$ signal (7.23 and 77.0 ppm) as internal reference. MS by hyphenated LC/MS LXQ Thermo Electron.

**Samples and extraction:** The analyzed samples were imported from Spain and sold as a dietary supplement in package containing white granules. The reported content of the granules was mainly sugar and a series of herbal extracts. Sildenafil, tadalafil and vardenafil were obtained from corresponding marketed products.

**HPTLC analysis:** The granules of a package (40 g) were grounded and extracted with ethyl acetate (50 mL) for 4 h. After filtration over 0.45 μm filter, the filtrate was evaporated and dissolved in 50 mL of ethyl acetate/H$_2$O 1:1 (v/v). The content of the organic phase was directly used for analysis, including HPTLC using sildenafil and vardenafil as reference standards. At UV lamp at 350 nm the RF value of the unknown constituent (0.81) resulted significantly higher than those of the two reference compounds (0.79 and 0.74 for sildenafil and vardenafil, respectively). The layers, treated with H$_2$SO$_4$ 2N spray reagent and subsequent warming at 110°C, showed other minor spots at lower R$_f$, probably due to the natural products. For complete identification and improve the quality of spectroscopic analyses, pure thiosildenafil was obtained after silica gel CC in CHCl$_3$:MeOH 40:1 of the above organic phase.

**Thiosildenafil:** The ethyl acetate extract was evaporated and the residue directly examined by NMR. $^1$H NMR (400 MHz, CDCl$_3$) and ITMS data were in accordance with reported ones [7].

**References**


Hypolipidemic Effect of Seed Oil of Noni (Morinda citrifolia)

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Morinda citrifolia, has been reported to possess different biological activities and almost all parts of this have been studied phytochemically. However there are few studies on the seeds of fruit. The objective of present study was investigated the effect to Noni Seed Oil (NSO) on serum lipid levels in normolipidemic and hyperlipidemic induced mice. We find that administration of noni oil causes a reduction in total cholesterol and triglycerides levels in both models. However hypolipidemic effect is higher when hyperlipidemia is presented.

Keywords: Morinda citrifolia seed oil, Atherogenic Index, Linolenic acid, hypolipidemic effect, tyloxapol.

Morinda citrifolia L. (Rubiaceae) commonly known as noni, is an evergreen tree may reach heights of 3 to 8 m tall. Its leaves range from 10 to 45 cm long and it bears tubular white flowers and a green fruit. This fruit turns yellow and then white as it ripens, has a pungent odor and contains seeds of about 3 mm in length. This plant is native to Asia, Australia and Polynesia [1]. The plant is used in the treatment of arthritis, headaches, digestive problems, diabetes mellitus, high blood pressure, and angina pectoris among others [2]. The Leaves, steam, root, fruit and seeds of noni are used in various forms such as capsules, teas, juice and oil [2,3].

Due to the great popularity of this plant many phytochemical studies have been carried out in which have reported compounds as iridoids, anthraquinones phenolics, glycosides of fatty acids and alcohols, cumarins, flavonoids, alkaloids and terpenes [4]. Regarding the biological activity of this specie, the antimicrobial effect was the first observed property [5], however other effects like antitubercular [6], hypoglycemic [7], anti-inflammatory [8], antitumor [9] and analgesic [10] have been reported.

Among these one evaluated the toxicity and nutritional value, as well as the determination of the fatty acid composition to assess if it is usable as edible vegetable oil. Seeds constitute 2.5% of the whole fruit and are considered a waste in the industrial process for making juice [3]. Although anthraquinones and fatty acids such as arachidonic and palmitoleic have been isolated from these seeds, it has not been established whether they show any biological activity [1].

Hyperlipidemic is defined as elevated lipid levels in plasma, and represent one of the factors associated with cardiovascular diseases, which are a worldwide death cause [11-13]. Treatment of dyslipidemia reduces cardiovascular events. The modern pharmacological therapy for abnormal lipids is effective but is expensive and it is associated with side-effects leading to patient incompliance. For this reason, our work evaluates the effect of NSO on lipid levels (total cholesterol (chol), triglyceride (Tg), high density lipoprotein (chol-HDL), in normolipidemic and hyperlipidemic mice. Castelli’s Atherogenic Index (AI) was calculated for determined risk factor of cardiovascular disease with noni seed oil consumed.

Table 1: Gas chromatographic retention times (Rt) and molecular weights of fatty methyl esters from NSO.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rt (min)</th>
<th>M+ (amu)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl palmitate</td>
<td>38.5</td>
<td>270</td>
<td>9.4</td>
</tr>
<tr>
<td>Methyl palmitoleate</td>
<td>39.2</td>
<td>268</td>
<td>0.7</td>
</tr>
<tr>
<td>Methyl stearate</td>
<td>42.5</td>
<td>298</td>
<td>4.2</td>
</tr>
<tr>
<td>Methyl oleate</td>
<td>43.1</td>
<td>296</td>
<td>15.9</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>44.2</td>
<td>294</td>
<td>67.8</td>
</tr>
</tbody>
</table>

Analysis of NSO
The total yield of oil for two extractions of dried seeds was 12%. GC-MS analysis of the FAME (fatty acid methyl esters) prepared by transesterification procedure indicated the presence of five fatty acids with the relative composition shown in Table 1. The FAME showed mass spectra with molecular ions at m/z 270, 268, 298, 296 and 294, corresponding to the retention times of 38.5, 39.2, 42.5, 43.1, 44.2 min. These times indicated the presence of palmitic (C16), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1)
Table 2: Hypolipidemic effect produced by NSO in normolipidemic mice.

<table>
<thead>
<tr>
<th>Doses (mg/kg/day)</th>
<th>Total-Chol (%)</th>
<th>Tg (%)</th>
<th>chol-HDL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>100±2.1</td>
<td>100±0.18</td>
<td>100±1.58</td>
</tr>
<tr>
<td>NSO 150</td>
<td>4.16±1.07</td>
<td>11.26±0.17</td>
<td>-13.91±1.49</td>
</tr>
<tr>
<td>NSO 300</td>
<td>-11.01±1.73</td>
<td>-12.2±0.22</td>
<td>-10.36±2.11</td>
</tr>
<tr>
<td>NSO 600</td>
<td>-19.09±2.69</td>
<td>-33.7±0.19</td>
<td>-22.42±3.2</td>
</tr>
</tbody>
</table>

Table 3: Hypolipidemic effect produced by NSO in hyperlipidemic mice male.

<table>
<thead>
<tr>
<th>Doses (mg/kg/day)</th>
<th>Total-chol (%)</th>
<th>Tg (%)</th>
<th>chol-HDL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>100±6.6</td>
<td>100±1.08</td>
<td>100±0.95</td>
</tr>
<tr>
<td>NSO 150</td>
<td>-22.13±1.72</td>
<td>-74.2±0.09*</td>
<td>33.54±1.06</td>
</tr>
<tr>
<td>NSO 300</td>
<td>5.73±7.16</td>
<td>-64.97±0.31</td>
<td>39.54±1.49</td>
</tr>
<tr>
<td>NSO 600</td>
<td>-15.57±1.8</td>
<td>-31.79±0.24</td>
<td>42.85±1.29</td>
</tr>
</tbody>
</table>

* Represents significant difference when compared with the control when analyzed by ANOVA ± standard error.

and linoleic (C18:2) fatty acids respectively, being the principal component linoleic acid. However further studies are necessary to determine the exact chemical composition.

**Biological assay:** Data on the effect on lipid-lowering activity of the three parameters evaluated are summarized in Tables 2 and 3. All animals showed good health during the period of administration. At necropsy no changes were found in the organs of the treated animals.

Animals administered with 600 mg/Kg (Table 2) registered the most important effect with a reduction of -19.09% and -33.7% for total cholesterol and triglycerides respectively. These results suggest a hypolipidemic activity of seed oil and a relationship between dose and effect.

Treatment of ICR mice with Triton WR 1339 (tyloxapol) resulted in a significant elevation in total serum cholesterol, HDL-cholesterol and triglycerides respect to control.

Tyloxapol is a non-ionic surfactant being widely used to explore possible mechanism of lipid lowering drugs, it causes drastic increase in serum triglycerides and cholesterol levels due to increase in hepatic cholesterol synthesis particularly by the increase in HMG Co-A (3-hydroxy-3-methyl-glutaryl Co-A) activity and by the inhibition of lipoprotein lipase responsible for hydrolysis of plasma lipids [14].

The Table 3 shows data about the use of NSO in hyperlipidemic mice. The lowest reduction values for cholesterol (-22.15%) and triglycerides (-74.2%) were obtained with dose of 150 mg/kg, exhibiting the best hypolipidemic effect. Not relationship dose-effect in cholesterol was observed. Nevertheless for triglycerides is notorious inverse relationship dose-effect.

Significant inhibition of lipid levels increase by noni seed oil of *Morinda citrifolia* in this model is indicative of the inhibition of cholesterol biosynthesis by inhibition of HMG Co-A. However, the failure of NSO to cause complete inhibition indicates the involvement of additional mechanisms.

Various indices have been used for the diagnosis and prognosis of cardiovascular disease, one of them was reported by Castelli [11]. Castelli’s Atherogenic index (AI) is total cholesterol/HDL ratio and it is considered that a value below than four units represents a low risk of cardiovascular disease [11-13]. Als were calculated for the different groups, being that it is smaller for normolipidemic group when administered NSO (Figure 1). While for the hyperlipidemic group a slight decrease regarding the control is observed only at dose of 300 mg/Kg. However the values for this group stay below the one it limits.

We find that the administration of noni oil causes a hypolipidemic effect, mainly evident when hyperlipidemia occurs. The results from this study rationalize the medicinal use of noni seed oil in dislipidemia. However further studies are required to prove efficacy of noni seed oil in dyslipidemia and to prove that it can be used as a potential medicine for cardiovascular diseases.

The main constituent of noni seed oil is linoleic acid, however hypolipidemic activity may be due to the presence of other compounds in oil.

**Experimental**

**Extraction seed oil (NSO):** The noni fruit was obtained from Córdoba Veracruz, México. The seeds were obtained from fresh fruits. The batch of seeds were washed and dried at room temperature. Dried seeds were ground and extracted by maceration with food-grade hexane at 1:5 ratio at room temperature for 24 h. The extract was filtered and the solvent removed by distillation under reduced pressure. The crude oil was used in the bioassay.
Oil analysis: Fatty acid composition was determined for gas chromatography (GC) of methyl ester obtained after transesterification of the crude oil [15] on a Hewlett-Packard 5890 Series II Gas Chromatograph equipped with a 5971A Mass Selective detector and using an HP-Innowax capillary column (30 m x 0.2 mm x 0.25 μm thick coating). Helium was used as the carrier gas at a flow rate of 1 mL/min with the injector set to split mode at 250°C. The oven was programmed from 100 to 140°C at 1.5°C/min and then from 140 to 250°C at 5°C/min and held at 250°C for 10 min. The detector was operated at 70 eV in scanning mode over the range of 50–550 amu. Mass spectra were compared with data in NIS library and % of fatty acid was calculated for integration value for chromatogram.

Hypolipidemic Evaluation: Hypolipidemic activity was studied in (ICR) male mice weighing 25-30g (Birmex, S. A., Mexico City). All animals were housed in hanging metal cages and maintained at 24±2 °C and 50±10% relative humidity, with 12 h light/dark cycle. They were fed on standard pellet diets (Rodent Diet 5001, PMI Nutrition International, Inc. Brenwood, MO) and drinking water was freely available. All animals appeared healthy throughout the dosing period, maintaining normal food intake and weight gain. At sacrifice, no gross abnormalities were observed in any treated mice.

All animals were treated in accordance with ethical principles and regulations specified by the Animal Care and Use Committee of our institution and the standards of the National Institutes of Health of Mexico.

The mice were randomly divided into groups of six animals. Hyperlipidemia was induced in the mice by administration of Triton WR 1339 (Tyloxapol) was dissolved in water at 400 mg/Kg. The seed oil was administered 1h before and 22 and 48 h after the tyloxapol injection [16].

Mice were treated with the oil seed suspended in a 1:4.5:4.5 tween 80: mineral oil: saline solution and administered orally by an incubation needle at doses of 150, 300 or 600 mg/Kg/day for 28 days. Animals receiving the vehicle were used as the non-cholesterol control group.

For tyloxapol-treated mice blood samples were taken 48 h after injection. On the other hand, animals receiving treatment for 28 days were fasted for 12 h before sacrifice. Blood samples were collected by periorbital plexus bleeding and centrifuged at 3000 rpm for 15 min. Total cholesterol (Col), high-density lipoprotein cholesterol (col-HDL), and triglycerides (Tg) levels were determined in the serum, using a Wiener lab, Selectra 2000 automatic analyzer.

All data are expressed as the percentage of the cholesterol group control (the mean± standard error) by using Student t test. P values less than 0.05 considered statistically significant.

Castelli Atherogenic index (AI) were calculated using the equation IA= Total cholesterol/HDL-chol [11].

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References


Composition of Egyptian Neroli Oil

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The bitter orange flower oil (or neroli) is an essential product, largely used in perfumery. Neroli is obtained by hydrodistillation or steam distillation, from the flowers of bitter orange (Citrus aurantium L.). Since a long time neroli production is limited and its cost on the market is considerably high. The annual production in Tunisia and Morocco is ca. 1500 Kg, representing more than 90% of the worldwide production. A small amount of neroli is also produced in Egypt, Spain and Comorros (not exceeding 150 kg totally). Due to the high cost, the producers and the users have tried to obtain less expensive products, with odor characters close to that of neroli oil to be used as substitute and sometimes as adulterants of the genuine oil. In this study are investigated five samples of Egyptian neroli oils produced in 2008 and 2009, in the same industrial plant, declared genuine by the producer. For all the samples the composition was determined by GC/FID and by GC/MS-LRI; the samples were also analyzed by esGC to determine the enantiomeric distribution of twelve volatiles and by GC-C-IRMS for the determination of the $\delta^{13}$CVPDB values of some mono and sesquiterpene hydrocarbons, alcohols and esters. The analytical procedures allowed to quantitatively determining 86 components. In particular the variation of the composition seems to be dependent on the period of production. In fact, the amount of linalool decreases from March to April while linalyl acetate presents an opposite trend, increasing in the same period. The RSD determined for the $\delta^{13}$CVPDB are very small (max. 3.89%), ensuring the authenticity of all samples. The results are also discussed in function of the limits provided by the European Pharmacopoeia (EP) (2004), AFNOR (1995) and ISO (2002) regulations for genuine neroli oils.

Keywords: Neroli oil, Citrus aurantium L., GC, GC/MS-LRI, GC-C-IRMS, es-GC.

The oil of neroli is obtained by hydrodistillation or by steam distillation of the flowers of bitter orange (C. aurantium L.).

Neroli, rose and jasmine are often cited as “the three pearls of perfumery”. Neroli is the classic ingredient of the most famous and prestigious perfumes and eau de cologne. It is also used as flavor ingredient in food and beverages. In traditional Chinese medicine the extracts from bitter orange flowers are used to treat digestive problems and insomnia.

Neroli is the product of a laborious work: the flowers, which bloom between the end of April and the beginning of June are collected manually during the first hours of the day; one worker can collect about 20 Kg per day for a period of 20 days; the flowers are hydro- or steam-distilled with a yield ranging from 0.08% at the beginning of the season to a maximum of 0.13% under the most favorable conditions. The production in the European Mediterranean Countries (mainly France) is subject to a strong decrease, mainly for the specialized working cost necessary to collect the flowers, and for the contraction of the cultivated fields of bitter orange.

The annual world production of neroli is today less than 2000 Kg; most of it is concentrated in Morocco and in Tunisia. Small amounts of neroli, about 150 Kg/year, are produced in Egypt, Spain, and Comorros. The market price of neroli is considerably high and the organic product can be sold at more than 4,500 USD/Kg. It is therefore predictable that this oil can be subject to adulteration by the addition of less valuable natural products, such as the oils obtained from flowers of citrus different from C. aurantium, or by addition of leaf oils or of synthetic compounds. The adulteration of neroli oil is not easily identifiable, mainly because the reference data available in literature, relative to oils produced industrially and extracted in laboratory [1-3], ranges widely and is also probably affected by the geographic origin of the trees. Based on the rules AFNOR (Association Francaise de Normalization) [4a], ISO (International Organization for Standardization) [4b] and EP (European Pharmacopeia) [5], some of the results available in literature should not indicate genuine samples. Very few results are available in literature on the enantiomeric distribution of volatiles determined in neroli oils [6-10]. In the authors knowledge the IRMS analysis was never performed before on neroli.
Usually essential oils quality assessment is obtained by traditional chromatographic techniques (GC-FID, GC-MS, Es-GC, HPLC) as recently reported by our research group [11], recognized for their validity in the quality control field. Advanced chromatographic techniques have been also exploited for different essential oils as fast GC/MS [12] and multidimensional GC-GC for quantitative [13] and enantiomeric ratio assessment [14,15]. Recently has gained importance the Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) that, determining small differences in the isotopic carbon composition of the matrices, can be exploited to discriminate between products of different origin [10,16-18a]. In this regards GC-C-IRMS can be an useful tool in the flavour and fragrance authenticity control, unveiling illicit essential oils production methods, such as the oils adulteration by the addition of synthetic or natural compounds, different from the genuine ones [18b,19,20].

To our knowledge neroli oil was never investigated by IRMS. It is therefore particularly interesting to provide information useful for the genuineness assessment of neroli, also in function of the geographic origin.

The present article reports the results relative to the composition of five samples of Egyptian neroli produced in 2008 and 2009, to the enantiomeric distribution and the isotopic ratio of selected components.

The samples analyzed are described below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hydrodistilled from Egypt (2008)</td>
</tr>
<tr>
<td>2</td>
<td>steam-distilled March 23th 2009</td>
</tr>
<tr>
<td>3</td>
<td>steam-distilled March 28th 2009</td>
</tr>
<tr>
<td>4</td>
<td>steam-distilled April 9th 2009</td>
</tr>
<tr>
<td>5</td>
<td>steam-distilled April 9-11th 2009</td>
</tr>
</tbody>
</table>

Table 1 reports the composition determined by GC-FID, of the samples analyzed. To facilitate comparison with information already available in literature the results are here reported as raw peak area %. The correction factors (C.F.) for each class of substances determined by GC-FID are however reported in Table 1 to provide complete information to the reader. In the case of distilled oils, as neroli, the volatile fraction should represent the whole oil. The quantitative results obtained from triplexes show CV% values always below 5%. The 86 components identified by GC/MS with the use of LRI as filters interactively applied during the mass spectral identification process [21] represent about 99% of the whole oils. In comparison with literature information this study led to the identification of numerous components (indicated by * in Table 1), while the presence of numerous minor components previously reported were not here confirmed.

Hydrocarbons range between 20-25%, oxygenated compounds vary between 73-78%; among these alcohols range from 58 to 70% and esters from 7 to 19%, while aldehydes are present at small amounts (0.16-0.26%). The main alcohol is linalool (44-53%), followed by α-terpineol (5-6%) and by geraniol (3-4%). The sesquiterpene alcohols (E)-nerolidol and (E,E)-2,6-farnesol are also well represented ranging together between 2-5%. The main ester is linalyl acetate (2-15%) followed by geranyl acetate (about 3%) and neryl acetate (about 1.5%). The most abundant monoterpene hydrocarbon is limonene (8-12%) followed by (E)-β-ocimene (3-5%), and by β-pinene (2-4%); the main sesquiterpene hydrocarbon is β-caryophyllene (0.6-0.9%).

The sample produced in 2008 by hydrodistillation, compared to all the other oils obtained by steam distillation, has the highest amount of linalool and of total alcohols, and the lowest amount of linalyl acetate and of total esters.

In the samples produced in 2009 the composition varies gradually but significantly during the productive season. The total monoterpene hydrocarbons and the single components of this class of compounds, the total monoterpenes alcohols and the single components of this class of compounds as well as the ratio linalool/linalyl acetate decrease during the season. Total esters and linalyl acetate present an opposite behavior, as well as the sesquiterpene hydrocarbons and aldehydes. Neryl and geranyl acetate remain constant during the whole season.

In Figure 1 are graphically described the seasonal variation of class of substances and some single components. The results confirm, as reported in literature, that the main components of neroli oil are linalool, linalyl acetate and limonene. The amount of these components determined in this study fall in the ranges hitherto determined for neroli oil. It should be however mentioned that in one Egyptian oil [1a] it was determined the 30% of linalool content and 1% of linalyl acetate; the highest value (74%) of linalool was reported for a Chinese oil [1a] which presented a very unusual low amount of limonene (1%); in some Spanish oils [3] were reported very low values of linalyl acetate (0.6%).

The results determined in the present study also confirm the presence of some key compounds such as methyl anthranilate, methyl N-methyl anthranilate, phenyl ethyl alcohol, (E)-nerolidol, and some newly identified components such as the (E,Z)- and (E,E)-2,6-farnesals, useful for the characterization of this product.

Table 2 reports the enantiomeric distribution of some components determined by es-GC. The values are determined from triplicates with CV% never exceeding 5.5% with the exception of that relative to the (-)-α-thujene isomer which is 8.9% due to the chromatographic behavior of this component. Figure 2 shows the chiral chromatogram of one of the samples analyzed.

The enantiomeric ratios of camphene, sabinene, α- and β-phellandrene and citronellal were determined in neroli oils for the first time. The values of the enantiomeric
Table 1: Composition of the five samples analyzed (% of peak areas) determined by GC-MS-LRI and GC-FID.

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-Germacrene D</td>
<td>0.03</td>
<td>0.07</td>
<td>0.07</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>0.44</td>
<td>0.79</td>
<td>0.52</td>
<td>0.57</td>
<td>0.59</td>
</tr>
<tr>
<td>9-Methyl anthranilate</td>
<td>0.04</td>
<td>0.10</td>
<td>0.12</td>
<td>0.09</td>
<td>0.06</td>
</tr>
<tr>
<td>Geranyl formate*</td>
<td>tr</td>
<td>tr</td>
<td>0.01</td>
<td>0.02</td>
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</tr>
<tr>
<td>Bornyl acetate*</td>
<td>tr</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Geranyl acetone*</td>
<td>tr</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Z,E-Elemene*</td>
<td>tr</td>
<td>0.02</td>
<td>0.03</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>-3-Carene</td>
<td>0.09</td>
<td>0.16</td>
<td>0.14</td>
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<td>0.16</td>
</tr>
<tr>
<td>Ketones</td>
<td>0.09</td>
<td>0.08</td>
<td>0.08</td>
<td>0.11</td>
<td>0.09</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Monoterpane</td>
<td>20.67</td>
<td>24.16</td>
<td>22.76</td>
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<td>18.30</td>
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<td>1.74</td>
<td>1.59</td>
<td>1.55</td>
<td>1.66</td>
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<td>Aliphatic</td>
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<td>0.05</td>
<td>0.04</td>
<td>0.06</td>
<td>0.03</td>
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<tr>
<td>ALDOL CYDES</td>
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<td>0.16</td>
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<td>0.26</td>
</tr>
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<td>0.08</td>
<td>0.10</td>
<td>0.11</td>
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</tr>
<tr>
<td>Sesquiterpane</td>
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<td>0.14</td>
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<td>0.16</td>
<td>0.16</td>
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<tr>
<td>Ketones</td>
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<td>0.08</td>
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<td>0.10</td>
<td>0.09</td>
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<td>Hydrocarbons</td>
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<tr>
<td>Monoterpane</td>
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<td>0.04</td>
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<tr>
<td>Aliphatic</td>
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<td>0.03</td>
<td>0.04</td>
<td>0.03</td>
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<tr>
<td>Alcohols</td>
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<td>1.55</td>
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<td>Monoterpane</td>
<td>1.6</td>
<td>1.33</td>
<td>1.55</td>
<td>1.76</td>
<td>1.95</td>
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<tr>
<td>Sesquiterpane</td>
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<td>0.02</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Aliphatic</td>
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<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Oxygenes + ethers</td>
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<td>0.22</td>
<td>0.30</td>
<td>0.32</td>
<td>0.37</td>
</tr>
<tr>
<td>Others</td>
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<td>0.14</td>
<td>0.16</td>
<td>0.14</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Notes: a. LRI measured on SLB-5MS column; b. Reference LRI reported in literature (FFNSC 1.5GC-MS library, Shimadzu, Japan; or Adams RP. Identification of essential oil components by gas chromatography/mass spectrometry, 4th Edn. Carol Stream, IL, USA: Allured Publishing Corp; or Hochmuth, D.H., Joulain, D., König, W.A., 2002. MassFinder Software and Data Bank, University of Hamburg); tr: ≤0.005; C.F. Correction Factor (FID response) for class of compounds; * identified, in the authors knowledge, for the first time in neroli oils; † correct isomer not identified.

Table 2: Enantiomeric distribution of some volatile components in the samples analyzed.

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>-(+)---Terpineol</td>
<td>71.23</td>
<td>71.02</td>
<td>71.12</td>
<td>70.84</td>
<td>70.79</td>
</tr>
<tr>
<td>N-(+)-Phellandrene</td>
<td>55.45</td>
<td>72.58</td>
<td>n.d.</td>
<td>85.63</td>
<td>67.99</td>
</tr>
<tr>
<td>-(+)---Pinene</td>
<td>39.77</td>
<td>27.42</td>
<td>n.d.</td>
<td>14.37</td>
<td>32.01</td>
</tr>
<tr>
<td>-(+)---Phellandrene</td>
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<td>72.25</td>
<td>66.46</td>
<td>73.54</td>
<td>50.69</td>
</tr>
<tr>
<td>S(+)-Limonene</td>
<td>1.65</td>
<td>2.47</td>
<td>2.42</td>
<td>2.58</td>
<td>2.60</td>
</tr>
<tr>
<td>S(+)-Linalool</td>
<td>98.35</td>
<td>97.46</td>
<td>98.54</td>
<td>97.42</td>
<td>98.45</td>
</tr>
<tr>
<td>S(-)-Limonene</td>
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<td>2.54</td>
<td>2.47</td>
<td>2.58</td>
<td>2.60</td>
</tr>
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<td>97.42</td>
<td>98.45</td>
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<td>98.54</td>
<td>97.42</td>
<td>98.45</td>
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<td>1.65</td>
<td>2.54</td>
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<td>2.58</td>
<td>2.60</td>
</tr>
<tr>
<td>S(-)-Limonene</td>
<td>98.35</td>
<td>97.46</td>
<td>98.54</td>
<td>97.42</td>
<td>98.45</td>
</tr>
</tbody>
</table>

Notes: n.d. not determined.

distribution of limonene, linalool, terpinen-4-ol, and α-terpineol here determined are in good agreement with literature results relative to genuine neroli oils [6,7a]; however, if compared to literature, the enantiomeric excess of -(+-) pinene is slightly lower, that of -(+-) pinene
by plotting the $\delta^{13}C_{VPDB}$ values for each component analysed in function of the period of production. The relative standard deviation of the $\delta^{13}C_{VPDB}$ values range for the samples analyzed between 3.52 ($(E)-\beta$-caryophyllene) and 0.96 ($\alpha$-terpinene). These low values indicate very narrow ranges of variation, therefore it is possible to assume that the $\delta^{13}C_{VPDB}$ can be considered characteristic of authenticity and of the geographic origin of the samples.

Table 4 provides a comparison of the ranges determined from the present results with the limits provided by the AFNOR, ISO and EP regulations [4, 5]. Some of the results fall within these limits; others fall only slightly outside them; in three of the samples analyzed linalool is present at levels sensibly higher than the limits provided by the aforementioned regulations; $\beta$-pinene is always below the minima reported for neroli oils. These behavior could be due to the geographic origin of the oils analyzed.

Considering the high commercial value of neroli, its limited production in different geographic areas and the high possibility that this product can be subject to adulteration, it is necessary to fix quality parameters in

**Table 3**: $\delta^{13}C_{VPDB}$ values calculated for the samples analyzed, average and relative standard deviation% (RSD).

<table>
<thead>
<tr>
<th>Component</th>
<th>Average</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-Pinene</td>
<td>27.67</td>
<td>1.46</td>
</tr>
<tr>
<td>Myrcene</td>
<td>24.74</td>
<td>3.89</td>
</tr>
<tr>
<td>Limonene</td>
<td>27.16</td>
<td>2.67</td>
</tr>
<tr>
<td>Linalool</td>
<td>27.67</td>
<td>1.45</td>
</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>27.67</td>
<td>1.45</td>
</tr>
<tr>
<td>Geranyl acetate</td>
<td>27.67</td>
<td>1.45</td>
</tr>
<tr>
<td>Neryl acetate</td>
<td>27.67</td>
<td>1.45</td>
</tr>
<tr>
<td>Caryophyllene*</td>
<td>27.67</td>
<td>1.45</td>
</tr>
<tr>
<td>Farnesol**</td>
<td>27.67</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Correct isomer identification: *(E)*; **(2E,6E)-**

**Figure 1**: Seasonal variation of class of components, of linalool and linalyl acetate in the four samples of neroli produced during 2009.

extremely lower and that of $(-)$-linalyl acetate is higher. With the exception of $\alpha$-pinene, the results here obtained for the components analyzed are overall in good agreement with the enantiomeric purity previously determined by Mosandl [6] considered characteristic of genuine neroli oils.

Table 3 reports the $(-)\delta^{13}C_{VPDB}$ values calculated for the neroli oils samples. This study reports for the first time the GC-C-IRMS analysis of selected volatile components in neroli oil. It is impossible to compare these values with literature information. Figure 3 shows the graph obtained

![Chromatogram](image-url)
consideration of its variability among different geographic areas. To accomplish this, for a correct evaluation, not only the indices given by these regulations should be taken into account, but also the δ\(^{13}\)C\(_{VPDB}\) of selected compounds and the chiral purity of more compounds than the two already indicated, thus providing adequate tools for quality and genuineness assessment.

**Experimental**

**Analysis of the essential oils:** On the five samples described in text the following analytical investigations have been carried out: GC/FID, GC/MS of the volatile fraction; direct enantio-GC for the determination of the enantiomeric distribution of some volatiles. Each analysis was performed in triplicates. Results are expressed as average peak area %.

**GC/FID:** The volatile fraction was analyzed by HRGC/FID as described. Gas chromatograph: Shimadzu GC2010 equipped with a Flame Ionization Detector, a split/splitless injector and an AOC-20i series auto-injector. Capillary column: 30 m x 0.25 mm I.D. 0.25 μm d\(_f\) coated with SLB-5MS [silphenylene polymer, virtually equivalent in polarity to poly (5% diphenyl/95% methyl)siloxane)] (Supelco, Milan, Italy); column temperature, 50-250°C (10 min) at 3°C/min; injector temperature: 250°C; detector temperature: 280°C; carrier gas, He at 99.5 kPa (30.0 cm/sec); injection mode: split; split ratio, 1:100; injected volume, 1.0 μL of diluted oil. Data handling was made by means of GCsolution software.

**GC/MS Analysis:** Samples were analyzed by GC/MS (EI) on a GCMS-QP2010 system equipped with commercially available libraries (see notes to Table 1) including the commercial version of the FFNSC ver. 1.3 (Shimadzu, Japan) database (created in the authors’ laboratory) consisting of about 2000 reference standards and their relative linear retention indices determined on apolar column, interactively used as filters for the spectral interpretation. GC conditions: capillary column and temperature program as in GC/FID; carrier gas, He delivered at a constant pressure of 30.6 kPa (30.1 cm/s); 1.0 μL of solution (1/10, v/v, essential oil/hexane) injected on a split/splitless injector; injector temperature, 250°C; injection mode, split; split ratio, 1:50. MS scan conditions: source temperature, 200°C; interface temperature, 250°C; E energy 70eV; mass scan range, 40-400 amu. Data was handled through the use of GCMSsolution software.

**Enantio-GC:** Shimadzu GC2010 gas chromatograph equipped with a Flame Ionization Detector, a split/splitless injector and an AOC-20i series autoinjector. Capillary chiral column was a Megadex DETTBS-β (diethyl-tert-butyl-silyl β-cyclodextrin) 25 m x 0.25 mm I.D. x 0.25μm d\(_f\) (Mega, Legnano, Italy). Temperature program: 50°-200°C at 2°C/min. Inlet pressure 96.6 kPa (220°C), split mode 1:20 (gas carrier He); injected volume, 1.0 μL; linear velocity, 30 cm/sec (constant). Data handling was made by means of GCsolution software

**GC-C-IRMS device and analyses:** Trace GC Ultra equipped with a TriPlus autosampler, retrofitted to the combustion interface GC/CIII and hyphenated to the isotope ratio mass spectrometer Delta V Advantage (all purchased from Thermo Fisher Scientific, Milan, Italy). GC: column: SLB-5ms (silphenylene polymer) 30 m x 0.25 mm i.d., 0.25 μm d\(_f\) (Supelco, Milan, Italy); temperature program: 50°C to 230°C at 3°C/min; split/splitless injector (250°C). Inlet pressure: 167 kPa; column flow: 2.0 ml/min (constant flow mode); carrier gas: He. GC/C III: ox. reactor (Cu/Ni/Pt): 980°C; red. reactor: 640°C; He: 1 bar; O\(_2\): 0.8 bar; CO\(_2\): 0.5 bar. IRMS: EI; electron voltage: 123.99 eV; electron current: 1.5 μA; 3 Faraday cup collectors at m/z 44, 45, and 46; peak center pre-delay and post-delay: 15 s, cup 3; reference: 60-80 s, 100-120 s, 140-160 s, 180-200 s; split: open; evaluation type: CO\(_2\) SS, ref. time: 155.90 s, δ\(^{13}\)C/δ\(^{12}\)C -60.30‰; integration time 0.2 s.

The GC-C-IRMS instrument achieves highly precise measurement of carbon isotopic abundance, converting the eluted volatile components, in CO\(_2\) and water into an oxidation chamber. After removing water, just behind the furnace, by a capillary-shaped phase separator, CO\(_2\) reaches an ionization chamber where it will be transformed into three ion traces for the different isotopomers: 12C\(^{16}\)O\(_2\), 13C\(^{16}\)O\(_2\) and 12C\(^{18}\)O\(_1\)\(^{16}\)O, with their corresponding masses at (m/z) 44, 45, 46. The three ion beams are registered simultaneously by means of an Universal Faraday collector that detects the different contributions of ionic fragments obtained. Isotopic ratios, 45/44 and 46/44, are expressed in ‰ and are related to a certified standard (VPDB-standard) of known value [20]. Exploiting the GC-Combustion backflush, the most concentrated components were not introduced into the combustion chamber.

The samples dilutions and the GC-Combustion conditions were as follows: concentration 1:10 (v/v), 1 μL split injection, 1:100 split ratio, backflush: off, for the determination of δ\(^{13}\)C\(_{VPDB}\) of limonene and linalool.

Concentration 1:10 (v/v), 1 μL split injection, 1:50 split ratio, backflush open: 780-830 s and 970-1060 s, for the determination of δ\(^{13}\)C\(_{VPDB}\) of β-pinene, myrcene, terpinen-4-ol, α-terpinol, nerol, neryl acetate, geranyl acetate, (E)-caryophyllene, nerolidol, (2E,6E)-farnesol. Data are collected in triplicate, by using the Isodat 2.5 software (Thermo Fisher Scientific).

**CO\(_2\) reference gas cylinder calibration:** The attained carbon isotope ratio of the unknown sample is compared to that of a calibrated CO\(_2\) reference. The CO\(_2\) reference gas was calibrated by injecting 1 μL of a carbon stable isotope ratio reference alkanes mixture comprising C\(_{16}\) to C\(_{30}\) (Indiana University, Bloomington, U.S.A.), calibrated against VPDB standard with a defined \(^{13}\)C content. Isotope ratios were expressed as δ values (%), versus a standard.
Tricosane ($C_{23}$) was arbitrarily chosen as reference alkane.

Acknowledgements - Authors wish the acknowledge Shimadzu for the continuous support.

References


Essential oil of *Nepeta x faassenii* Bergmans ex Stearn (*N. mussinii* Spreng. x *N. nepetella* L.): A Comparison Study

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*School of Pharmacy and Pharmaceutical Sciences, Faculty of Health Sciences, Trinity College Dublin, Ireland*

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Analysis (GC and GC/MS) of an essential oil sample obtained from dry leaves of *Nepeta × faassenii* Bergmans ex Stearn, a hybrid species produced by crossbreeding *N. mussinii* Spreng. with *N. nepetella* L., led to the identification of 109 constituents that represented 95.9% of the oil. The major constituents were 4aa,7aa-nepetalactone (67.8%), 1,8-cineole (6.6%), germacrene D (4.8%), β-pinene (2.7%), (E)-β-ocimene (2.6%), 4aa,7β-nepetalactone (2.3%) and (E)-β-farnesene (1.0%). Chemical composition of the oil was compared, using multivariate statistical analyses (MVA) with those of the oils of other *Nepeta* taxa, in particular *N. mussinii* and *N. nepetella*. This was done in order to explore the mode of inheritance of the monoterpene biosynthetic apparatus of *N. faassenii*. Chemical composition of the volatiles of a *Nepeta* taxon (different populations) can be subject to variation due to environmental and geographical factors. To accommodate this fact in the MVAs, along side with *N. faassenii* essential oil, additional 6 oils (3 different populations of *N. nuda* L. and *N. cataria* L. from Serbia) were included in this study (isolated and analyzed (chemically and statistically)). The MVA analyses recognized *N. faassenii* as being closely related to both *N. mussinii* and *N. nepetella*. If the relative content of oil constituents per plant and not per chromatogram were used as variables in the MVA (this was done by simple multiplication of the yields and relative percentages of components) a higher degree of mutual similarity (in respect to the monoterpene biosynthesis) of *N. faassenii* to *N. mussinii*, than to the other parent species, was observed.


The genus *Nepeta* L., Lamiaceae, includes over 200 species of perennial plants and some annuals [1]. The members of this genus are known as catnip or catmint because of the purported effect on cats, pleasantly stimulating cats’ pheromonic receptors, typically resulting in temporary euphoria [1]. The active substances of catnip plants, some of which have a sedative effect on humans, have been widely used in medicine, food industry and perfumery. They are also used in folk medicine as remedies for bronchitis, flu, cold and other illnesses. Number of catnips have sudorific, diuretic and bacteriostatic properties, reduce fever and help treat stomach-aches [1,2]. Generally speaking, *Nepeta* species are essential oil-rich taxa, and some of them contain more than 1% of the essential oil [2,3]. Moreover, many of them are cultivated as garden plants. In order to produce new, interesting garden/ornamental plants, many *Nepeta* species have been crossbred repeatedly. The aim of the breeding processes is not only creating plants with an appalling look, but also producing larger amounts of biologically active compounds [2]. One of the outcomes of these experiments is the sterile hybrid *Nepeta × faassenii* Bergmans ex Stearn, which has been produced by crossbreeding *N. mussinii* Spreng. with *N. nepetella* L. [4]. *Nepeta × faassenii* is cultivated in Europe, C Asia and Persia, and is used as a spice and an ornamental plant [4]. There is only limited data on the composition of the volatile oil of the mentioned hybrid taxon [3a]. For this reason, the aim of this study was set to analyze in detail (GC and GC/MS) the essential oil of *N. faassenii* and to compare its chemical composition, using multivariate statistical analyses (MVA: agglomerative hierarchical cluster analysis (AHC) and principal component analysis (PCA)) with those of the oils of other *Nepeta* taxa, in particular *N. mussinii* and *N. nepetella*. Having in mind that the monoterpene diversity and morphology could serve as a mirror of the gene flow [5], multivariate analyses were primarily done in order to provide an insight into the inheritance of the chemical characters for this taxon.

The chemical composition of the essential oils of a *Nepeta* taxon (different populations) can be subject to variation due to environmental and geographical factors. To accommodate this fact in the MVAs, along side with *N. faassenii* essential oil, additional 6 oils (3 different populations of *N. nuda* L. and *N. cataria* L. from Serbia) were included in this study (isolated and analyzed (chemically and statistically)).
<table>
<thead>
<tr>
<th>RT</th>
<th>Compound</th>
<th>NF</th>
<th>NC</th>
<th>NN</th>
<th>Class</th>
</tr>
</thead>
<tbody>
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<td>765</td>
<td>(Z)-2-Penten-1-ol</td>
<td>tr</td>
<td>tr</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>778</td>
<td>3-Methyl-2-butenal</td>
<td>tr</td>
<td>tr</td>
<td>O</td>
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</tr>
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<td>tr</td>
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<td>O</td>
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<td>801</td>
<td>Hexanal</td>
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<td>tr</td>
<td>O</td>
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<td>Furural</td>
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<td>tr</td>
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</tr>
<tr>
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<td>MM</td>
<td></td>
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<td>α-Terpine</td>
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<td>tr</td>
<td>O</td>
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</tr>
<tr>
<td>1029</td>
<td>Methyl 1-cyclohexenyl ketone</td>
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<td>O</td>
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<td>1,8-Cineole</td>
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<td></td>
<td></td>
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<tr>
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<td>37.5 ± 4.9</td>
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</tr>
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<td>O</td>
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<td>2-Phenylacetaldheyde</td>
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<td>γ-Terpine</td>
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### Table 1 (contd.)

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<td>Hexacane</td>
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<td>O</td>
</tr>
<tr>
<td>Octacane</td>
<td>tr</td>
<td>O</td>
</tr>
</tbody>
</table>

| Total | 95.9 | 98.5±±1.1 | 98.0±±1.5 |

**p-Menthan-9-one (MM) 7.4 0.1±±0.0 47.3±±1.7**

**Sesquiterpenoids (S) 9.5 3.4±±0.5 30.6±±5.2**

**Hydrocarbons 8.6 2.7±±0.4 26.4±±4.2**

**Oxygenated 0.9 0.7±±0.4 26.5±±3.1**

**Others (O) 1.4 0.9±±0.5 0.6±±0.1**

- Compounds listed in order of elution from a DB-5MS column with retention indices (RI) determined experimentally by co-injection of a homologous series of C7-C28 n-alkanes.
- Percentages given as average values ± absolute deviations of the components’ relative abundances (three oil samples).
- ‘tr’-trace (<0.05%); ‘All compounds present below 0.05% in at least one of the analyzed N. nuda and N. cattaria oil samples are given as tr; ‘The identity of the constituent was determined by comparison of MS and RI matching and confirmed by co-injection of an authentic sample; ‘Probably a contaminant of the isolation procedure; synonym.

#### Figure 1: Typical TIC chromatograms of the herein analyzed N. cattaria, N. nuda and N. faassenii essential oil samples (for sample designation see Table 2).

Almost 200 different compounds, representing 95.9-99.1% of the total oils, were identified in the 7 presently analyzed samples (Table 1). Typical TIC chromatograms of the analyzed samples are depicted in Figure 1. Chemical compositions of the analyzed N. nuda and N. cattaria oils were in general agreement with the literature data [3a,g]. Major compounds identified in N. faassenii oil were 4αa,7α,7αa-nepeta lactone (67.8%), 1,8-cineole (6.6%) and germacrene D (4.8%). Additional 4 compounds with the relative amount higher than 1% of were: β-pinene (2.7%), (E)-β-ocimene (2.6%), 4αa,7β,7αa-nepeta lactone (2.3%) and (E)-β-farnesene (1.0%).
As already mentioned, monoterpene profiles of hybrids and their parent taxa could serve as a mirror of the gene flow [5]. We wanted to verify this hypothesis in the case of *N. faassenii*, and decided to use essential oil composition as a reflection of the inheritance of the biosynthetic apparatus of this hybrid taxon.

For that reason, chemical compositions of the herein analyzed samples and 29 other oils originating from different *Nepeta* taxa (Table 2), including the two previously studied oils of *N. faassenii* and 8 oils of the parent species *N. nepetella* and *N. mussinii*, were compared using multivariate statistical analysis (MVA: AHC and PCA), Figures 2-5. The exact identity of the *Nepeta* taxa other than *N. faassenii*, *N. nepetella* and *N. mussinii* was unimportant for the present study and these were chosen randomly (either from the literature or different chemotypes of one taxon).

Table 2: List of the essential oil samples used in the statistical analyses.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Sample designation</th>
<th>Ref.</th>
<th>Yield* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. faassenii</em> Bergmans ex Stearn</td>
<td>N. faas1 p.s.</td>
<td>[3a]</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>N. faas2</td>
<td>[3a]</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>N. faas3</td>
<td>[3a]</td>
<td>0.2</td>
</tr>
<tr>
<td><em>N. mussinii</em> Spreng.</td>
<td>N. muss1</td>
<td>[3b]</td>
<td>-</td>
</tr>
<tr>
<td>(syn. <em>N. racemosa</em> Lam.)</td>
<td>N. muss2</td>
<td>[3c]</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>N. muss3</td>
<td>[3d]</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>N. muss4</td>
<td>[3e]</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>N. muss5</td>
<td>[3e]</td>
<td>0.1</td>
</tr>
<tr>
<td><em>N. nepetella</em> L.</td>
<td>N. nep1</td>
<td>[3c]</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N. nep2</td>
<td>[3a]</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>N. nep3</td>
<td>[3f]</td>
<td>1.2</td>
</tr>
<tr>
<td><em>N. nuda</em> L.</td>
<td>N. nuda1</td>
<td>p.s.</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>N. nuda2</td>
<td>p.s.</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>N. nuda3</td>
<td>p.s.</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>N. nuda4</td>
<td>[3a]</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>N. nuda5</td>
<td>[3a]</td>
<td>0.2</td>
</tr>
<tr>
<td><em>N. cataria</em> L.</td>
<td>N. cat1</td>
<td>p.s.</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>N. cat2</td>
<td>p.s.</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>N. cat3</td>
<td>p.s.</td>
<td>0.4</td>
</tr>
<tr>
<td><em>N. cat1</em></td>
<td>N. cat4</td>
<td>[3e]</td>
<td>-</td>
</tr>
<tr>
<td><em>N. cat3</em></td>
<td>N. cat5</td>
<td>[3e]</td>
<td>0.1</td>
</tr>
<tr>
<td><em>N. cat6</em></td>
<td>N. cat6</td>
<td>[3a]</td>
<td>0.2</td>
</tr>
<tr>
<td><em>N. macrocephalum</em> Boiss.</td>
<td>N. mac</td>
<td>[3h]</td>
<td>0.2</td>
</tr>
<tr>
<td><em>N. ucrainica</em> L. ssp. <em>kopetdaghenis</em></td>
<td>N. ucr</td>
<td>[3i]</td>
<td>0.04</td>
</tr>
<tr>
<td><em>N. oxyodonata</em> Boiss.</td>
<td>N. oxy</td>
<td>[3j]</td>
<td>0.1</td>
</tr>
<tr>
<td>N. foliosa Moris</td>
<td>N. fofi</td>
<td>[3k]</td>
<td>0.1</td>
</tr>
<tr>
<td><em>N. septemcrenata</em> Erem</td>
<td>N. sept</td>
<td>[3l]</td>
<td>0.4</td>
</tr>
<tr>
<td><em>N. sibirica</em> L.</td>
<td>N. sib</td>
<td>[3a]</td>
<td>1.0</td>
</tr>
<tr>
<td><em>N. rianjensis</em> Diklic &amp; Milosjevic</td>
<td>N. rianj</td>
<td>[3m]</td>
<td>1.0</td>
</tr>
<tr>
<td><em>N. cadmea</em> Boiss.</td>
<td>N. cadmea</td>
<td>[3o]</td>
<td>2.1</td>
</tr>
<tr>
<td><em>N. crispa</em> Willd.</td>
<td>N. crispa</td>
<td>[3o]</td>
<td>-</td>
</tr>
<tr>
<td><em>N. mahanensis</em> Jamzad &amp; Simmonds</td>
<td>N. mahan</td>
<td>[3o]</td>
<td>-</td>
</tr>
<tr>
<td><em>N. ispahanica</em> Boiss.</td>
<td>N. ispahan</td>
<td>[3o]</td>
<td>-</td>
</tr>
<tr>
<td><em>N. eremophila</em> Haaske. &amp; Bomm.</td>
<td>N. erem</td>
<td>[3o]</td>
<td>-</td>
</tr>
<tr>
<td><em>N. rivalaris</em> Bomm.</td>
<td>N. rival</td>
<td>[3o]</td>
<td>-</td>
</tr>
<tr>
<td><em>N. ciliaris</em> Boiss.</td>
<td>N. ciliaris</td>
<td>[3o]</td>
<td>-</td>
</tr>
</tbody>
</table>

*Given as in the original references (% w/w or v/v); b p.s.-present study; c Yield not given in the original references; d Reference [3e] plus personal correspondence with K. H. C. Baser; syn.-synonym.*

The dendrogram depicted in Figure 2, obtained as the result of AHC analysis performed using original variables (percentage composition of the oils, AHC1), delimitates three statistically different classes of samples, C1-C3. Class C1 (4αα,7α,7αα-nepetalactone class) groups all samples with a high relative amount of 4αα,7α,7αα-nepetalactone (more than 65%). Oils obtained from *N. nepetella* (N. nep1-N. nep3), as well as the two *N. faassenii* samples (N. faas1 and N. faas2) were also grouped within this class. The mutual feature of the oils, including two *N. mussinii* samples, clustered within the C2 (4αα,7α,7αβ-nepetalactone class) was the
Nepeta x faassenii essential oil

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Figure 3: A-Dendrogram (AHC2 analysis; transformed variables – summation of the nepetalactones) representing chemical composition dissimilarity relationships of 36 Nepeta essential oil samples (observations) obtained by Euclidian distance dissimilarity (dissimilarity within the interval [0, 50000], using aggregation criterion-Ward's method). Three groups of samples (C4-C6) were found (from left to right); B-Expanded section of dendrogram A, showing the dissimilarity interval [0, 5500].

Figure 4: A-Dendrogram (AHC3 analysis; transformed variables (information about the oil yields included to give the per plant content)) representing chemical composition dissimilarity relationships of 27 Nepeta essential oil samples (observations) obtained by Euclidian distance dissimilarity (dissimilarity within the interval [0, 30000], using aggregation criterion-Ward's method). Three groups of samples (C7-C9) were found (from left to right); B-Expanded section of dendrogram A, showing the dissimilarity interval [0, 11000].

According to the results of AHC1, one could speculate that in general, the hybrid N. faassenii is, as far as the monoterpene biosynthesis is concerned, generally more similar to N. nepetella than to its other parent species (C1). The placement of one N. faassenii oil sample outside of the C1 class might be possibly explained by the existence of more than one chemotype of this hybrid.

It could be even possible that the crossbreeding might not give identical N. nepetella x N. mussinii hybrids all of the time. However, although N. faassenii, N. nepetella and high relative amount of 4α,7α,7αβ-nepetalactone (>70%). The remaining class (C3) was much more heterogenic with respect to the dominant components of its comprising samples. One sample of N. faassenii (N. faas3) and 3 samples of the N. mussinii oil were placed within the same subclass of C3.

Germacrene D, (E)-β-ocimene, 4αα,7α,7αβ-nepetalactone, 4αα,7α,7αα-nepetalactone, 4αα,7β,7αα-nepetalactone and 1,8-cineole were among the dominant components of these N. faassenii and N. mussinii oils.
N. mussinii oils didn’t share the same amounts of (different) nepetalactones. For this reason, additional MVA analyses, using transformed variables were performed to answer this situation. In the first transformed set of variables (MVA2: AHC2 and PCA2) 4 nepetalactone diastereoisomers were not used as individual variables, but were replaced with one collective variable (the sum of the relative abundances of the 4 nepetalactones). The results of AHC 2 are given in Figure 3. The three observed statistically different groups of samples (C4-C6) are not identical with those from AHC1, but can be correlated to a certain level. Once again, the N. faassenii oils were not placed within the same class of the resulting dendrogram. The sample N. faas3 (collected from the same botanical garden as N. faas2), characterized with a high percentage of germacrene D, (E)-β-ocimene, was again recognized as statistically different, as might be expected. Except for this sample and N. muss5, all other N. faassenii, N. nepetella and N. mussinii oils were not recognized as statistically different (C4, AHC2).

It has been previously shown that not only the identity of the dominant essential oil constituents is important for the comparison and classification of plant species, but the oil yield as well [6]. Therefore, an additional set of variables, taking into account the yields of the compared oils, was used for the MVAs (MVA3, for each sample, the relative amount of the every original variable was multiplied with the corresponding oil yield, to reflect the per plant and not per chromatogram relative amount of the volatile constituents). MVA3 were performed using a slightly reduced set of observations, as the yields of all oils listed in Table 2 were not given in the original references. The resulting dendrogram of the AHC3 is given in Figure 4. This time, all oil samples of N. faassenii were placed within the same class (C7), together with N. mussinii oils. Contrary to that, N. nepetella samples were recognized as statistically different and were grouped within C9. As can be seen from Table 2, the yields of N. faassenii and N. mussinii oils were comparable and fall within the interval 0.1-0.2% (the only exception was N. muss4), whereas the yields of N. nepetella oils were significantly higher (0.8-1.2%).

Alongside with the AHCes, the samples listed in Table 2 were analyzed by PCA, using the same three sets of variables (original and transformed). The results of PCA1-PCA3 were almost identical. As can be seen from a typical byplot of the PCA (PCA1, original variables), given in Figure 5, values of F1 and F2 factors for all analyzed N. faassenii, N. nepetella and N. mussinii oils were very similar, suggesting a high level of likeness of the mentioned samples.

It could be interesting to mention that all performed MVA analyses (AHC1-AHC3 and PCA1-PCA3) generally showed that oils of the “control” species (N. nuda and N. cataria) were not statistically different (except for the N. cat4) within the taxon.

Thus, one could conclude that the oil composition of these Nepeta taxa were not susceptible, at least not significantly, to external factors (environmental and geographical). For this reason, it is not unreasonable to assume that the statistically significant differences in the chemical composition of the all herein analyzed Nepeta oils could, at least partially, reflect the differences of corresponding taxa on the biosynthetic/genetic level. Having all the above stated in mind, some general conclusions regarding the inheritance of the monoterpene biosynthetic apparatus of N. faassenii could be drawn. Considering only the chemical composition of the analyzed oils, both AHC and PCA (Figures 2, 3 and 5) recognized, as expected, N. faassenii as closely related to both N. mussinii and N. nepetella. It could be even speculated that the hybrid can unselectively inherit the monoterpene biosynthetic apparatus from both of its parent species (AHC1 and AHC2). Nevertheless, when the chemical composition of the compared oils was not the only compositional data taken into account, but also their corresponding yields (AHC3), a higher degree of mutual similarity (with respect to the monoterpene biosynthesis) of N. faassenii to N. mussinii, than to its other parent species, was observed.

Experimental

Plant material: Above-ground parts of N. faassenii were collected in the botanical gardens, Trinity College, Dartry, Dublin 6. Nepeta nuda was collected from three different locations in SE Serbia (slopes of the Suva planina mountain (Donji Dušnik), the Ploče highland and the vicinity of the city of Niš (village Knez selo)), in August, 2010. Above-ground parts of N. cataria were collected in the vicinity of the city of Pirot, the slopes of the mountain Rtanj and the vicinity of the city of Niš (village Knez...
Isolation of the essential oil: Air-dried, to constant weight, aboveground parts of *Nepeta × faassenii, N. cataria* and *N. nuda* (batches of 25 – 150 g) were subjected to hydrodistillation with *ca.* 1 l of distilled water for 2.5 h using the original Clevenger-type apparatus [7]. Yields of the obtained oils (w/w) are given in Table 2. The obtained oils were separated by extraction with diethyl ether and dried over anhydrous magnesium sulphate. The solvent was evaporated under a gentle stream of nitrogen at room temperature in order to exclude any loss of the essential oil and immediately analyzed. When the oil yields were determined, after the bulk of ether was removed under a vacuum, the residue was exposed to a temperature for a short period to eliminate the solvent completely. The pure oil was then measured on an analytical balance and multiple gravimetric measurements were taken during 24 h to ensure that all of the solvent had evaporated.

**GC and GC/MS analyses:** The GC/MS analyses were repeated three times for each sample using a Hewlett-Packard 6890N gas chromatograph. The gas chromatograph was equipped with a fused silica capillary column DB-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250° and 300°C, respectively. The oven temperature was raised from 70° to 290°C at a heating rate of 5°C/min and then isothermally held for 10 min. As a carrier gas helium at 1.0 mL/min was used. The samples, 1 µL of the oil solutions in diethyl ether (1 : 100), were injected in a pulsed split mode (the flow was 1.5 mL/min for the first 0.5 min and then set to 1.0 mL/min throughout the remainder of the analysis; split ratio 40 : 1), which enabled sufficient separation (narrower peaks due to a pulsed injection mode despite starting the run at 70°C) and positive identification of numerous components with RI lower than 900. The identification of partially overlapping peaks was also aided by NIST AMDIS (Automated Mass Spectral Deconvolution and System) Software version 2.4, supplied by National Institute of Standards and Technology (NIST, USA). Mass selective detector was operated at the ionization energy of 70 eV, in the 35–500 amu range with a scanning speed of 0.34 s. GC (FID) analyses were carried out under the same experimental conditions using the same column as described for the GC/MS. The percentage composition was computed from the GC peak areas without the use of correction factors. Qualitative analyses of the essential oil constituents were based on several factors. Firstly, the comparison of the essential oils linear retention indices relative to the retention times of C₅-C₂₉ n-alkanes on the DB-5MS column [8] with those reported in the literature [9]. Secondly, by comparison of their mass spectra with those of authentic standards, as well as those from Wiley 6, NIST02, MassFinder 2.3. Also, a homemade MS library with the spectra corresponding to pure substances and components of known essential oils was used, and finally, wherever possible, the identification was achieved by GC coinjection with an authentic sample (see Table 1). Relative standard deviation (RSD) of peak areas of the repeated measurements (independent sample preparations and GC-MS) was for all substances below 1%. The only exceptions which had higher RSD were minor components such as bicyclogermacrene, (E)-β-ocimene, (Z)-2-hexenal, α-terpineol, β-caryophyllene, cis-sabinene hydrate and β-sesquiphellandrene where RSD was 6, 3, 4, 9, 8, 2 and 13%, respectively. In repeated measurements, no significant deviation of the component retention times was observed.

**Multivariate statistical analyses:** Principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) were performed using the Excel program plug-in XLSTAT version 2010.03.5. Both methods were applied utilizing three sets of variables: original variables (percentages of individual oil constituents (only constituents with the percentage higher than 1% in at least one sample were taken into account)) and transformed variables (a-original variables were transformed in such a way that 4 nepetalactone isomers were summed into one collective variable; b-for each sample, the relative amount of the every original variable was multiplied by the corresponding oil yield). AHC was determined using Pearson dissimilarity where the aggregation criterion were simple linkage, unweighted pair-group average and Euclidean distance where the aggregation criterion were weighted pair-group average, unweighted pair-group average and Ward’s method. PCA of the Pearson (n) type was performed.

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**References**


Chemical Composition and Biological Activity of Salvia verbenaca Essential Oil

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Salvia verbenaca L. (syn. S. minore) is a perennial herb known in the traditional medicine of Sicily as “spaccapetri” and is used to resolve cases of kidney stones, chewing the fresh leaves or in decoction. The chemical composition of the essential oil obtained from aerial parts of S. verbenaca collected in Piano Battaglia (Sicily) on July 2009, was analyzed by GC and GC-MS. The oil was strongly characterized by fatty acids (39.5%) and carbonylic compounds (21.2%), with hexadecanoic acid (23.1%), (Z)-9-octadecenoic acid (11.1%) and benzaldehyde (7.3%) as the main constituents. The in vitro activity of the essential oil against some microorganisms in comparison with chloramphenicol by the broth dilution method was determined. The oil exhibited a good activity as inhibitor of growth of Gram + bacteria.

Keywords: Salvia verbenaca, Lamiaceae, volatile components, hexadecanoic acid, (Z)-9-octadecenoic acid, benzaldehyde, β-phellandrene, antibacterial activity.

Salvia species are used in folk medicine all around the world for their antibacterial, antitumor [2], antidiabetic [3], and antioxidant [4] activities. Members of this genus produce many useful secondary metabolites including terpenes and phenolics and their derivatives that have been in the center of pharmacopoeias of many countries [5].

The genus Salvia (sage) is one of the largest and the most important aromatic and medicinal genus of the Lamiaceae family, comprising about 900 species widespread throughout the world [1]. Salvia species are used in folk medicine all around the world for their antibacterial, antitumor [2], antidiabetic [3], and antioxidant [4] activities. Members of this genus produce many useful secondary metabolites including terpenes and phenolics and their derivatives that have been in the center of pharmacopoeias of many countries [5].

Salvia verbenaca L. is known in Italy as Salvia minore and it’s a tall herbaceous perennial plant, 20-50 cm high, with bluish purple flowers of about 1 cm length arranged in verticillasters (each of which generally contains six flowers). The calyx (green, 4-8 mm long) encloses a 6-10 mm long corolla. Nutlet fruits contain 1-4 seeds. The verticillasters are close together on the stern at flowering, but move further apart by fruit set. Flowering commences in mid-April and finishes towards the end of May [6]. The species is distributed in the Mediterranean area and in Italy is found frequently throughout the territory with the exclusion of the Alps. In Sicily, the plant is spread in scrublands and grasslands throughout all the island, from sea level to 1.200 m. above sea level [7]. In the Sicilian traditional medicine is known as “spaccapetri” and its leaves and flowering aerial parts are used to resolve cases of kidney stones, chewing the fresh leaves or in decoction. The plant is also known as bactericide against respiratory ailments, as healing in wounds and ulcers, and above all as eyedrops, because fruits or seeds when applied on the eyes remove impurities or dust particles.

As part of our extensive screening program of Salvia species from Mediterranean Area [2,4,8,9], we report in this paper the qualitative and quantitative analyses of the essential oil of wild population of S. verbenaca collected in Sicily and compare it with those previously reported.

A total of 76 constituents, representing 91.8% of the total oil, have been identified from the essential oil extracted from the aerial parts of S. verbenaca. In Table 1 the
Table 1: Volatile components of aerial parts of *Salvia verbenaca*.

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>LRI ¹</th>
<th>LRI ²</th>
<th>% ³</th>
<th>Identification ⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrocarbons</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonane</td>
<td>900</td>
<td>900</td>
<td>4.7</td>
<td>R, MS</td>
</tr>
<tr>
<td>α-Ionone</td>
<td>1208</td>
<td>1208</td>
<td>1.2</td>
<td>R, MS</td>
</tr>
<tr>
<td>Tetracosane</td>
<td>2400</td>
<td>2400</td>
<td>6.0</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>Pentacosane</td>
<td>2500</td>
<td>2500</td>
<td>0.8</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>Heptacosane</td>
<td>2700</td>
<td>2700</td>
<td>0.7</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>Nonacosane</td>
<td>2900</td>
<td>2900</td>
<td>0.5</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td><strong>Carboxylic compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E)-2-Hexenal</td>
<td>854</td>
<td>1209</td>
<td>1.5</td>
<td>R, MS</td>
</tr>
<tr>
<td>Decanal</td>
<td>1204</td>
<td>1508</td>
<td>0.3</td>
<td>R, MS</td>
</tr>
<tr>
<td>(E)-2-Decenal</td>
<td>1200</td>
<td>1655</td>
<td>0.5</td>
<td>R, MS</td>
</tr>
<tr>
<td>(E,E)-2,4-Decadienal</td>
<td>1315</td>
<td>1827</td>
<td>0.3</td>
<td>R, MS</td>
</tr>
<tr>
<td>(E)- Geranylacetone</td>
<td>1453</td>
<td>1867</td>
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<td>R, MS</td>
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<tr>
<td>(E)-β-Ionone</td>
<td>1484</td>
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<tr>
<td><strong>Terpenoids</strong></td>
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<tr>
<td>Monoterpenic hydrocarbons</td>
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<tr>
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<tr>
<td>β-Pinene</td>
<td>978</td>
<td>1118</td>
<td>0.5</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>α-Terpine</td>
<td>1012</td>
<td>1189</td>
<td>0.9</td>
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</tr>
<tr>
<td>β-Caryophyllene oxide</td>
<td>1418</td>
<td>1612</td>
<td>1.2</td>
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<td>α-Humulene</td>
<td>1455</td>
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<td>allo-Aromadendrene</td>
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<td>1661</td>
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<td>α-Amorphene</td>
<td>1475</td>
<td>1679</td>
<td>t</td>
<td>R, MS</td>
</tr>
<tr>
<td>β-Selinene</td>
<td>1475</td>
<td>1715</td>
<td>0.1</td>
<td>R, MS</td>
</tr>
<tr>
<td>Germacrene D</td>
<td>1477</td>
<td>1726</td>
<td>t</td>
<td>R, MS</td>
</tr>
<tr>
<td>γ-Cadinene</td>
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<td>1776</td>
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<td>R, MS</td>
</tr>
<tr>
<td>α-Calacorene</td>
<td>1542</td>
<td>1918</td>
<td>t</td>
<td>R, MS</td>
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<td><strong>Sesquiterpenic hydrocarbons</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>1,8-Cineole</td>
<td>1034</td>
<td>1213</td>
<td>0.2</td>
<td>R, MS, Co-GC</td>
</tr>
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<td>Linalool</td>
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<td>1553</td>
<td>0.7</td>
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</tr>
<tr>
<td>Camphor</td>
<td>1143</td>
<td>1532</td>
<td>t</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>Pinocarvone</td>
<td>1154</td>
<td>1587</td>
<td>1.1</td>
<td>R, MS</td>
</tr>
<tr>
<td>Bornol</td>
<td>1167</td>
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</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>1176</td>
<td>1611</td>
<td>t</td>
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<tr>
<td>p-Cymen-8-ol</td>
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<td>1856</td>
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<td>α-Terpine</td>
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<td>R, MS, Co-GC</td>
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<td>Safranal</td>
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<td>Carvone</td>
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<td>R, MS</td>
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<td><strong>Oxygenated monoterpenes</strong></td>
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<td>Isocaryophyllene oxide</td>
<td>1527</td>
<td>2001</td>
<td>t</td>
<td>R, MS</td>
</tr>
<tr>
<td>Germacrene D 4-ol</td>
<td>1575</td>
<td>2065</td>
<td>0.3</td>
<td>R, MS</td>
</tr>
<tr>
<td>Spathulenol</td>
<td>1577</td>
<td>2148</td>
<td>1.7</td>
<td>R, MS</td>
</tr>
<tr>
<td>Caryophyllene oxide</td>
<td>1581</td>
<td>2208</td>
<td>1.9</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>Caryophylladienol I</td>
<td>1640</td>
<td>2316</td>
<td>t</td>
<td>R, MS</td>
</tr>
<tr>
<td><strong>Fatty acids and esters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bornyl angelate</td>
<td>1566</td>
<td></td>
<td>t</td>
<td>R, MS</td>
</tr>
<tr>
<td>Dodecanoic acid</td>
<td>1566</td>
<td>2503</td>
<td>0.4</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>1769</td>
<td>2713</td>
<td>0.9</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>Pentadecanoic acid</td>
<td>1873</td>
<td>2740</td>
<td>0.5</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>Methyl hexadecanoate</td>
<td>1925</td>
<td>2208</td>
<td>0.7</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>Hexadecanoic acid</td>
<td>1972</td>
<td>2931</td>
<td>23.1</td>
<td>R, MS, Co-GC</td>
</tr>
<tr>
<td>Ethyl hexadecanoate</td>
<td>1994</td>
<td>2245</td>
<td>2.6</td>
<td>R, MS</td>
</tr>
<tr>
<td>(Z)-9-Octadecenoic acid</td>
<td>2117</td>
<td>3157</td>
<td>11.1</td>
<td>R, MS</td>
</tr>
<tr>
<td>(E,Z)-9,12-Octadecadienoic acid ethyl ester</td>
<td>2162</td>
<td>2532</td>
<td>0.2</td>
<td>R, MS</td>
</tr>
</tbody>
</table>
Retention indices, percentage composition and identification methods are given; the components, grouped in class of substances, are listed in order of elution on a HP 5MS column. Carboxylic compounds (21.2%) and fatty acids (39.5%) were the main fractions of the oil, while the terpenoidic fraction of the oil amounted to 20.9%, with monoterpenes accounting to 14.8% and sesquiterpenes to 6.1%. The most abundant compound was hexadecanoic acid (23.1%), followed by (Z)-9-octadecenoic acid (11.1%), benzaldehyde (7.3%) and the monoterpenic hydrocarbon β-phellandrene (5.9%).

The essential oil of *Salvia verbenaca* from Sicily presented noticeably different qualitative and quantitative results compared with the other studied oils. Pitarokili *et al*. (Greece) [10] detected as main compounds β-phellandrene (30.3%) and (E)-caryophyllene (16.1%), whereas Al-Howiriny (Saudi Arabia) [11] reported sabine (16.0%), cadinene (7.9%), 4-terpineol (7.4%) and pinene (7.3%) as the dominating compounds. *S. verbenaca* essential oil from Morocco presents terpineol (19.2%), camphor (6.6%) and β-thujone (6.1%) as main compounds [12], while Ben Taarit *et al*. [13,14] showed that *S. verbenaca* wild-growing in different locations in Tunisia is particularly rich in oxygenated sesquiterpenes and monoterpenic hydrocarbons, particularly viridiflorol (21.8%), tricyclene (18.8%), (Z)-β-cóimene (29.5%) for the samples collected in Sabelet Ben Ammar, Soemais and Sers respectively and β-caryophyllene (23.1%) and caryophyllene oxde (15.9%) for the samples collected in the northeast region of Tunisia. Previous papers [15, 16] showed that many factors affect the yield and the composition of essential oils of *Salvia* species, including plant source, individual plant chemotypes, time of harvest, the environmental conditions and the proportion of plant parts distilled.

The in vitro antibacterial activity of the essential oil of *S. verbenaca* against eight bacterial strains was evaluated by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) using the broth method. The oil appeared to be not active against Gram – bacteria (MICs >100 μg/mL), while it showed antibacterial activity against the Gram + bacteria tested. In particular, *Staphylococcus aureus* and *Streptococcus faecalis* were slightly sensitive to the action of the oil (MIC = 100 μg/mL), while the oil exerted an appreciable activity against *Bacillus subtilis* and *Staphylococcus epidermidis* (MIC = 50 μg/mL for both)

### Table 1: continued.

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Total amount of compounds</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymol methyl ether</td>
<td>1239 1611</td>
<td>0.2 Ri, MS</td>
</tr>
<tr>
<td>Thymol</td>
<td>1290 2198</td>
<td>0.8 Ri, MS, Co-GC</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>1297 2239</td>
<td>0.6 Ri, MS, Co-GC</td>
</tr>
<tr>
<td>Eugenol</td>
<td>1353 2186</td>
<td>0.7 Ri, MS, Co-GC</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td>3.2 Ri, MS, Co-GC</td>
</tr>
<tr>
<td>1-Octen-3-ol</td>
<td>977 1452</td>
<td>0.4 Ri, MS</td>
</tr>
<tr>
<td>2-Pentylfuran</td>
<td>1002 1243</td>
<td>0.9 Ri, MS</td>
</tr>
<tr>
<td>2-Phenyethanol</td>
<td>1115 1934</td>
<td>0.4 Ri, MS</td>
</tr>
<tr>
<td>(Z)-Phytol</td>
<td>1949 2622</td>
<td>1.5 Ri, MS</td>
</tr>
<tr>
<td>(E)-Phytol</td>
<td>2132 2625</td>
<td>t Ri, MS</td>
</tr>
<tr>
<td>Squalene</td>
<td>2828 3408</td>
<td>t Ri, MS</td>
</tr>
</tbody>
</table>

3 Linear Retention Index on a HP-5 MS column, 4 Linear Retention Index: retention index on an Innowax column, t: trace, less than 0.05%, R: retention index matches with bibliography; MS: identification based on comparison of mass spectra; Co-GC: comparison of retention time of authentic compounds.

### Experimental

**Plant material:** Aerial parts of *S. verbenaca* were collected at the full flowering stage on July 2009 from plants growing in Piano Battaglia (Sicily). Voucher specimens were deposited at the Herbarium of the Botanical Gardens of Palermo (Italy) under the number PAL 09-876.

**Isolation of the volatile components:** The fresh samples were cut into small pieces, then subjected to hydrodistillation according to the standard procedure described in the European Pharmacopoeia [17]. The yield (w/w) was 0.18%. The oil was dried over anhydrous sodium sulphate and then stored in sealed vials, at -20°C, ready for the GC and GC-MS analyses.

**Gas chromatography:** Analytical gas chromatography was carried out on a Perkin-Elmer Sigma 115 gas chromatograph fitted with a HP-5 MS capillary column (30 m x 0.25 mm i.d.; 0.25 μm film thickness). Column temperature was initially kept at 40°C for 5 min, then gradually increased to 250°C at 2°C min⁻¹, held for 15 min and finally raised to 270°C at 10°C min⁻¹. Diluted samples (1/100 v/v, in n-pentane) of 1 μL were injected manually at 250°C, and in the splitless mode with a 1 minute purge-off due to the small amount of oil partially utilized for biological tests. Flame ionization detection (FID) was performed at 280°C. Analysis was also run by using a fused silica HP Innowax polyethylenglycol capillary column (50 m x 0.20 mm i.d.; 0.20 μm film thickness). Helium was the carrier gas (1 mL min⁻¹) in both cases.

**Gas chromatography - mass spectrometry:** GC-MS analysis was performed on an Agilent 6850 Ser. II apparatus, fitted with a fused silica HP-1 capillary column (30 m x 0.25 mm i.d.; 0.33 μm film thickness), coupled to an Agilent Mass Selective Detector MSD 5973; ionization energy voltage 70 eV; electron multiplier voltage energy 2000 V. Mass spectra were scanned in the range 35-450 amu, scan time 5 scans/s. Gas chromatographic conditions were as reported above; transfer line temperature, 295°C.
Identification of components: Most constituents were identified by gas chromatography by comparison of their retention indices (LRI) with either those of the literature [18, 19] or with those of authentic compounds available in our laboratories. The retention indices were determined by GC-FID mode in relation to a homologous series of n-alkanes (C6-C32) under the same operating conditions on both columns. Further identification was made by comparison of their mass spectra on both columns with either those stored in NIST 02 and Wiley 275 libraries or with mass spectra from the literature [19, 20] and our home made library. Component relative concentrations were calculated based on GC-FID peak areas without using correction factors.

Antimicrobial activity: The antibacterial activity was evaluated by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) using the broth dilution method as previously described [2]. Eight bacteria species, selected as representative of the class of Gram positive and Gram negative, were tested: *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus faecalis* (ATTC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), *Proteus vulgaris* (ATCC 13315) and *Pseudomonas aeruginosa* (ATCC 27853).

Acknowledgments – The GC and GC-MS analyses were performed at the "C.S.I.A.S." of the University "Federico II" of Napoli. The assistance of the staff is gratefully appreciated.

References


Chemical Composition and Antimicrobial Activities of the Essential Oils from Ocimum selloi and Hesperozygis myrtoides

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Ocimum selloi, a traditional medicinal plant from Brazil, is sold in open-air markets at Rio de Janeiro State. Hesperozygis myrtoides is a very aromatic small bush found in the State of Minas Gerais, Brazil, growing at an altitude of 1800m. The chemical composition of both essential oils was analyzed as well as their antimicrobial activity against fungi and bacteria. For all specimens of Ocimum selloi obtained at open-air markets, methylchavicol was major compound found (93.6% to 97.6%) in their essential oils. The major compounds identified in the oil of H. myrtoides were pulegone (44.4%), isomenthone (32.7%), and limonene (3.5%). Both oils displayed antimicrobial activity against all tested microorganisms but Candida albicans was the most susceptible one. Combinations of the two oils in different proportions were tested to verify their antimicrobial effect against C. albicans, which, however, was not modified in any of the concentrations tested. The minimum inhibitory concentration (MIC) was determined to confirm the antimicrobial activity against C. albicans as well as other clinical isolates (C. glabrata, C. krusei, C. parapsilosis and C. tropicalis).

Keywords: Lamiaceae, Ocimum, Hesperozygis, essential oils, methylchavicol, pulegone, antimicrobial activity.

The family Lamiaceae is composed by 220 genera and about 3500-4000 species, many of them used in folk medicine and as aromatic herbs in the cosmetic, food and perfumery industries [1]. The most abundant secondary metabolites of Lamiaceae are terpenoids (mono, sesqui, di and triterpenes) and flavonoids, but the medicinal use of this family is primarily related to their essential oil content [1]. Ocimum and Hesperozygis belong to the Lamiaceae family and comprise about 160 and 6 species, respectively. Ocimum selloi Benth. is widely used in traditional medicine, including in the mountain region of Rio de Janeiro State, where it is sold in open-air markets [2]. This species has a pleasant fragrance, similar to that of anise and fennel’s fruits. Hesperozygis myrtoides (A.St.-Hil.) Epling is a very aromatic small bush found in the region of Aiuruoca (Minas Gerais State, Brazil) where it grows at an altitude of 1800m high. This plant, locally called “poejo” (“pennyroyal”) because of its strong mint odor, is also used for the treatment of respiratory disorders. Another traditional use of this plant is in the preparation of a drink with “cachaça”, the Brazilian sugar cane spirit, where the plant is soaked into the bottle’s spirit and buried for one year before it is consumed (personal communication to the author by local people of Aiuruoca.). This work aimed to investigate the chemical composition of the essential oils obtained from these species as well as to evaluate their antimicrobial activity.

The essential oils of O. selloi were obtained from fresh leaves of individuals purchased at different open markets in the State of Rio de Janeiro. Colorless oils, with a characteristic odor of anise and with yields ranging from 0.2% to 0.5% (Table 1) were obtained. From chromatographic analysis by GC-FID and GC-MS a quite similar chemical composition was observed among different individuals of O. selloi. The propenylphenol methylchavicol was identified in high concentration in all analyzed samples (93.6% to 97.6%). The oil of O. selloi from Rio de Janeiro can be considered a good new source of this substance in a practically pure form. The compound methylchavicol, also called estragol, is a propenylphenol responsible for the anise flavor in some plant species and
Table 1: Essential oil yields of Ocimum selloi and Hesperozygis myrtoides according to the collection sites.

<table>
<thead>
<tr>
<th>Plants</th>
<th>Collection sites</th>
<th>Plant collection Month/Year</th>
<th>Essential oil yield (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. selloi</td>
<td>Itaguaí (RJ)</td>
<td>September/2009</td>
<td>0.5</td>
</tr>
<tr>
<td>O. selloi</td>
<td>Petrópolis (RJ)</td>
<td>November/2009</td>
<td>0.4</td>
</tr>
<tr>
<td>O. selloi</td>
<td>Petrópolis (RJ)</td>
<td>January/2010</td>
<td>0.5</td>
</tr>
<tr>
<td>O. selloi</td>
<td>Itaguaí (RJ)</td>
<td>February/2010</td>
<td>0.5</td>
</tr>
<tr>
<td>O. selloi</td>
<td>Madureira (RJ)</td>
<td>March/April/2010</td>
<td>0.5</td>
</tr>
<tr>
<td>H. myrtoides</td>
<td>Aiuruoca (MG)</td>
<td>April/2010</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*Average of three extractions.

Table 2: Chemical composition (relative percentage %) of the essential oils from O. selloi purchased at different open markets in Rio de Janeiro.

<table>
<thead>
<tr>
<th>Substance</th>
<th>RIm</th>
<th>RIo</th>
<th>Itaguaí</th>
<th>Madureira</th>
<th>Petrópolis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylchavicol</td>
<td>1204</td>
<td>1195</td>
<td>95.9</td>
<td>94.9</td>
<td>94.7/97.6</td>
</tr>
<tr>
<td>Byciclogermacrene</td>
<td>1497</td>
<td>1494</td>
<td>0.8</td>
<td>0.7</td>
<td>0.8/0.4</td>
</tr>
<tr>
<td>β-Caryophyllene</td>
<td>1420</td>
<td>1418</td>
<td>0.6</td>
<td>0.9</td>
<td>0.9/0.6</td>
</tr>
<tr>
<td>α-Copaene</td>
<td>1377</td>
<td>1376</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1/0.1</td>
</tr>
<tr>
<td>Methylcyclogeranic</td>
<td>1406</td>
<td>1401</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2/0.1</td>
</tr>
<tr>
<td>3-Octen-1-ol</td>
<td>980</td>
<td>978</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2/0.1</td>
</tr>
<tr>
<td>β-Bisabolene</td>
<td>1510</td>
<td>1509</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1/0.1</td>
</tr>
<tr>
<td>Total Identified Compounds</td>
<td></td>
<td></td>
<td>97.8</td>
<td>97.3</td>
<td>97.0/99.0</td>
</tr>
</tbody>
</table>

*not identified.

Table 3: Identified compounds in the essential oil from fresh leaves of H. myrtoides.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>RIm</th>
<th>RIo</th>
<th>Fresh Leaves (%)</th>
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</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>927</td>
<td>939</td>
<td>0.2</td>
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<tr>
<td>Sabinene</td>
<td>976</td>
<td>976</td>
<td>0.2</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>979</td>
<td>980</td>
<td>0.3</td>
</tr>
<tr>
<td>Myrceene</td>
<td>992</td>
<td>991</td>
<td>0.4</td>
</tr>
<tr>
<td>Limonene</td>
<td>1031</td>
<td>1031</td>
<td>3.5</td>
</tr>
<tr>
<td>cis-β-Ocimene</td>
<td>1040</td>
<td>1040</td>
<td>0.2</td>
</tr>
<tr>
<td>Heptanyl acetate</td>
<td>1113</td>
<td>1113</td>
<td>0.2</td>
</tr>
<tr>
<td>Menthone</td>
<td>1156</td>
<td>1154</td>
<td>0.2</td>
</tr>
<tr>
<td>Isomenthone</td>
<td>1167</td>
<td>1164</td>
<td>32.7</td>
</tr>
<tr>
<td>α-Terpeneol</td>
<td>1189</td>
<td>1189</td>
<td>0.1</td>
</tr>
<tr>
<td>Pulegone</td>
<td>1242</td>
<td>1242</td>
<td>44.4</td>
</tr>
<tr>
<td>Isomenthyl acetate</td>
<td>1307</td>
<td>1306</td>
<td>7.0</td>
</tr>
<tr>
<td>Isopulegol acetate</td>
<td>1312</td>
<td>1308</td>
<td>0.7</td>
</tr>
<tr>
<td>Terpinyl acetate</td>
<td>1350</td>
<td>1350</td>
<td>0.4</td>
</tr>
<tr>
<td>N1*</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valencenc</td>
<td>1493</td>
<td>1491</td>
<td>0.2</td>
</tr>
</tbody>
</table>

H. myrtoides furnished a colorless essential oil with a pleasant mint odor and an average yield of 1.6% (Table 3). The major compounds were identified as the oxygenated monoterpenes pulegone (44.4%) and isomenthone (32.7%), besides limonene in small amount (3.5%). To the best of our knowledge, this is the first report on the chemical composition of H. myrtoides essential oil. Comparison of the chemical composition of the essential oil of H. myrtoides with essential oils from other species of this genus [10], showed that H. ringens (collected in the State of Rio Grande do Sul, Brazil) also has a high concentration of pulegone (79.2%), while H. rhododon (collected in the Paraná State, Brazil) has lower levels of this compound. Isomenthone, the other monoterpane present in relevant concentration in the oil of H. myrtoides (32.7%), is absent in H. ringens and present only at low concentration in the oil of H. rhododon (2.2%). On the other hand, menthone, which was characterized in tiny proportions in H. myrtoides (0.3%) and H. ringens (2.2%), was one of the major compounds in the essential oil of H. rhododon (43.4%).

The antimicrobial activity of the essential oils of O. selloi and H. myrtoides were evaluated for a series of microorganisms (Table 4). Both oils displayed antimicrobial activity against all tested microorganisms but the major inhibition halos were shown for the resistant strain of C. albicans (Table 4). Due to the interesting results of these oils against C. albicans and the possibility to combine the odor of fresh mint of H. myrtoides with the anise odor of O. selloi in a future pharmaceutical formulation to treat oral candidiasis, we tested the combination of the two oils in order to verify the effect in their antimicrobial activity. The proportions tested aimed to spare the oil of H. myrtoides in respect to that of O. selloi, since H. myrtoides is native to regions of high altitudes, where it grows wild (making it hard to harvest), and the species O. selloi can be easily grown [5,9]. The proportions tested and the results obtained are shown in Table 4. The combination of the two oils did not modify the activity against C. albicans strain, in any of the concentrations tested, but it is worth to note that the inhibition zone (cm) remained quite the same even with a relatively low concentration of the essential oil of H. myrtoides.

Nowadays, opportunistic fungal infections that assault immunocompromised patients are frequently resistant to ordinary clinical drugs and, hospital-acquired infections and antibiotic-resistant bacteria continue to be major health...
Essential oils of *Ocimum selloi* and *Hesperozygis myrtoides*

Conservation concerns worldwide [11-13]. Therefore, there is a constant search for new antifungal agents. The determination of the minimum inhibitory concentration (MIC) has demonstrated that essential oils have a varied ability to inhibit fungal growth and which justifies susceptibility studies [14]. Since the essential oils tested positive against *C. albicans* resistant strain ATCC 24433 in a preliminary test (drop test) the minimum inhibitory concentration (MIC) was determined to confirm the antimicrobial activity on *Candida* clinical strains. The MIC data are summarized in Table 5.

### Table 4: Antimicrobial activity of essential oils from *O. selloi* (A) and *H. myrtoides* (B), and their association at different proportions (A + B):

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>C. albicans</em></th>
<th><em>S. aureus</em></th>
<th><em>E. coli</em></th>
<th><em>A. niger</em></th>
<th><em>L. casei</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. selloi</em> (A)</td>
<td>1.2</td>
<td>0.3</td>
<td>0.5</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td><em>H. myrtoides</em> (B)</td>
<td>1.5</td>
<td>1.2</td>
<td>0.8</td>
<td>---</td>
<td>0.8</td>
</tr>
<tr>
<td>A + B (4:1)</td>
<td>1.3</td>
<td>0.6</td>
<td>0.6</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>A + B (3:1)</td>
<td>1.2</td>
<td>0.6</td>
<td>0.6</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>A + B (2:1)</td>
<td>1.2</td>
<td>0.8</td>
<td>0.6</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>A + B (1:1)</td>
<td>1.2</td>
<td>0.6</td>
<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*O. selloi* was the most susceptible to both essential oils followed by *C. albicans*, which was the most susceptible to the oil of *O. selloi*. On the other hand, the other *Candida* strains - *C. krusei*, *C. parapsilosis* and *C. tropicalis* were more susceptible to the oil of *H. myrtoides*. The antifungal activity of pulegone, the major compound present in the essential oil of *H. myrtoides*, has already been described for *C. albicans* [15,16] which leads to infer that the activity of this oil is may be due to its high content of this substance. As for what concerns the essential oils of *O. selloi*, all analyzed samples bear more than 94% of methylevchavicol. Even if we cannot rule out the possible antimicrobial activity of minor compounds, it is reasonable to suggest that methylevchavicol may be the principal antifungal agent in these oils. It has been suggested that the antimicrobial action of rich propenylphenol essential oils can arise from the complexation between the protein or other components of the cell membrane of the microbes and the phenolic components [17]. Essential oils rich in methylevchavicol generally show weak antimicrobial activity [18] but our results points out interesting results demonstrated for *Candida* clinical strains.

### Experimental

**Plant material:** Samples of *Ocimum selloi* Benth. were purchased from producers at open markets at Petropolis and Itaguai cities, Rio de Janeiro State, as well as at the Madureira fair (“Mercadão de Madureira”), at Rio de Janeiro city. Plants were identified by Dr. R. M. Harley and voucher specimens are deposited at Laboratorio de Fitosanitaria and voucher specimens are deposited at Laboratorio de Laboratorio de Fitoquimica e Farmacognosia, Faculdade de Farmácia, UFRJ. Samples of *Hesperozygis myrtoides* (A.St.-Hil.) Epling were collected in Aiurcuoa, Minas Gerais State. *H. myrtoides* was identified by Dr. R. M. Harley and voucher specimens are deposited at Universidade Estadual de Feira de Santana Herbarium (Feira de Santana, Bahia State, Brazil) under the number 1333584.

**Essential oil extraction:** The essential oils were extracted separately from fresh leaves (150 g, *O. selloi*, 3,802 g *H. myrtoides*) by hydrodistillation in a Clevenger-type apparatus for 2 h. Yields are reported in Table 1.

**GC and GC-MS analyses:** Gas chromatographic analyses were performed using an Agilent 7890A gas chromatograph (Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a HP-5 (5% phenyl/95% dimethylpolysiloxane) fused silica capillary column (30m x 0.32mm x 0.25μm). Hydrogen was the carrier gas (1.5 mL min⁻¹). The injector temperature was kept at 250°C and the oven temperature programmed from 60° to 240°C at a rate of 3°C min⁻¹. Detector (FID) was operated at 280°C. One microliter of a 1% solution of the oil in dichloromethane was injected in split mode (1:100). GC-MS analyses were performed in an Agilent 5973N mass selective detector coupled to an Agilent 6890 gas chromatograph (Palo Alto, CA), equipped with a HP-5MS capillary column (30m X 0.25mm X 0.25μm), operating in electron impact (EI) mode at 70eV, with transfer line maintained at 260°C, while mass analyzer and ion source temperature were held at 150°C and 230°C respectively. Helium (1.0 mL min⁻¹) was used as carrier gas. Oven temperature program, injector temperature and split rate were the same as stated for GC analyses. A standard solution of n-alkanes (C₁₇-C₂₅), injected in the same column and conditions as above, was used to obtain the retention indices [19]. Individual volatile components were identified by comparison of their mass spectra (MS) and retention indices (RI) with those reported in literature [20] and also to the Wiley Registry of Mass Spectral Data, 6th Edition [21].

**Antimicrobial Assay:**

**Drop Test:** The antimicrobial activity of the essential oils was preliminarily evaluated by agar diffusion technique (drop test) [22] against resistant strains of *Candida albicans* B type ATCC 36802, *Staphylococcus aureus* MRSA (BMB9393), *Escherichia coli*, *Aspergillus niger* and *Lactobacillus casei*. The inhibition zone generated after application of 1 μL of pure essential oil obtained from each species or in combination (Table 4) was measured in centimeters.

**Minimum inhibitory concentration (MIC):** Minimum inhibitory concentrations were determined by broth microdilution method according to the document M27-A3 (*Candida albicans*) of the Clinical and Laboratory Standard Institute (CLSI, 2008) [23], using resazurin as indicator for cell viability [24]. All determinations were
performed in triplicate and two independent experiments lead to concordant results. Positive and negative growth controls were included in all assays.

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References


The essential oil obtained from the leaves of *Lantana camara var. moritziana* (Otto & Dietr.) López-Palacios collected at Rubio, Táchira State, Venezuela, was obtained by hydrodistillation in a Clevenger trap (0.1% yield). The oil was analyzed by gas chromatography-mass spectrometry (GC/MS) on HP GC-MS System, model 5973, identifying 33 compounds (97.1%) of which the major components were germacrene D (31.0%), followed by β-caryophyllene (14.8%), α-phellandrene (6.7%), limonene (5.7%) and 1,8-cineole (5.2%). Evaluation of the antibacterial activity by agar diffusion method with discs against international reference bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella Typhi*, *Pseudomonas aeruginosa*) showed growing inhibition of *E. faecalis* and *S. aureus* at MIC of 350 mg/mL and 400 mg/mL, respectively.

**Keywords:** *Lantana camara*, Verbenaceae, essential oil, antibacterial activity.

*Lantana camara* Linn (Verbenaceae) is an ornamental grass with aromatic leaves, orange to bright red flowers and blue or black fruits (drupes). It is native to tropical and warm regions worldwide [1-3]. These plants are used in ethnomedicine to treat various diseases of the gastrointestinal tract, respiratory tract, as well as tranquilizers, anti-tumor, rheumatism, hypertension, uterine bleeding, and applied externally as an antiseptic to treat leprosy, scabies, tetanus, pustules [4-8].

In Venezuela we found 17 species of *Lantana* Linn among which *Lantana camara* L. commonly known as red cariaquito, is frequently found as three varieties *L. camara var. aculeata* (L.) Moldenke (L.), *L. camara var. mista* (L.) L H Bailey, and *L. camara var. moritziana* (Otto and Dietr.) Lopez-Palacios [2,9-11]. The last one is a shrub sometimes of unpleasant smell with yellow to orange or red flowers which are small and fragrant [10,11].

Previous studies on the analysis of the chemical composition of the essential oil of *Lantana camara* from different parts of the world have been reported: studies of the essential oil of *Lantana camara* growing in Brazil showed the presence of monoterpenes, sesquiterpenes and bisabolone derivatives. Germacrene D is one of the mayor component; even at different times of collection [12-14]. From Madagascar, Africa, the main constituents found were β-caryophyllene (19%), an unknown sesquiterpene (16%), and δ-3-carene (10%) [15]. From Calicut, India, the major ones were β-caryophyllene (34.8%), geranyl acetate (22.1%) terpinyl acetate (5.8%), bornyl acetate (4.1%) and D-limonene (2.3%).

*Lantana* essential oil is used in traditional medicine, for example, with insecticidal and nematicidal properties [16,17]. Insecticidal activity of the essential oil of *Lantana camara* was found against *Tribolium castaneum* (LC₅₀ 0.45mg/cm²) [18]. This oil showed antibacterial activity against *Pseudomonas aeruginosa* MBC 10 μg/mL, *Staphylococcus aureus* MBC 25 μg/mL, *E. coli* 1.25 μg/mL and antifungal *Aspergillus niger*, *Fusarium solani*, *Candida albicans* [3,6,8,19,20].

In the present investigation, the study of the composition and antibacterial activity of the essential oil of *Lantana camara var. moritziana* is presented. Leaves of *Lantana camara var. moritziana* were hydrodistilled yielding 0.1% of the essential oil. Analysis of this by GC-MS allowed the identification of thirty three compounds (97.1% of whole sample), which are listed in Table 1. The three major ones were Germacrene D (31.0%), β-caryophyllene (14.8%) and α-phellandrene (6.7%). Germacrene D and β-caryophyllene were also found as main constituents in the composition of the essential oils of *Lantana camara*.
from several countries [6,8,13,14,16,21-23]. Citral has been detected as the common major compound from five varieties of L. camara from Egypt [24].

Bacterial resistance is a growing phenomenon driven primarily by indiscriminate and irrational use of antibiotics. Resistant Staphylococcus aureus and E. faealis has emerged as a serious public-health problem that demands increased vigilance in the diagnosis and investigation of new alternative treatments [25].

The antibacterial activity of the essential oil of Lantana camara var. moritziana was evaluated. The oil had a weak activity against S. aureus ATCC (25923) and E. faealis ATCC (29212) with MIC values of 400 and 350μg/mL, respectively. The complete results are shown in Table 2.

**Experimental**

**Plant material:** The leaves of Lantana camara var. moritziana were collected (April, 2010) at Rubio, Táchira State, Venezuela, located at 101 m.s.m 8°53'07"N 64°89'11"O. A voucher specimen Nº NR005, has been deposited at the Herbarium of the Faculty of Pharmacy and Bioanalysis, University of the Andes (MERF herbarium).

**Isolation of the essential oil:** Fresh leaves (1000g) were cut into small pieces and subjected to hydrodistillation for 6h using a Clevenger-type apparatus. One mL of pale yellow essential oil (0.1% yield) was obtained. The oil was kept at -4°C until used for biological tests.

**Gas chromatography:** The volatile components of essential oil were analyzed by gas chromatography using a Perkin-Elmer chromatograph. A 5% phenylmethyl polysiloxane fused-silica column (AT-5, Alltech Associates Inc., Deerfield, IL), 60 m x 0.25 mm, film thickness 0.25 μm, was used. The initial oven temperature was 60°C; it was then heated to 260°C at 4°C/min, and the final temperature maintained for 20 min. The injector and detector temperatures were 200°C and 250°C, respectively. The carrier gas was helium at 1.0 mL/min. The sample (1 μL) was injected using a Hewlett-Packard ALS injector with a split ratio of 50:1. The identification of each of the compounds was performed by GC-MS with a Hewlett Packard Model 5973, equipped with a HP-5MS column 30 m long x 0.25 diameter, film thickness 0.25 μm Hewlett-Packard. The oven temperature program was the same as that used for the HP-5 column for GC analysis; the transfer line temperature was programmed from 150°C to 280°C; Injector temperature 230°C, interphase temperature 150°C, helium carrier gas at a linear speed of 34 m/s, ionization energy 70 eV, scan range of 40-50 amu, 3.9 scan/s. Injection volume 1 μL of a solution diluted to 2% in diethyl ether. The identification of compounds was based on database Wiley and NIST MS Data Library 05, logarithmic retention indices (LRI) were compared with values available in the literature (6th edition) [26].

**Table 1:** Chemical composition of essential oil of Lantana camara var. moritziana.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compounds</th>
<th>Area (%)</th>
<th>LRI</th>
<th>LRI [26]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(Z)-3-Hexenol</td>
<td>0.8</td>
<td>854</td>
<td>851</td>
</tr>
<tr>
<td>2</td>
<td>α-Hexanol</td>
<td>0.6</td>
<td>866</td>
<td>837</td>
</tr>
<tr>
<td>3</td>
<td>α-Pinene</td>
<td>1.9</td>
<td>938</td>
<td>936</td>
</tr>
<tr>
<td>4</td>
<td>Sabinene</td>
<td>1.4</td>
<td>975</td>
<td>973</td>
</tr>
<tr>
<td>5</td>
<td>β-Pinene</td>
<td>1.9</td>
<td>979</td>
<td>978</td>
</tr>
<tr>
<td>6</td>
<td>Myrcene</td>
<td>0.7</td>
<td>990</td>
<td>991</td>
</tr>
<tr>
<td>7</td>
<td>α-Phellandrene</td>
<td>6.7</td>
<td>1005</td>
<td>1005</td>
</tr>
<tr>
<td>8</td>
<td>δ-3-Carene</td>
<td>2.2</td>
<td>1011</td>
<td>1011</td>
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<tr>
<td>9</td>
<td>α-Cymene</td>
<td>2.3</td>
<td>1026</td>
<td>1022</td>
</tr>
<tr>
<td>10</td>
<td>Limonene</td>
<td>5.7</td>
<td>1031</td>
<td>1031</td>
</tr>
<tr>
<td>11</td>
<td>1,8-Cineole</td>
<td>0.9</td>
<td>1091</td>
<td>1091</td>
</tr>
<tr>
<td>12</td>
<td>(E)-β-Ocimene</td>
<td>0.8</td>
<td>1050</td>
<td>1041</td>
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<tr>
<td>13</td>
<td>Terpinolene</td>
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<td>1091</td>
<td>1092</td>
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<tr>
<td>14</td>
<td>Linalool</td>
<td>1.0</td>
<td>1101</td>
<td>1086</td>
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<tr>
<td>15</td>
<td>Camphor</td>
<td>0.5</td>
<td>1152</td>
<td>1143</td>
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<tr>
<td>16</td>
<td>α-Copaene</td>
<td>0.5</td>
<td>1383</td>
<td>1379</td>
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<tr>
<td>17</td>
<td>β-Bourbonene</td>
<td>0.5</td>
<td>1391</td>
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<td>18</td>
<td>β-Cubebene</td>
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<td>β-Elemene</td>
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<td>β-Caryophyllene</td>
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<td>1.3</td>
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<td>1468</td>
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<tr>
<td>25</td>
<td>Altoaromadendrene</td>
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<td>1461</td>
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<tr>
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<td>t-Murolene</td>
<td>0.6</td>
<td>1491</td>
<td>1480</td>
</tr>
<tr>
<td>27</td>
<td>Germacrene D</td>
<td>31.0</td>
<td>1497</td>
<td>1480</td>
</tr>
<tr>
<td>28</td>
<td>α-Chamigrene</td>
<td>5.1</td>
<td>1511</td>
<td>1503</td>
</tr>
<tr>
<td>29</td>
<td>α-Farnesene</td>
<td>1.6</td>
<td>1519</td>
<td>1516</td>
</tr>
<tr>
<td>30</td>
<td>δ-Cadinene</td>
<td>0.8</td>
<td>1535</td>
<td>1524</td>
</tr>
<tr>
<td>31</td>
<td>Germacrene B</td>
<td>2.4</td>
<td>1566</td>
<td>1560 [13]</td>
</tr>
<tr>
<td>32</td>
<td>Spathulenol</td>
<td>0.8</td>
<td>1584</td>
<td>1576</td>
</tr>
<tr>
<td>33</td>
<td>Caryophyllene oxide</td>
<td>0.9</td>
<td>1589</td>
<td>1581</td>
</tr>
</tbody>
</table>

Compounds were identified by comparison of the mass spectrum of each component with the Wiley GC/MS library data base and from logarithmic retention index (LRI) data. Area % was determined by GC-FID.

**Table 2:** Antimicrobial activity of the essential oil of Lantana camara var. moritziana.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Inhibition zone (mm)*</th>
<th>MIC μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus ATCC (25923)</td>
<td>8* 35*</td>
<td>400</td>
</tr>
<tr>
<td>Enterococcus faealis ATCC (29212)</td>
<td>8* 21*</td>
<td>350</td>
</tr>
<tr>
<td>Escherichia coli ATCC (29222)</td>
<td>NA 24*</td>
<td>NT</td>
</tr>
<tr>
<td>Klebsiella pneumoniae ATCC (23357)</td>
<td>NA 32*</td>
<td>NT</td>
</tr>
<tr>
<td>Salmonella Typhi CDC57</td>
<td>NA 40*</td>
<td>NT</td>
</tr>
</tbody>
</table>

E: Erythromycin (150μg), VA: Vancomycin (30 μg), SAM: Sulbactam - Ampicillin (16μg/10μg), AZT: Aztreonam (30μg), CIP: Ciprofloxacín (30μg), CAZ: Cefazidime (30 μg). NA: non active, NT: not tested.

*Inhibition zone, diameter measured in mm, disc diameter 6 mm, average of two consecutive assays.

MIC: Minimal inhibitory concentration, concentration range 10-600 μg/mL.
Microbiological analysis

**Bacterial strains:** *Staphylococcus aureus* ATCC 25923, *Enterococcus fecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 23357, *Salmonella Typhi* CDC57 y *Pseudomonas aeruginosa* ATCC 27853 were used in this study, provided by the Department of Microbiology and Parasitology, Faculty of Pharmacy and Bioanalysis, University of the Andes.

**Antibacterial method:** Antibacterial activity was evaluated according to the agar diffusion method with disks [27]. The strains were maintained in agar conservation at room temperature. Every bacterial inoculum (2.5 mL) was incubated in Mueller-Hinton broth at 37°C for 18 h. The minimum inhibitoty concentration (MIC) was determined only against organisms that showed inhibition zone, preparing dilutions of the oil with dimethylsulfoxide at concentrations of 10 to 600 μg/mL. The reference antibiotics employed were: Erythromycin® 150μg, Vancomycin® 30 μg, Sulbactam-Ampicillin® 10 μg/10 μg, Aztreonam® 30 μg, Ciprofloxacin® 30 μg, and Ceftaxidime® 30 μg. The tests were performed in duplicate.

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References


Activity against *Streptococcus pneumoniae* of the Essential Oil and 5-(3-Buten-1-ynyl)-2, 2'-bithienyl Isolated from *Chrysactinia mexicana* Roots

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The essential oil of *Chrysactinia mexicana* retrieved from the root bark was characterized by gas chromatography coupled to a mass detector. The compounds silphiperfol-5-ene, 7-epi- silphiperfol-5-ene, modheph-2-ene, \(\alpha\)-isocomene, \(\beta\)-isocomene and methyl-linoleate were identified. The principal compound (76.42%) could not be identified by the library and was further isolated through a reverse phase C-18 chromatography followed by silica gel chromatography and identified as 5-(3-buten-1-ynyl)-2,2'-bithienyl. Both the oil and the isolated compound were tested for their antimicrobial activity against two strains of *Streptococcus pneumoniae* resistant to \(\beta\)-lactam antibiotics. MICs were 250 \(\mu\)g/mL and 125 \(\mu\)g/mL respectively. This is the first report about extraction of oil and compound 5-(3-buten-1-ynyl)-2, 2'-bithienyl from roots of *Chrysactinia mexicana* as well as the determination of antimicrobial activity against *S. pneumoniae*.

**Keywords:** *Chrysactinia mexicana*, essential oil, 5-(3-buten-1-ynyl)-2, 2'-bithienyl, antimicrobial activity, *Streptococcus pneumoniae*.

Essential oils are used in alternative medicine as a remedy for many infectious diseases, antimicrobial properties have been long established and several studies have confirmed that they have activity against bacteria, yeasts and fungi [1-3]. Essential oils have been traditionally used for respiratory tract infections [4].

*Chrysactinia mexicana*, is a fragrant plant that contains essential oils in aerial part, commonly known as calanque, damiana or hierba de San Nicolás or falsa damiana. It is distributed throughout northern Mexico and Texas, USA. It use is known for to treat respiratory illnesses and skin infections. The leaves and stems are used as a diuretic, to counteract stomach ailments or postpartum pain, using the root as a home remedy [5]. The activity of an ether extract of the roots of *Chrysactinia mexicana* against two strains of *Streptococcus pneumoniae* resistant to penicillin have been reported [6].

*Streptococcus pneumoniae*, is a major causative agents of infectious diseases of the respiratory tract, causing a significant number of deaths worldwide, in both hospital and community, affecting mainly children and elderly. This situation is exacerbated by the increasingly frequent emergence of resistant strains. This infectious disease is the leading cause of death in developing countries, followed by heart disease, diarrhea, HIV/AIDS and stroke. Pneumonia is the leading cause of death in children under 5 years and about two million deaths each year among this group of people around the world, mainly in low resource environments, poor nutrition and poor hygiene conditions [7,8]. In the last decades has increased the incidence of pneumococcal infections in adults, despite the wide availability of effective antibiotics, continue to cause high morbidity and mortality, reporting mortality rates of up to 20% in adult patients with bacterium [9,10]. Due to the great problem posed by bacterial infections, was reviewed the importance of using traditional medicine, mainly using natural products of plant origin, to counteract these diseases, including respiratory tract infections [11]. Our aim was to obtain the essential oil from the root of *Chrysactinia mexicana*, characterize their composition, to isolate some key components and evaluate their antimicrobial activity against two strains of *Streptococcus pneumoniae* resistant to \(\beta\)-lactam antibiotics.

Since there are reports of *Chrysactinia mexicana* use in folk medicine for the treatment of respiratory diseases and scientific studies of activity of both the shoot and root against various microorganisms [5,6,12], our aim in this...
work was, to characterize their composition, to isolate some key components and to evaluate their antimicrobial activity against two strains of *Streptococcus pneumoniae* resistant to β-lactam antibiotics.

In preliminary experiments we tested the activity of essential oil from the root bark and root without bark of *Chrysactinia mexicana* against two strains of *Streptococcus pneumoniae* resistant β-lactam antibiotics (ATCC 49619 and 24-CCPN-02) and we found major activity in root bark (MIC of 250 μg/mL against both strains), therefore we decided to work specifically with the root bark. We obtained a dark yellow essential oil with yield of 0.41%.

Cárdenas *et al.* reported the characterization of the essential oil from the leaves of this plant showing that the main components are terpenes, including eucalyptol (41.3%) and piperitone (37.7%) [12]. So far, no studies are available on the characterization of extract components or essential oil from root of *Chrysactinia mexicana*. This work is the first report on the study of the root of this plant, specifically of the essential oil obtained from the bark. GC/MS analysis showed the presence of 12 compounds. The main component had a retention time of 59.85 minutes and represented the 76.42%, other component with retention time of 66.43 minutes, represented the 8.34%. However, neither could be identified using the NIST library. Only six of the 12 compounds were identified from GC/MS spectra using the NIST library and they represented the 8.50% of total area. Five of them were sesquiterpenes (silphiperfol-5-ene, 7-epi-silphiperfol-5-ene, modheph-2-ene, α-isocomene and β-isocomene) and one was the ether methyl-linoleate. The sesquiterpenes have been reported in roots from *Silphium perfoliatum* and *Echinops giganteus* [13,14]. Other three minor component representing 15.08% of the total area were no identified (Table 1). Because the main component could not be identified by NIST library, we decided to isolate it and identified by spectroscopic techniques.

Essential oil obtained from the root bark of *Chrysactinia mexicana* was further purified by means of reverse phase low-pressure liquid chromatography, to afford the active compound six fractions were obtained (F1 to F6). The most important compound was found in fraction F5 and the yield was 34%. A gravitational column chromatography on silica gel was performed from F5 to give 4 fractions (F5a to F5d) and, pure compound was obtained in F5b representing a yield of 64.4% from the F5 initially placed in this column.

Compound was subjected to structural analysis, CG/MS analysis displayed one peak at 59.85 minutes with m/z 216 (M+). IR showed several important signals at ν max 3000-3104 cm⁻¹ (C-H), 2192.53 cm⁻¹ (triple bound C-C), 1636.14 cm⁻¹ and 1602.86 cm⁻¹ (terminal olefin) and 785 y 838 cm⁻¹ (corresponded to derivatives 2,2'-bithienyl 5 substituted).

<table>
<thead>
<tr>
<th>Position</th>
<th>Type</th>
<th>Chemical shifts, (ppm)</th>
<th>Coupling constants, Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>1'</td>
<td>C</td>
<td>139.01</td>
<td></td>
</tr>
<tr>
<td>2'</td>
<td>C</td>
<td>136.70</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CH</td>
<td>132.86</td>
<td>7.11 (d) 3.80</td>
</tr>
<tr>
<td>3</td>
<td>CH</td>
<td>127.97</td>
<td>7.02 (dd) 5.09, 3.60</td>
</tr>
<tr>
<td>CH₁</td>
<td>C, terminal olefin</td>
<td>127.04</td>
<td>5.74 (dd) 17.50, 1.90</td>
</tr>
<tr>
<td>4'</td>
<td>C</td>
<td>125.04</td>
<td>7.25 (d) 5.12</td>
</tr>
<tr>
<td>3'</td>
<td>CH</td>
<td>124.27</td>
<td>7.18 (d) 3.60</td>
</tr>
<tr>
<td>4</td>
<td>CH</td>
<td>123.54</td>
<td>7.04 (d) 3.80</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>121.75</td>
<td></td>
</tr>
<tr>
<td>CH₂</td>
<td>C, terminal olefin</td>
<td>116.82</td>
<td>6.04 (dd) 17.52, 11.20</td>
</tr>
<tr>
<td>C, alkyne</td>
<td>C</td>
<td>92.98</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>83.26</td>
<td></td>
</tr>
</tbody>
</table>

H NMR spectrum showed three signals at δ 5.56 ppm (dd, J =11.20, 1.90 Hz), δ 5.74 ppm (dd, J = 17.50, 1.90 Hz) and δ 6.40 ppm (dd, J = 17.50, 11.20 Hz) for terminal olefin. Signals between δ 7.04 ppm and δ 7.25 ppm were assigned to olefinic protons from heterocycles according to J values (J = 3.60, 3.80, 5.09, 5.12 Hz) (Table 2). The 13C NMR spectra exhibited 12 signals, including one methylene, six methines and five quaternary carbons, as seen determined by a DEPT experiment. Structure of 5-(3-buten-1-ynyl)-2,2'-bithienyl (figure 1) was established by 2D NMR experiments: COSY, HMQC and HMBC. The compound has been reported in aerial part of *Chrysactinia mexicana* and roots of other plants [15-18].

Compound was subjected to structural analysis, CG/MS analysis displayed one peak at 59.85 minutes with m/z 216 (M+). IR showed several important signals at ν max 3000-3104 cm⁻¹ (C-H), 2192.53 cm⁻¹ (triple bound C-C), 1636.14 cm⁻¹ and 1602.86 cm⁻¹ (terminal olefin) and 785 y 838 cm⁻¹ (corresponded to derivatives 2,2'-bithienyl 5 substituted).

![Figure 1: Structure of 5-(3-buten-1-ynyl)-2,2'-bithienyl.](image)

5-(3-buten-1-ynyl)-2,2'-bithienyl have been isolated from several plants and it antimicrobial and nematidical activities [16] have been reported (antimicrobial activity against the plants pathogens: *Colletotrichum sp*, *Fusarium oxysporum*, *Phomopsis viticola*, and *Peltula. obscurans* [19] or against the human pathogens: *Candida albicans*, *Escherichia coli* and *Sarcina lutea* [20]). We studied the activity of the pure compound 5-(3-buten-1-ynyl)-2,2'-bithienyl against two resistant strains of *Streptococcus pneumoniae* (ATCC 49619 and 24-CCPN-02) and the MIC value was equal for both strains (Table 3). This is the first
report of the activity of 5-(3-buten-1-ynyl)-2, 2'-bithienyl on the pathogen causing respiratory tract infectious diseases in humans. The activities found for both, the compound isolated and essential oil were similar, this finding could be explained because the main component in the essential oil is 5-(3-buten-1-ynyl)-2, 2'-bithienyl that representing 75% of the total area.

On the other hand, we did an analysis of the essential oil of leaves but the compound 5-(3-buten-1-ynyl)-2, 2'-bithienyl was not detected. Therefore, we decide to prepare again extracts of the leaves, root bark, twigs and bark of the trunk (side closest to the root) using the solvents hexane, methylene chloride and methanol (1:1:1), agree with the report by Domínguez [15]. Our results showed that the compound was not present in the aerial part. The difference in the composition of the oil could be attributed to the date and place of collection.

The antimicrobial activity displayed by *Chrysactinia mexicana* essential oil against *Streptococcus pneumoniae* supports the traditional use of this plant for the treatment of infectious diseases. The isolation biodirected afforded the 5-(3-buten-1-ynyl)-2, 2'-bithienyl as the principal compound responsible of the antimicrobial activity shown by this plant. The results presented here are important in the search for new antimicrobial agents against bacteria responsible for respiratory diseases, especially those resistant to conventional antibiotics.

**Experimental**

**Bacterial culture:** *Streptococcus pneumoniae* (InDRE 24-CCpn-02) and *Streptococcus pneumoniae* (InDRE 49619), resistant to oxacillin and sensitive to vancomycin respectively, were obtained from the “Instituto Nacional de Diagnóstico y Referencia Epidemiológicos” (InDRE, México D.F., México). Microorganisms were maintained on agar supplemented with bovine blood (BBL, Becton Dickinson de México) until use.

**Plant material:** *Chrysactinia mexicana* roots were collected in Arteaga, Coahuila, Mexico, in November 2009. A voucher specimen (UNL 024102) was deposited in the herbarium of the Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León.

**Isolation of essential oil:** The essential oil was obtained by hydrodistillation of ground fresh root bark for 4 h, using a Cleverenger-type apparatus. Essential oil was conserved at –4°C until use.

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**Table 3: Minimal Inhibitory Concentration of essential oil and 5-(3-buten-1-ynyl)-2, 2'-bithienyl obtained from the root bark of *Chrysactinia mexicana* against *Streptococcus pneumoniae* strains**

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC (µg/mL)</th>
<th><em>Streptococcus pneumoniae</em> Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATCC 49619</td>
</tr>
<tr>
<td>Essential oil</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>5-(3-buten-1-ynyl)-2,2'-bithienyl</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Cephalotin</td>
<td>31.25</td>
<td>31.25</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>&lt;1.95</td>
<td>&lt;1.95</td>
</tr>
</tbody>
</table>

**CG/MS analysis:** Analysis of the essential oil was performed using an Agilent Technologies 6890N gas chromatograph equipped with an HP-5ms column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and a 5973 INERT selective mass spectrometer. The carrier gas was helium (99.999%) at a flow rate of 0.5 mL/min; ionization energy was 70 eV. Data acquisition was scan mode. Ionization source temperature was 230°C, quadrupole temperature was 150°C, and the injector temperature was 220°C. Oven temperature was programmed to 35°C for 9 min, then from 35°C to 150°C at 3°C/min and held for 10 min, then at 10°C/min to 250°C, and finally at 3°C/min to 270°C and held for 10 min. The samples were injected using the splitless mode. The injection volume was 2 µL. Components were identified by comparison of their retention indices (Kovats indices) relative to C₈–C₂₀ n-alkanes, and their mass spectra were compared with mass spectra from the US National Institute of Standards and Technology (NIST) library and reference data [21]. Relative percentages of components were calculated based on GC peak areas without using correction factors.

**Isolation of 5-(3-buten-1-ynyl)-2, 2'-bithienyl:** Essential oil (17.3 mg) obtained from the *Chrysactinia mexicana* root bark by hydrodistillation was subjected to inverse phase column chromatography over C-18. Elution was started with MeOH:H₂O 90:10 to 0.5 mL/min. until the principal compound was eluted and then with MeOH. Finally 6 fractions were obtained (F1 to F6). On the basis of the antimicrobial activity, F5 (5.9 mg) was further fractioned in column chromatography over silica gel with hexane and then with MeOH to give 4 fractions (F5a to F5d). According to its antimicrobial activity, fraction F5b (3.8 mg) was further analyzed by CG/MS, IR, ¹H NMR, ¹³C NMR and 2D NMR (COSY, HMQC and HMBC) and identified as 5-(3-buten-1-ynyl)-2, 2'-bithienyl. NMR Spectra were recorder on Bruker Avance DPX 400 equipment.

**Antimicrobial activity:** Both the oil and the isolated compound were tested for their antimicrobial activity. *Streptococcus pneumoniae* strains resistant to β-lactamic antimicrobials, were tested with microdilution assays according to the National Committee for Clinical Laboratory Standards [22]. In order to prepare the inocula, *Streptococcus pneumoniae* strains were cultured in Petri dishes containing blood agar (Bacto, Becton Dickinson). Plates were incubated overnight at 37°C and suspensions were prepared by transferring colonies to 0.85% NaCl solution until the turbidity of the 0.5 McFarland standard was reached. The suspensions were diluted 1:50 with cation-adjusted Mueller–Hinton broth supplemented with 5% lysed horse blood (CAMHB-LHB; BBL, Becton Dickinson) to make the working suspensions of *Streptococcus pneumoniae*. The essential oil was prepared at a concentration of 2 mg/mL in 20% of DMSO in CAMHB-LHB. The antimicrobial activity assay was performed in flat-bottom 96-well polystyrene microplates.
covered with a low evaporation lid. The culture medium was CAMHB-LHB. The concentration of the essential oil ranged from 500,000 to 15.625 µg/mL. Oxacillin and vancomycin were used as antimicrobial drug controls (64–4 µg/mL). The final concentration of microorganisms was \(1 \times 10^4\) UFC. Plates were incubated at 37°C for 24 h and bacterial growth was examined. The MIC was defined as the minimum concentration of essential oil that stops growth. Every biological assay was conducted in duplicate.

Acknowledgements - The authors are grateful to the biologists Marco Antonio Guzmán Lucio and M.C. María del Consuelo González de la Rosa for definitive taxonomic identification of species reported here. We thank Dr. Adolfo Caballero Quintero for support in IR analysis and Ivonne Carrera for her technical assistance in the extraction procedures and acknowledge grants 103.5/08/3125 and 103.5/09/4913 from PROMEP-Mexico.

References

Antimycotic Effect of the Essential Oil of *Aloysia triphylla* against *Candida* Species Obtained from Human Pathologies

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The research of alternative substances to treat infections caused by *Candida* species is a need. Aromatic plants have the ability to produce secondary metabolites, such as essential oils (EO). The antimicrobial properties of *Aloysia triphylla* (L’Her.) Britton (cedrón) EO has been previously described. The aims of this work were to determine the antimicrobial activity and the effect on the cell structure of the EO of *A. triphylla* against *Candida* sp isolated from human illnesses. The EO was obtained by hydrodistillation of *A. triphylla* leaves. The minimum inhibitory concentration (MIC) was performed with microdilution method and the minimum fungicidal concentration (MFC) was determined. *A. triphylla* EO’s showed antifungal activity against all yeast: *C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. guillermondii, C. parapsilosis* and *C. tropicalis* which were resistant to fluconazol (150 mg/mL). The range of MIC values was from: 35 to 140 µg/mL and the MFC: 1842 to 2300µg/mL. The time of killing at the MFC against *C. albicans* (3 x 10⁵ UFC/mL) was 140 min. The dates of OD₆₂₀ and OD₂₆₀ suggest lysis and loss of absorbing material, respectively. The HROM shows distortion in morphology and shape of the cell, with large vacuoles in the cytoplasm. These studies clearly show that *A. triphylla* EO is a promising alternative for the treatment of candidiasis.

Keywords: candidiasis, *Aloysia triphylla*, essential oil, antimycotic activity.

*Candida* species are commonly part of the normal flora in the digestive tract of healthy humans; however they have been described as responsible for opportunistic infections, particularly in neonates and immunocompromised patients [1]. These infections are difficult to treat with traditional drugs because they have multiple side effects, high toxicity and yeasts develop resistance against antifungal chemotherapics. Thus, searching for alternative antifungal compounds has been a major concern in recent years [2,3]. The investigation of alternative substances to treat these infections is necessary to find a solution to these problematic. Several medicinal plants have been extensively studied in order to find more effective and less toxic compounds [4].

Aromatic plants constitute an interesting group of vegetables with ability to produce secondary metabolites, such as essential oils (EO). *Aloysia triphylla* (L’Her.) Britton, (*Aloysia citriodora* Palau,) popularly known as “cedrón”, is a member of the Verbenaceae Family. It is perennial and grows widely in North and South America and also in northeast, northwest and central regions of Argentina. It is cultivated from Mexico till the South region of the continent. It is a bush with white flowers and fruits, with an intense scent lemon-like, sweet, lightly floral, and herbaceous [5,6]. This specie is used in folk medicine to treat many digestive disorders, as anti-inflammatory, analgesic, antipyretic, tonic and stimulating. It shares an important place on the international herbal market due to the sensory and medicinal properties of it EO. These attributes determine its use as a primary ingredient for infusions and nonalcoholic beverages as well as aromatic ingredient for the flavor and fragrance industries. The pharmaceutical industry uses *A. triphylla* for its carminative, antispasmodic and sedative properties [7,8]. EO are constituted by a complex mixture of organic compounds including monoterpens, diterpenes, carbonylated products and polyenes. There are many studies that suggest the antibacterial and antifungal activity of these compounds [9,10]. *A. triphylla* could be used to treat infections produced by *Candida* species.

The aims of this work were to determine the antimicrobial activity and the effect on the cell structure of the EO of *A. triphylla* against *Candida* species isolated from human illnesses.

The *A. triphylla* EO’s were analyzed with GC-MS. The average yield obtained in the hydrodistillation process was 0.4% (w/v) and the main components identified were: limonene (2.9%), neral (20%), geranial (29.2%), spathulenol (8.9%) and caryophyllene oxide (7%), in concordance with other authors that previously described...
all of them as the characteristic constituents of the EO of *A. triphylla* [6,10-12].

The antifungal activity of *A. triphylla* EO was tested using a microdilution broth method. The EO presented antifungal activity against all yeast. The range of MIC values was from 35 µg/mL to 143 µg/mL (Table 1). It is interesting to note low values of MIC necessary to inhibit *C. albicans* and *C. dubliniensis* (MIC: 35 µg/mL). In addition to this, the EO was able to cause the death of all *Candida* species. The fungicidal effect of the EO against the yeasts reached values of MFC from 230 µg/mL to 1842 µg/mL. (Table 1)

Table 1: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of *A. triphylla* essential oil against *Candida* species

<table>
<thead>
<tr>
<th>Species</th>
<th>MIC (µg/mL)</th>
<th>MFC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>35</td>
<td>460</td>
</tr>
<tr>
<td><em>C. dubliniensis</em></td>
<td>35</td>
<td>921</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>143</td>
<td>230</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>71</td>
<td>230</td>
</tr>
<tr>
<td><em>C. guillermondii</em></td>
<td>71</td>
<td>1842</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>71</td>
<td>1842</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>143</td>
<td>230</td>
</tr>
</tbody>
</table>

For more accurate evaluation of the antifungal activity of the EO, time-kill assays were performed using the yeast *C. albicans*. The killing time was tested at different cell concentration and results are shown in Figure 1. For 3 x 10^5 CFU/mL, killing time was 140 min. For a concentration of 3 x 10^6 CFU/mL the killing time was 300 min, for 3 x 10^8 CFU /mL was 480 min. and for 3 x 10^12 CFU /mL was 1140 min. (Figure 1). These data shows that it is necessary more time to kill a bigger cell population; this means that killing time of the EO is directly proportional to number of cell. In all cases the viability control of the yeast presented a macroscopically visible growth while treated cells did not show visible growth at each killing time.

Table 2 shows the results obtained in the cellular lyses assay with *C. albicans* cells treated at the MFC. The control showed an increase in OD_{620} what means that the yeast continued with its cellular growth. In contrast, in treated cells the OD_{620} diminished almost at not detectable values; this last result could be explained by the possibility that cellular lysis caused by the EO occurred.

In the suspensions obtained from the centrifugation of *C. albicans* inoculums treated with EO and not treated (control), the OD_{260} of the treated samples increased (OD_{260} = 0.270) compared to the control suspensions (OD_{260} =0.008). This suggests that there is a lost of 260-nm-absorbing material.

The HROM showed in control cells that the morphology and the cytoplasmic density were characteristic of a normal cell (Figure 2). After EO treatment at the MFC
(460 µg/mL), morphology and shape of the cell was distorted and a notable structural disorganization was seen within the cytoplasm with the observation of large vacuoles. In addition, the contents of some treated cells appeared depleted and amorphous. Lost of cellular material was also observed (Figure 3).

*A. triphylla* EO showed antifungal activity against all *Candida* species, particularly in those that acquire resistance to conventional chemotherapics, like *C. albicans* and *C. dubliniensis*. The last one was recently identified as an opportunistic pathogen associated with oral candidiasis, particularly in individuals who are positive for human immunodeficiency virus (HIV) and immunocompromised patients [1,13]. *C. albicans* is the most common fungal pathogen in humans, responsible for skin, oral, esophagic, intestinal tract, vaginal and circulatory diseases commonly affecting immunologically compromised patients and those undergoing prolonged antibiotic treatment [14].

The results presented in this work demonstrate that *A. triphylla* EO had the potential to kill *Candida* cells causing lysis. What is more, this study clearly demonstrates that there was a direct relationship between number of cells and the time that the EO needed to cause killing effect.

*C. albicans* suspensions treated with *A. triphylla* EO lost significant 260-nm-absorbing material, suggesting that nucleic acids were released through a damaged cytoplasmic membrane. These data are coincident to experiences made with *C. albicans* treated with tea tree oil [15]. Marked leakage of cytoplasmic material is considered indicative of gross and irreversible damage to the cytoplasmic membrane. Many antimicrobial compounds that act on the bacterial cytoplasmic membrane induce the loss of 260-nm absorbing material. Some antimicrobial agents cause gross membrane damage and provoke whole-cell lysis and this has been reported previously for essential oils from oregano, rosewood, and thyme [16].

EO components have the capability to alter cell permeability by entering between the fatty acyl chains making up membrane lipid bilayers and disrupt the lipid packing. Due to this, the membrane properties like permeability, fluidity and consequently its functions may get changed [17]. This may also affect the regulation and function of the membrane bound enzymes that alter the synthesis of many cell wall polysaccharide components and alter the cell growth morphogenesis [18].

The data obtained by Vataru Nakamura, et al. 2004. with electronic microscopy showed that *C. albicans* as well as *C. tropicalis*, *C. parapsilosis*, and *C. krusei* underwent remarkable ultrastructural alterations which were visible by electron microscopy, when treated with the essential oil of *O. gratissimum* [4]. This study is in concordance with our experience in which changes in the morphology of *C. albicans* cells were observed, as well as the formation of large vacuoles in the cytoplasm when they were exposed to the MFC of *A. triphylla* EO. Tyagy et al. 2010, worked with *C. albicans* cells treated with lemongrass EO and they observed similar results in experiences with different techniques of electronic microscopy [18].

*A. triphylla* EO is a promising natural product for the treatment of candidiasis. Therefore further studies on their pharmacokinetics and toxicological behavior are warranted. The results obtained represent a contribution to the characterization of the anti-*Candida* activity of EO of traditional medicinal plants from the Argentinian flora.

**Experimental**

**Plant material and EO extaction:** The EO was obtained by hydrodistillation of samples of *A. triphylla* collected from plants growing in farms (plantations) located in La Paz, Córdoba Province (Argentina).

**Gas Chromatography:** The EO were analyzed with a Shimadzu GC-R1A gas chromatograph equipped with a fused silica column (30 m x 0.25 mm) coated with CBP-1. The temperature of the column was programmed from 60°C to 240°C at 4°C/min. The injector and detector temperatures were at 270°C. The gas carrier was He, at a flow rate of 1 mL/min. Peak areas were measured by electronic integration. The relative amounts of the individual components are based on the peak areas obtained, without FID response factor correction. Programmed temperature retention index of the compounds were determined relative to n-alkanes. GC analysis was still performed using a column Supelcowax-10 with the same conditions as described above [19].

**Gas Chromatography-Mass Spectrometry:** GC-MS analyses were performed on a Perkin Elmer Q-910 using a 30 m x 0.25 mm capillary column coated with CBP-1. The temperature of the column and the injector were the same than those from GC. The carrier gas was He, at a flow rate of 1 mL/min. Mass spectra were recorded at 70 eV. The oil components were identified by comparison of their retention indices, mass spectra with those of authentic samples, by peak enrichment, with published data, mass spectra library of National Institute of Standards and Technology (NIST 3.0) and our mass spectra library which contains references mass spectra and retention indices of volatile compounds. GC-MS analysis was still performed using a column Supelcowax 10 with the same conditions as describe above [20].

**Microorganisms:** The activity of the EO was tested against the following yeasts: *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. guillermondii*, *C. parapsilosis* and *C. tropicalis*. These strains were resistant to fluconazole (150 mg/mL). The strains were isolated in the
Central Hospital of Rio Cuarto and identified in the Mycology Area of Department of Microbiology and Immunology of the National University of Rio Cuarto.

**Antimicrobial activity:** The minimum inhibitory concentration (MIC) of the *A. triphylla* EO was evaluated against yeast species with the broth microdilution method described by Mann and Markham (1998) [21]. The minimum fungicidal concentration (MFC) was determined [23].

**Culture methods:** Tubes containing Sabouraud Broth (SB) (Britania) with 0.1% (w/v) agar (SBA) were prepared at pH 7 inoculated with each microorganism and incubated overnight (18 h) at 37ºC. Optical densities were measured at 620nm in a spectrometer and number of cells was confirmed by the viable plate count on Sabouraud Agar (SA) (Britania).

Firstly, the cell concentration necessary to cause reduction of resazurin within 3.30 h was determined for each of the test microorganisms. Serial 10 fold dilutions of the overnight culture were prepared in SBA and aliquots (170 μL) from these dilutions were dispensed into microplates containing 20 μL of diluent (Dimethylsulphoxide-distilled water 1:1). The resazurin solution (10 μL) was added; then they were incubated for 3.30 h at 37ºC. The appropriate dilution to work was the last one unable to reduce resazurin (blue), which was tested, by the plate count method. Resazurin is a redox indicator that is blue in its oxidized form and pink in its reduced form [21].

**Determination of the Minimum Inhibitory Concentration (MIC):** Serial two fold dilutions of the EO were prepared by vortexing it in the diluent at room-temperature. The resazurin assay medium, SBA, was inoculated with the test organism to yield a final cell density ≈ 1 log cycle lower than the cell density required to reduce resazurin (usually 10⁶ cfu/mL). The inoculum density was confirmed by plate count. A sterile 96-well microtitre tray was set up with each of the tested microorganisms. Serial 10 fold dilutions of the test microorganisms were processed in SBA and aliquots (170 μL) of the dilution belonging to the MIC and the previous dilutions were inoculated in SA and incubated at 37ºC for 24 h. The MFC was considered as the last dilution that did not show cell growth [22].

**Yeast killing assays:** The time of killing of *A. triphylla* EO against the yeast *C. albicans* were evaluated by measuring the cellular viability at the MFC. The treatment consisted on a suspension of cells (UFC/mL) in SBA plus EO dilution (MFC) was incubated at 37ºC, 120 rpm. A sample (0.1 mL) was removed at 30 min intervals and plated on SA (viability control) and incubated overnight. A suspension of cells (UFC/mL) in SBA without EO was incubated at the same conditions (suspension control).

**Cellular lysis:** Suspensions of *C. albicans* (10⁴ UFC/mL) were prepared in SB (control) and SB supplemented with EO at the MFC and incubated at 37ºC for 18 h. The OD₆₂₀nm was measured at 0 min and at 18 h. Then both of them were centrifuged at 10000 rpm for 5 min. The pellet was resuspended in PBS (Phosphate buffer saline) and OD₆₂₀nm was measured. Each experience was made by triplicate.

**Loss of 260-nm-absorbing material:** The supernatant from the suspensions of *C. albicans* prepared for cellular lysis assay was used to measure the loss of OD 260-nm-absorbing material.

**High Resolution Optic Microscopy (HROM):** Thin cuts (± 0.25 μm) obtained using a manual ultramicrotome (Sorvall MT-1A, DuPont) of *C. albicans* were processed for high resolution optic microscopy. They were placed on a slide and stained with toluidine blue on a thermic platine, allowing the income of the dye in the fungal cell. Thin stained cuts were mounted in DPX (Merk®) and observed with an optic microscope Axiohort (Carl Zeiss, Alemania). Images were obtained with a digital camera Powershot G6, 7.1 megapixels (Canon INC, Japón) joined to the optic microscope. Software AxioVision Release 4.6.3 (Carl Zeiss, Alemania) was used to process the images [23].

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Antimycotic activity of the essential oil of *Aloysia triphylla*  
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Secretory Cavities and Volatiles of *Myrrhinium atropurpureum* Schott var. *atropurpureum* (Myrtaceae): An Endemic Species Collected in the Restingas of Rio de Janeiro, Brazil

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In this study, we investigated the leaf anatomy and the composition of volatiles in *Myrrhinium atropurpureum* var. *atropurpureum* endemic to Rio de Janeiro restingas. Particularly, leaf secretory structures were described using light microscopy, and histochemical tests were performed from fresh leaves to localize the secondary metabolites. To observe secretory cavities, fixed leaf samples were free-hand sectioned. To evaluate lipophilic compounds and terpenoids the following reagents were employed: Sudans III and IV, Red oil O and Nile blue. Leaf volatiles were characterized by gas chromatography after hydrodistillation (HD) or simultaneous distillation-extraction (SDE). Leaf analysis showed several cavities in mesophyll that are the main sites of lipophilic and terpenoid production. Monoterpenes, which represented more than 80% of the major volatiles, were characterized mainly by α- and β-pinene and 1,8-cineole. In order to provide tools for *M. atropurpureum* identification, the following distinguishing characteristics were revealed by the following data: 1) adaxial face clear and densely punctuated by the presence of round or ellipsoidal secretory cavities randomly distributed in the mesophyll; 2) the presence of cells overlying the upper neck cells of secretory cavities; 3) the presence of numerous paracytic stomata distributed on the abaxial leaf surface, but absent in vein regions and leaf margin; and 4) non-glandular trichomes on both leaf surfaces. Our study of the compounds produced by the secretory cavities of *M. atropurpureum* led us to conclude that volatile terpenoid class are the main secretory compounds and that they consist of a high concentration of monoterpenes, which may indicate the phytotherapeutic importance of this plant.

**Keywords:** Atlantic rain forest, histochemistry, volatile compounds, secretory cavity, restinga, leaf anatomy, Myrtaceae.

The Atlantic Rain Forest is among the world’s foremost biodiversity hotspots \([1a]\). From evolutionary and conservation view points, the Atlantic coastal vegetation of Brazil, particularly in the case of the state of Rio de Janeiro, should be treated as a mosaic comprising all forest types and also the neighboring open vegetation \([1b,1c]\). Within this environment, a plant community represented by usually arbustive-herbaceous vegetation, known as a restinga, is located in part in Rio de Janeiro State. Restinga ecosystems are areas of coastal plains covered by marine deposits, which are the result of geological processes that occurred during the Quaternary, around 5 thousand years ago \([2]\). In this peculiar environment, plants are influenced by the Atlantic Ocean and thus subjected to high salinity and high temperatures. This ecosystem presents different physiognomies and diverse flora where Myrtaceae is one of the most important families. Plants in this area are under high risk of extinction as a consequence of increasing land development.

*Myrrhinium atropurpureum* Schott var. *atropurpureum* (Myrtaceae), which is restricted to Rio de Janeiro restingas, is a native source of medicinal compounds used as astringents and antimicrobials. A different variety, *M. atropurpureum* var. *octandrum* Bentham (syn. *M. loranthoides* Hook. et Arn.), may be found in Brazil’s southland to Uruguay and northwestern Argentina to Ecuador \([3]\). *M. atropurpureum* var. *atropurpureum* differentiates itself by having ovaries with 10-14 ovules per locule, leaves 1.7-2.5 times longer than wide, with thick blades and a leathery margin that is strongly revolute. This species is a large evergreen shrub, or small tree, reaching from 1.5 to 5.0 m high. In Brazil, *M. atropurpureum* is known as “pau-ferro”, “carrapato” and “murtillo”. It is an ornamental plant with aromatic leaves and fleshy, sweet and swollen flower petals commonly used as food for birds \([4]\). Similar to other Myrtaceae species, *M. atropurpureum* presents large secretory cavities in leaves, a peculiar structure that is a taxonomic
feature responsible for the accumulation of important secondary metabolites involved in plant defense [5,6]. In order to register a phytotherapeutic, it is necessary to perform a macroscopic and microscopic characterization of plant material to help identify a given species, as well as define its phytochemical features. Therefore, the purpose of the current study was to characterize the leaf anatomy and localize the leaf volatile secretory structures of *M. atropurpureum*, in addition to investigating the composition of volatiles produced in such secretory structures.

*M. atropurpureum* specimens are described as shrubs up to 5 m with opposite phyllotomy (Figure 1A, B) Adult leaves are elliptical, obtuse or rounded apex; base acute or rounded; margin revolute, leathery and discolored, with the adaxial face clear and densely punctuated by the presence of secretory cavities. *M. atropurpureum* produces flowers with petals elliptical to oval, 4-5 x 2-3 mm, red in floral bud, becoming purple or pink, juicy and sweet in full anthesis (Figures 1B-D) [7]. As discussed by Roitman et al. [4], this floral type is very uncommon and seems to be restricted to some genera of Myrtaceae and Scrophulariaceae, both of which occur in South America.

In frontal view, both faces of *M. atropurpureum* leaves are covered by an epidermis composed of common cells with straight and thickened walls (Figure 2A). In cross section, the epidermis is uniseriate and covered with a thick cuticular layer, especially on adaxial surface, as revealed by Sudan III and IV tests (Figure 21-J). These features are common to species occurring in dry places under high temperature and intense light [8a], as well as leaves of plants found in areas with saline soil near the sea (halophytes and psamophilous plants) [8b,8c]. In environments where the availability of nutrients in the substrate is reduced, such as Brazilian salt marshes, or restingas, the cuticular layer and dense wax deposits observed in leaves of some Myrtaceae [9] can serve a protective function by reducing the loss of phosphorus and potassium by leaching [10].

While numerous paracytic stomata are randomly distributed only on the abaxial leaf surface, they are absent in the vein regions and leaf margin (Figures 2E-F). On both surfaces of the epidermis, isolated cells, or groups with two, three, and even four colorless cells, are observed, separated from each other by a strongly cutinized wall (Figure 2A-B). The cross section evidences that these cells are related to oil secretory cavities in mesophyll. Around these sets, or groups of cells, the remaining epidermal cells occur concentrically. These cells, called overlying cells [9,12], can occur frequently, either in isolation or in pairs, and they are distinguished by low affinity for histological staining. In *M. atropurpureum*, the cell walls between overlying cells are straight and strongly cutinized (Figure 2A). It is possible that these special cells are related to the elimination of secretions, which are mainly composed of volatile compounds accumulated in the secretory cavities, by the intense reaction of cell wall thickening to reagents for lipophilic substances. Similar anatomical results were observed by Donato and Morretes [6] for *Eugenia brasilienisis*. The authors cite the study of Lilset et al. [12], in which *Melaleuca alternifolia* (Myrtaceae) leaves, when subjected to a vacuum, release drop of oil on the surface through overlying cells. Judging from studies of other Myrtaceae species, such as *Myrcia* sp. and *Campomanesia* sp. [11] we suggest that overlying cells are involved in the process of aroma elimination on the leaf surface, based on the strong olfactory properties of Myrtaceae population in natural habitats (personal observations).

Non-glandular trichomes are observed on both leaf surfaces (Figure 3). A second trichome type was observed and it was pluricellular, rounded-shape, and accumulate phenolic substances (Figures 2D, G; 3). These trichomes are composed of two or three cells in the basal region with one apical enlarged cell (Figures 2E-F). Some of these trichomes can occur near the overlying cells. Only phenolic substances are detected in these epidermal appendages. No mention of these types of trichomes is found in the literature, but, given the saline environment in which the studied plants live, these trichomes could be related to the elimination of salts impregnated in plant tissues, as proposed for *Eucalyptus* species living in salinized soils [13].

The cross section shows that the secretory cavities are round or ellipsoidal and outlined by slightly thickened cell walls. Internally, the secreting cells have a thin wall, and they show positive reaction to tests for lipophilic compounds (Figures 21-N). The cavities are randomly distributed in the mesophyll, and they accumulate a yellow-greenish secretion in fresh material (Figure 2A). Under the adaxial surface, the secretory cavities are located more deeply, and they connect with the epidermal.
layer through a series of cells that form a "neck" (Figures 2B-C). As suggested previously, the set formed by the neck cells is also possibly related to the elimination of secretions that are mainly composed of volatile compounds accumulated in the secretory cavities. On the abaxial face, the cavities are near the epidermis, or separated from it by a pair of cells. The histochemistry analysis confirmed that the content of secretory cavities of *M. atropurpureum* is lipophilic in nature, as revealed by the positive reaction with Sudan IV and Nile blue tests (Figures 2I-N). The Nile blue test showed that the lipids produced in the secretory cavities have an acidic composition (Figures 2L-N), and the positive reaction with Sudan confirms their volatile property (Figures 2I-J).

The number of secretory cavities in the leaf may be affected by environmental conditions. For instance, in *Eugenia brasiliensis*, which inhabits two contrasting environments in the Atlantic Rain Forest (Brazil), the number of secretory cavities in both faces of foliar blade was lower in individuals inhabiting moist shaded areas than individuals collected in the mostly dry and brightly lit areas at sea level, suggesting that the development of some tropical plants in restinga areas may reflect a greater production of essential oils and other compounds associated with therapeutic effects, in contrast to plants growing in shaded areas [6].

The palisade is formed by two layers of narrow, thin-walled cells, and the spongy parenchyma has up to 12 layers of voluminous cells with thin walls, presenting, a leaf with isolateral structure. Tiny druse-type crystals were observed in the chlorenchyma cells. Phenolic reaction was observed in the chlorenchyma cells. Phenolic reaction was observed in all mesophyll. Such features are typically found in xeromorphic plants growing in saline or dry environments, where a reduction in nutrients and water is common [8b]. The vascular system of the leaf blade is composed of vascular bundles with phloem proportionally more abundant than xylem (Figure 2H). Some morpho-anatomical and phytochemical features of resistance to
restinga environment conditions are recognized in *M. atropurpureum*: thick cuticle and walls in the epidermis, secretory structures, thick leaves, possibly accumulating water, and phenolic compounds. The lipophilic substances that accumulate in the cavities may also be a chemical defense that confer protection against herbivory.

The presence of lipophilic content observed in the secretory cavities led us to a phytochemical analysis of the composition of the volatiles, which is summarized in Table 1. The lipophilic nature of the secretion was detected after reaction with Nile blue, which showed blue staining indicative of lipid acids, in agreement with the presence of 1,8-cineole, which is weakly acid with a hydroxyl in its chemical structure. Positive reaction to Sudan confirmed the lipophilic character of the contents of secretory cavities. The leaf volatiles were found to be very poor in sesquiterpenes. Using both extraction methods, the components found in greater concentration among the volatiles were α and β-pinene and 1,8-cineole. On the other hand, the oil composition of the var. *atropurpureum* from southern Brazil presented limonene (35%), 1,8-cineole (23%) and α-pinene (12%) as the main compounds [14]. Based on our studies comparing volatiles from plants of the Grumari and Marambaia restingas, we found the concentration of α-pinene to be equally high. Similarly, α-pinene (75%) was found to be the main component in the volatile compounds of a different variety (*M. octandrum*) from Argentina [15]. This result may indicate that α-pinene is a plant marker compound in this genus.

We compared volatiles from *M. atropurpureum* collected from two distinct restingas during the same period (July, 2010). Using SDE, we showed variations in concentrations of the monoterpenes β-pinene, 1,8-cineole and γ-terpinene between Marambaia and Grumari restingas. In samples from Marambaia, there was a significant increase in 1,8-cineole and γ-terpinene concentrations, while in Grumari, an increase in β-pinene was revealed. However, no difference was found in α-pinene concentration, as noted above. These data demonstrate how the environment affects the production of volatiles, even though both restingas are in the same State, Rio de Janeiro.

According to Araújo [16], the restingas that occur in Rio de Janeiro can be divided on the basis on their flora and the physiognomy of ten types of vegetation, as influenced by both topography and climate. The Marambaia restinga is formed by a largely well-preserved extension of sand dunes approximately 40 km long. This area forms an east-west peninsular-like extension of the continent into the Atlantic Ocean linked to Marambaia Island. In contrast, Grumari is one of the smallest remaining restinga fragments located within the metropolitan area of Rio de Janeiro which has experienced increasing degradation by the expansion of farmland areas, contributing, in turn, to the loss of this habitat.

The hydrodistillation method was used to obtain essential oil. Similar to SDE, HD revealed the following major components: pinenes (35.0%) and 1,8-cineole (33.0%), β-caryophyllene and its oxide, spathulenol, globulol and guiol were the main sesquiterpenes found, totaling 16.9%. Therefore, considering the different methods of extraction, HD and SDE, for samples collected in April (HD) and July (SDE) in the same restinga - Grumari, the main volatile...
constituents, α and β-pinene and 1,8-cineole, presented significant differences in relative concentrations. That is, by using HD, α-pinene (25.5%) and β-pinene (9.5%) presented reduced concentrations, while the concentration of 1,8-cineole (33.0%) was high when compared to the SDE method where a reduction of only 1.7% for 1,8-cineole was found, but an increase in both α-pinene (54.0%) and β-pinene (22.0%). These differences in volatile composition may have resulted from the extraction method used or the influence of relative humidity and precipitation in accordance with seasonal variation in April and in July.

Overall, therefore, phytochemical analyses of the volatiles showed the presence of compounds with pharmacological interest in that the lipophilic content of secretory cavities and phenolics have previously been reported to demonstrate antibacterial, antifungal, anti-inflammatory and antioxidant activity [17,18a].

In conclusion, the leaf secretory system has numerous cavities with circular lumen close to the interface between the epidermis and the parenchymas. Histochemical reagents, such as Nile blue and Sudan, showed that these secretory cavities are responsible for the production of essential oil and lipophilic compounds. This finding agrees with volatile composition obtained by GC-FID and GC-MS, in which it was found that the main compounds were monoterpens, such as pinenes and 1,8-cineole, representing more than 50%. These morpho-anatomical and phytochemical data based on our study of Myrrhinium atropurpureum var. atropurpureum from restinga areas may help in species identification, and the volatile composition of this plant, consisting of pinenes and 1,8-cineole, has specific implications in the context of phytotherapeutics.

**Experimental**

**Plant material:** Leaves from Myrrhinium atropurpureum var. atropurpureum were collected between 10-11 h in two Brazilian restingas: Grumari, Municipality of Rio de Janeiro (23°02′94″S, 43°31′98″W), which is located in the Grumari Protection Environmental Area, and Marambaia Restinga - “Linha 4”, 23°02′75″S, 43°35′68″W (Municipality of Mangaratiba), both in Rio de Janeiro City, Brazil. The collection occurred in April, 2009 (HD), and in July, 2010 (SDE). A voucher specimen is deposited at the Herbarium of Rio de Janeiro Botanical Garden under accession number RB 415731. Myrrhinium atropurpureum leaves were collected from three georeferenced specimens growing in open shrub formation. Restingas are at sea level and present the following environmental conditions: temperature (± 24-23°C), annual precipitation (1172-1240 mm) and sandy soil.

**Leaf anatomy and histochemistry:** Mature leaves of two to three individuals were taken from the third or fourth nodes from the apex of the branch. The leaves were fixed in FAA70 and preserved in 70% ethanol. The leaves were free-handed, or, by using a Ranvier microtome, sectioned in longitudinal and transverse planes. The sections were clarified in sodium hypochlorite and rinsed in 1% acetic acid and distilled water. Afterwards, the samples were stained with alcian blue and fucsin 0.1%. The epidermis was described using leaf segments separated by the solution of Franklin [18b], stained with 0.25% fuchsin in 50% ethanol. All samples were mounted in 50% glycerin on slides with cover slips. For scanning electron microscopy (SEM), fixed leaves were dehydrated in graded ethanol series, submitted to critical point drying with CO2 (Leica EM CPD-030), mounted on stubs, and coated with a thin layer of gold (Denton vacuum Desk IV, LLC). The samples were analyzed with a JEOL JSM-6490LV scanning electron microscopy (JEOL, Tokyo, Japan).

Histochemical tests were performed on fresh leaves from the Marambaia restinga, sectioned by the free-hand method, and submitted to Sudan III, Sudan IV [19a] and Red oil O staining to determine lipophilic compounds [19b], followed by the Nile blue test for acid and neutral lipophilic compounds [19c]. Histochemical tests were adapted and carried out following Victório et al. [19d]. Control procedures for histochemical tests were carried out. All samples were rinsed with distilled water before mounting in glycerin on slides with cover slips. To confirm the nature of calcium oxalate crystals, tests were carried out with acetic acid and hydrochloric acid [19a]. All samples were rinsed with distilled water and mounted in glycerin on slides with cover slips. Observations were carried out and captured on light microscopy Olympus® (BX-41).

**Volatile extraction and gas chromatography analyses:** Two extraction methods were used: hydrodistillation (HD) and simultaneous distillation-extraction (SDE). For the HD method, fresh leaves (50 g) of Myrrhinium atropurpureum were cut and submitted to a Clevenger-type apparatus for 2 h, yielding 0.4% v/w of the oil. For the second extraction method, fresh leaves (5 g) of Myrrhinium atropurpureum were homogenized with 50 mL of distilled water and submitted to SDE for 2 h [20a]. A 2 mL volume of dichloromethane was used as an organic collecting solvent and placed in the solvent flask. Boiling chips were added to both flasks. Mineral oil bath under stirrer/heat plate was used to apply heat to flasks. The heating temperatures for the sample and solvent flasks were controlled at 110-130°C and 55-60°C, respectively, so that boiling in sample flasks began, and extraction was carried out for 2 h. The vapors were condensed as a result of the circulation of cooling water pumped to the apparatus. HD and SDE samples were introduced to GC/FID and GC/MS for analysis.

Analytical GC (gas chromatography) was carried out on a Varian Star 3400 gas chromatograph fitted with a DB-1-MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and equipped with flame ionization detection (FID). Temperature was programmed from 60°C to 240°C at
3°C/min. The injection consisted of 1 μL of distilled oil diluted with dichloromethane obtained by SDE. And, the essential oil obtained by HD was diluted in dichloromethane (1:1). Hydrogen was used as the carrier gas at a flow rate of 1 mL min⁻¹. The injector temperature was 260°C, with interface temperature of 200°C. Leaf volatile samples were analyzed in splitless mode. GC-MS analyses were carried out on a Shimadzu Model GC-MS-QP 5000 fitted with a HP-5/MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness). GC-MS conditions were the same as above, except for 1) helium which was used as the carrier gas at a flow rate of 1 mL/min and 2) the mass spectrometer which was operated on electron impact mode at 70 eV, at a scan rate of 0.5 scans/s and fragments from 40 to 500 Da. Quantification was performed from GC-FID profiles using relative areas (%). Identification of components in the volatiles was based on retention indices relative to n-alkanes (C₈ - C₁₀) and computer matching with the National Institute of Standards and Technology (NIST 98) library, as well as by comparison between mass fragmentation patterns and those reported in the literature [14, 20b].

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References


Chemical Composition and in vitro Antibacterial Activity of the Essential Oil of *Phthirusa adunca* from Venezuelan Andes

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In this paper, preliminary studies on the chemical characterization of *Phthirusa adunca* Meyer essential oil, obtained by hydrodistillation, is presented. The separation of the components was performed by GC-MS. Twenty-three compounds (94.5% of the sample) were identified of which the three major ones (76% of the sample) were β-phellandrene (38.1%), germacrene D (26.8%) and β-pinene (11.5%). The essential oil showed a broad spectrum of activity against *Salmonella Typhi* CDC 57 (100 µg/mL), *Staphylococcus aureus* ATCC 25923 (200 µg/mL), *Enterococcus faecalis* ATCC 29212 (250 µg/mL), *Escherichia coli* ATCC 25922 y *Klebsiella pneumoniae* ATCC 23357 (500 µg/mL). This is the first report on the composition and activity of the essential oil of this species.

Keywords: *Phthirusa adunca* (Meyer), Loranthaceae, essential oil, antibacterial activity, β-phellandrene.

Loranthaceae family comprises intertropical plants, hemiparasite on different species. It is composed for more than one thousand species. The main characteristic of this family is the connexion with xilematic vases of the host from which obtain water and nutrients but with a life cycle independent of the host. They possess unisex flowers, berry fruits and seeds protected for sticky substances [1,2].

Several references relate the ethnobotanical use of plants of the genus *Phthirusa*. Among them, *P. pyrifolia* (HBK) Eichl is used for stomach burning relief and bone fractures [3-5]. From *P. pyrifolia* a leaf lectin (PpyLL) with antimicrobial activity have been isolated [6]. The protector effect on gastric lesions induced by ethanol at 1 g/Kg weigh on rats have been reported [7] as well as its effect on the inhibition of fatty acid synthase and reduction of weight on rats [8].

*Phthirusa adunca* G. (Meyer) Maguire, known in Venezuela as guatepajarito, grows on a variety of shrubs and trees as well on rocky substrates [9]. Several of its physiological characteristics structure of their haustorium will depend on its host. It is used in Peru for delivery and fracture bone treatment [10]. In the current report the chemical composition of the essential oil of *P. adunca* collected in Venezuela and its antibacterial activity by the agar diffusion method on international reference bacteria is shown. Leaves of *P. adunca* were hydrodistilled yielding 0.8% of essential oil. Analysis of this by GC-MS allowed the identification of Twenty-three compounds which are listed in Table 1 (94.5% of the sample). However, 76% of the sample is represented by three compounds: β-phellandrene (38.1%), germacrene D (26.8%) and β-pinene (11.5%). These results are preliminary, and further study to characterize this essential oil is necessary. The essential oil showed a weak antimicrobial activity, as shown in table 2, against *Salmonella Typhi* CDC 57 (100 µg/mL), *Staphylococcus aureus* ATCC 25923 (200 µg/mL), *Enterococcus faecalis* ATCC 29212 (250 µg/mL), *Escherichia coli* ATCC 25922 y *Pseudomonas aeruginosa* (ATCC 27853) y *Klebsiella pneumoniae* ATCC 23357 (500 µg/mL).

Even thought the presence of β-phellandrene as the major compound of an essential oil has not been associated with good antimicrobial activity [11], the presence of germacrene D in the oils seems to be responsible for some antimicrobial activity [12]. Therefore the mix of the components in the oils seems to have a potential effect on the activity.

Experimental

Plant material: The aerial parts of *Phthirusa adunca* were collected (May 2008) at El Caucho, Mérida State, Venezuela, located at 2000 m.a.s.l. A voucher specimen Nº FMB501, collected by Bladimiro Silva, has been deposited at the Herbarium of the Facultad de Farmacia y Bioanálisis, University of Los Andes (MERF herbarium).
A Perkin-Elmer AutoSystem gas chromatograph equipped with a flame ionization detector and data handling system. A 5% phenylmethyl polysiloxane fused-silica column (AT-5, Alltech Associates Inc., Deerfield, IL), 60 m x 0.25 mm, film thickness 0.25 μm, was used. The initial oven temperature was 60°C; it was then heated to 260°C at 4°C/min, and the final temperature maintained for 20 min. The injector and detector temperatures were 200°C and 250°C, respectively. The carrier gas was helium at 1.0 mL/min. The sample (1μL) was injected using a Hewlett-Packard ALS injector with a split ratio of 50:1. Retention indices were calculated relative to C8-C24 n-alkanes, and compared with values reported in the literature [14].

Gas chromatography–mass spectrometry: GC-MS analyses were carried out on a Model 5973 Hewlett-Packard GC-MS system fitted with a HP-5MS fused silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm, Hewlett-Packard). The oven temperature program was the same as that used for the HP-5 column for GC analysis; the transfer line temperature was programmed from 150°C to 280°C; source temperature, 230°C; quadrupole temperature, 150°C; carrier gas, helium, adjusted to a linear velocity of 34 cm/s; ionization energy, 70 eV; scan range, 40:500 amu; 3.9 scans/s. The sample was diluted with diethyl ether (20μL in 1 mL) and 1μL was injected using a Hewlett-Packard ALS injector with a split ratio of 50:1. The identity of the oil components was established from their GC retention indices, by comparison of their MS spectra with those of standard compounds available in the laboratory, and by a library search (Nist, 05) [14-16].

Microbiological analysis

Bacterial strains: Staphylococcus aureus (ATCC 25923), Enterococcus faecalis (ATCC 29212), Escherichia coli (ATCC 25922), Klebsiella pneumoniae (ATCC 23357), Salmonella Typhi (CDC 57) and Pseudomonas aeruginosa (ATCC 27853) were used in this study.

Antimicrobial method: The antimicrobial activity was tested according to the disc diffusion assay described by Velasco et al. [17]. The strains were maintained in agar conservation at room temperature. Every bacterial inoculum (2.5 mL) was incubated in Mueller-Hinton broth at 37°C for 18 h. The bacterial inoculum was diluted in sterile 0.85% saline to obtain a turbidity visually comparable to that of a McFarland N° 0.5 standard (10^5 CFU/mL). Every inoculum was spread over plates containing Mueller-Hinton agar and a paper filter disc (6 mm) saturated with 10 μL of essential oil. The plates were left for 30 min at room temperature and then incubated at 37°C for 24 h. The inhibitory zone around the disc was measured and expressed in mm. A positive control was also assayed to check the sensitivity of the tested organisms using the following antibiotics: Erythromycin® (15 μg), Vancomycin® (30 μg), Sulbactam-Ampicillin® (10 μg/10 μg), Aztreonam® (30 μg), Ciprofloxacin® (30 μg), Ceftazidime® (30 μg) (Table 2).

The minimum inhibitory concentration (MIC) was determined only with microorganisms that displayed resistance.

Table 1: Chemical composition of the essential oil of Pithirusa adunca.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peak Area (%)</th>
<th>LRI</th>
<th>LRI Literature</th>
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<tr>
<td>α-Pinene</td>
<td>1.3</td>
<td>937</td>
<td>939</td>
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<tr>
<td>Sabine</td>
<td>5.0</td>
<td>976</td>
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<tr>
<td>β-Pinene</td>
<td>11.5</td>
<td>980</td>
<td>980</td>
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<tr>
<td>Myrcene</td>
<td>1.2</td>
<td>990</td>
<td>991</td>
</tr>
<tr>
<td>1-Phellandrene</td>
<td>1.2</td>
<td>1004</td>
<td>1005</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>0.2</td>
<td>1018</td>
<td>1018</td>
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<tr>
<td>β-Terpineol</td>
<td>38.1</td>
<td>1036</td>
<td>1031</td>
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<tr>
<td>trans-β-Octine</td>
<td>0.3</td>
<td>1050</td>
<td>1050</td>
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<tr>
<td>γ-Terpineol</td>
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<td>1061</td>
<td>1062</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>0.2</td>
<td>1090</td>
<td>1090</td>
</tr>
<tr>
<td>1-Terpineol</td>
<td>0.1</td>
<td>1145</td>
<td>1144</td>
</tr>
<tr>
<td>4-Terpineol</td>
<td>0.8</td>
<td>1183</td>
<td>1174</td>
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<tr>
<td>α-Terpineol</td>
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<tr>
<td>δ-Elemene</td>
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<tr>
<td>α-Copaene</td>
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<td>β-Cubebene</td>
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<tr>
<td>β-Caryophyllene</td>
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<td>α-Humulene</td>
<td>0.9</td>
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<tr>
<td>Germacrene-D</td>
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<td>Bicyclogermacre</td>
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<td>T-Murolol</td>
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<tr>
<td>Kaur-16-ene</td>
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<td>2039</td>
<td>2042</td>
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<tr>
<td>Total</td>
<td></td>
<td></td>
<td>94.5</td>
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Table 2: Antimicrobial activity of the essential oil of Pithirusa adunca.

<table>
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<th>Microorganism</th>
<th>Inhibition zone (mm)*</th>
<th>MIC (μg/mL)</th>
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<tr>
<td></td>
<td>Oil</td>
<td>E</td>
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<tr>
<td>Staphylococcus aureus (ATCC 25923)</td>
<td>17*</td>
<td>35*</td>
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<tr>
<td>Enterococcus faecalis (ATCC 29212)</td>
<td>10*</td>
<td>21*</td>
</tr>
<tr>
<td>Escherichia coli (ATCC 25922)</td>
<td>10*</td>
<td>24*</td>
</tr>
<tr>
<td>Klebsiella pneumoniae (ATCC 23357)</td>
<td>7*</td>
<td>32*</td>
</tr>
<tr>
<td>Salmonella Typhi (CDC 57)</td>
<td>12*</td>
<td>40*</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (ATCC 27853)</td>
<td>NA</td>
<td>35*</td>
</tr>
</tbody>
</table>

E: Erythromycin® (15 μg), VA: Vancomycin® (30 μg), SAM: Sulbactam-Ampicillin® (10μg/10μg), AZT: Aztreonam® (30 μg), CIP: Ciprofloxacin® (30 μg), CAZ: Ceftazidime® (30 μg), NA: non active, NT: not tested.

Isolation of the essential oil: Fresh leaves (1000 g) were cut into small pieces and subjected to hydrodistillation for 3 h using a Clevenger-type apparatus. The oil (0.8% yield) was dried over anhydrous sodium sulfate and stored at 4°C [13].

Gas chromatography: GC analyses were performed using a Perkin-Elmer AutoSystem gas chromatograph equipped with a flame ionization detector and data handling system.
inhibitory zones. MIC was determined by dilution of the essential oil in dimethylsulfoxide (DMSO), pipetting 10 μL of each dilution onto a filter paper disc. Dilutions of the oil within a concentration range of 10-600 μg/mL were also carried out. MIC was defined as the lowest concentration that inhibited the visible bacterial growth. A negative control was also included in the test using a filter paper disc saturated with DMSO to check possible activity of this solvent against the bacteria assayed. The experiments were repeated at least twice.

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