Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **26** (6): 846-51 (2007)

Trabajos originales

Recibido el 8 de septiembre de 2007
Aceptado el 14 de septiembre de 2007

In vivo Evaluation of the Mutagenic Potential of Estragole and Eugenol Chemotypes of *Ocimum selloi* Benth. Essential Oil

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SUMMARY. The aim of this work was to investigate the chemical composition and mutagenicity of the *O. selloi* essential oils native from the South of Brazil. The essential oil designated as chemotype A showed a total of 10 components, which constitute more than 99% of the volatile fraction. The major chemicals were methyl chavicol or estragole (46.33%), *trans*-anethole (31.20%) and germacrene D (6.37%). The chemotype of *O. selloi* called sample B was constituted by only 4 detected components as the most abundant compounds, with more than 99% of the composition of the essential oil characterized. The major constituents were methyl eugenol (45.17%), *trans*-caryophyllene (43.52%) and isoaromadendrene (4.83%). The results obtained in the *in vivo* evaluation of mutagenicity indicated that none of chemotype studied presents mutagenic activity. These results support existing information about the low toxicological risk in the *O. selloi* use as a traditional medicine.

RESUMEN. "Evaluación *In Vivo* del Potencial Mutagénico de los Quimiotipos Estragol y Eugenol del Aceite Esencial de *Ocimum selloi* Benth." Se determinó la composición química y la mutagenicidad de los aceites esenciales obtenidos de las hojas del *Ocimum selloi* Benth, perteneciente a la familia Lamiaceae, nativos de la región sur de Brasil. Para el estudio se utilizaron técnicas de cromatografía gaseosa capilar acoplada a espectrometría de masas (GC/MS). Para la evaluación de la mutagenicidad *in vivo* se utilizó el ensayo de micronúcleos en médula ósea de ratones Wistar. Del aceite esencial nominado quimiotipo A, fueron identificados 10 constituyentes, que correspondieron a más de 99% de la fracción volátil. Los constituyentes mayoritarios que se identificaron fueron metil chavicol o estragol (46,33%), trans-anetol (31,20%) y germacreno D (6,37%). La composición del quimiotipo B fue de solamente 4 compuestos, que correspondieron a 99% da composición de la esencia. Los compuestos más abundantes fueron metil eugenol (45,17%), *trans*-cariofileno (43,52%) e isoaromadendreno (4,83%). Los resultados de la evaluación *in vivo* de la mutagenicidad indicaron que ninguno de los quimiotipos estudiados presentó aumento estadístico significativo de las aberraciones cromosómicas, lo que sugerí que estos aceites esenciales no son mutagénicos. Estos resultados reafirman la información existente del bajo riesgo toxicológico del uso del *O. selloi* en medicina tradicional.

INTRODUCTION

The genus *Ocimum* L. belongs to the Lamiaceae and comprises annual and perennial herbs and shrubs native to the tropical and subtropical regions of Asia, Africa and South America. *Ocimum* taxa are an economically important group of plants used extensively for the pharmaceutical, food, flavour and perfumery industries [1]. The taxonomy of *Ocimum* is complex due to interspecific hybridization and polyploidy of the species in the genus [2]. Pushpangadan and Bradu recognized more than 150 species [3]; however, Paton *et al.* [4] proposed that *Ocimum* had only 65 species and other attributions should be considered as synonyms.

Among the native medicinal plants of Brazil, *Ocimum selloi* Benth., commonly known as alfavaca [5] has been wildly used as an anti-diarrheic, antispasmodic, analgesic and anti-inflammatory medicine [6-8]. Paula *et al.* [9] performed a study to provide data on the skin irritant potential and mosquito repellency of the estragole chemotype of *O. selloi* essential oil. None volunteer exposed to undiluted oil (4-h patch test)

KEY WORDS: Essential oil; Lamiaceae; Mutagenicity; *Ocimum selloi*.
PALABRAS CLAVE: Aceite esencial; Lamiaceae; Mutagenicidad; *Ocimum selloi*.

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ISSN 0326-2383

showed a positive skin irritant reaction. Also, results demonstrated that the analyzed oil is an effective repellent against *Anopheles braziliensis* Chagas, by reducing markedly the number of bites registered in a thirty min interval. Additional data reported the repellent activity of the eugenol chemotype of *O. selloi* essential oil against the same mosquito [10].

Farago *et al.* [11] studied the antibacterial activity of the estragole and eugenol varieties of *O. selloi* essential oil, where both chemotypes showed low response against *Escherichia coli* and *Staphylococcys aureus* strains.

Because of the frequent use of *O. selloi* as a traditional medicine and the lack of reports concerned with a comprehensive toxicological evaluation of essential oils [12], this study attempts to evaluate the chemical composition and mutagenicity of estragole and eugenol chemotypes of the *O. selloi* essential oils native to the South of Brazil.

EXPERIMENTAL

Plant material

The leaves of *O. selloi* were collected in Ponta Grossa - PR - Brazil (50° 07' W/25° 07' S) in January and February 2005 from three cultivated taxa in the downtown area, chosen as sample A, and from three wild herbs on the outskirts of the Caverna do Olho d'Água, designated as sample B. The plant materials were identified by Prof. Inês Janete Matozzo Takeda and voucher specimens were deposited at the Herbário of the State University of Ponta Grossa as HUPG 10,718 and HUPG 12,226, respectively.

Essential oil extraction

Essential oil was extracted by steam distillation of the dry leaves in a commercial Clevenger apparatus [13]. After 6 h of distillation, essential oils were obtained from 50 g of crude drug. The essential oil content was determined on a volume to dry weight basis. The values for essential oil content of the three replications were averaged. The essential oil samples, referred to as A for the urban material and as B for the wild material, were stored in glass vials with Teflon-sealed caps at 4 ± 0.5 °C in the absence of light.

Gas chromatography/mass spectrometry analysis (GC/MS-MSD)

Essential oil samples were analyzed by a Shimadzu GC-17A gas chromatograph coupled with a Shimadzu QP5050 mass spectrometer. Mass spectra and gas chromatograms were obtained as electron impact spectra. The constituents of the volatile oils were identified by comparing their GC retention times and the MS fragmentation pattern with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns with the mass spectra libraries and with those in the literature [14]. The analysis was carried out using a fused silica column DB-1 (inner diameter 0.25 mm, length 30 m, film thickness 0.25 µm). Operating conditions were as follows: injector temperature 250 °C; FID temperature 250 °C, carrier (helium) flow rate 1.2 mL/min and split injection with split ratio 1:43. Oven temperature was initially 60 °C and then raised to 180 °C at a rate of 4 °C/min, then raised to 230 °C at a rate of 5 °C/min and finally held at that temperature for 10 min. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 40 mA; scan rate, 1 1000 m/z per sec; mass range, 10-550 m/z; ion source temperature, 250 °C.

Biological assays

Experimental Animals

Wistar-EPM-1 male and female rats *(Rattus norvegicus)* weighing 150-300 g, 7-12 weeks old, were provided by the Biotério Central of the State University of Ponta Grossa. All animals were housed in individual cages and received a normal laboratory pellet diet and water *ad libitum*. The animals used in the present study were maintained in accordance with the guidelines of the Colégio Brasileiro de Experimentação Animal, Brazil. This protocol was approved by the Ethical Committee of the State University of Ponta Grossa, Brazil.

Acute intra-peritoneal toxicity

As a preliminary assay, an acute intra-peritoneal toxicity trial was carried out. Tests were conducted to determine the dose range of the essential oils, estragole chemotype - sample A and eugenol chemotype - sample B, in particular, the maximum tolerated dose (MTD). Each undiluted *O. selloi* essential oil was administered intraperitoneally (i.p.) to 60 rats (30 male and 30 female) randomized into 6 groups. From these, appropriated volumes of each sample containing 5,000, 2,500, 2,000, 1,500, 1,375 and 1,250 $mg.kg^{-1}$ were given to 6 groups of 5 rats each. The animals were observed for symptoms of acute toxicity, morbidity and mortality within 14 days. The MTD was determined as the concentration for which no toxicological sign was observed [15].

In vivo evaluation of the mutagenic potential

For the mutagenic assay, rats in the experimental groups received 100 and 50% of the MTD in a single i.p. injection [15]. For the sample A (estragole chemotype), considering preliminary data from the procedure described above, 2,000 and 1,000 mg.kg^{-1} were administered to the male rats. Female rats received 1,375 and 750 mg.kg^{-1} of this essential oil. For the sample B (eugenol chemotype), in turn, acute intraperitoneal toxicity indicated concentrations of 1000 and 500 mg.kg^{-1} to be administrated on rats of both sexes. Mitomycin C (Sigma, USA) 1 mg.kg^{-1} (i.p.) in 0.9% sodium chloride aqueous solution and a food-grade corn oil (Bunge Alimentos, Brazil) were used as positive and negative control groups, respectively.

Bone marrow was used for the micronucleus test [16]. Briefly, all rats were humanly sacrificed 36 h after the injection, when femur bone marrow was collected in fetal bovine serum (Laborclin, Brazil). After centrifugation at 1000 *g* for 5 min and redispersion, smears were prepared and slides were Giemsa stained. A total of 2000 reticulocytes were analyzed per rat.

Statistical analysis

The mutagenic potential data were submitted to one-way analysis of variance (ANOVA) and the t-test was then performed. Results were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

In keeping with the proposed goal of searching for Brazilian herbal medicine plants with mutagenic potential, this paper reports data on two chemotypes of *O. selloi,* motivated mainly by their frequent use by the population to treat pain and gastrointestinal tract disorders.

Essential oil content and chemical composition

Tables 1 and 2 show the *O. selloi* essential oil compositions determined by the combined GC/MS-MSD analysis for the samples evaluated.

The essential oil designed as A, an estragole chemotype of *O. selloi,* after undergoing steam distillation, appeared as pale yellow oil with a warm, spicy, anise-like odour in a mean yield of 1.3%. A total of 10 components were identified, which constitute more than 99% of the volatile

Peak	Compound [a]	ID method [b]	RT [c]	RA [d]
1	Methyl chavicol	MS, GC	16.52	46.33
2	*cis*-anethole	MS, GC	18.45	0.73
3	*trans*-anethole	MS, GC	20.56	31.20
4	*trans*-ocimene	MS	22.69	0.44
5	α-neoclovene	MS	24.12	1.23
6	*trans*-caryophyllene	MS	25.77	4.72
7	Germacrene D	MS	27.88	6.37
8	δ-elemene	MS	28.51	5.67
9	α-farnesene	MS	28.83	1.79
10	spathulenol	MS	31.18	1.50

Table 1. Essential oil composition of *O. selloi* - estragole chemotype - by GC/MS-MSD. [a] compounds are listed in order of elution from a DB-1 column. [b] MS, peaks identified on MS comparison with file spectra; GC/MS, peak identified on comparison with pure reference standards. [c] RT, retention time on a DB-1 column in min. [d] RA%, relative area percentage (peak area relative to total peak area %).

Peak	Compound [a]	ID method [b]	RT [c]	RA [d]
1	Methyl eugenol	MS, GC	24.97	45.17
2	isoaromadendrene	MS	28.05	4.83
3	*trans*-caryophyllene	MS, GC	31.28	43.52
4	isoelemicin	MS	31.60	5.69

Table 2. Essential oil composition of *O. selloi* - eugenol chemotype - by GC/MS-MSD. [a] compounds are listed in order of elution from a DB-1 column. [b] MS, peaks identified on MS comparison with file spectra; GC/MS, peak identified on comparison with pure reference standards. [c] RT, retention time on a DB-1 column in min. d RA%, relative area percentage (peak area relative to total peak area %).

fraction. Compounds involved in the biosynthetic phenylpropanoids pathway accounted for a total of 78.26% (Table 1). The major constituent was methyl chavicol (46.33%), also named estragole. The second and third most abundant constituents were *trans*-anethole (31.20%) and germacrene D (6.37%), respectively.

In contrast, an eugenol chemotype of *O. selloi,* chosen as sample B, appeared as pale green oil, spicy and with anesthetic responses, clove-like odour in a mean yield of 2.0%. In this case, only 4 components were detected as the most abundant chemicals. Phenylpropanoids and terpenoids displayed similar levels in the global essential oil composition (Table 2), with more than 99% of the volatile fraction characterized.

Many members of the Lamiaceae such as *Mentha* L., *Salvia* L. and *Origanum* L. spp., are also cultivated to be used as medicinal and as a source of essential oils. The essential oils of these species and of many other taxa from the Lamiaceae are mostly composed of mono- and sesquiterpenes. Similarly, many plants of the genus *Ocimum* contain essential oils based primarily on monoterpene derivatives such as camphor, limonene, thymol, citral, 1,8-cineole, geraniol, geranyl acetate and linalool [2,8,17]. However, the chemotypes of *O. selloi,* studied in this paper, showed higher proportions of phenolic derivates, such as methyl chavicol, *cis*- and *trans*-anethole and methyl eugenol (Tables 1 and 2), but also combined with different proportions of terpenoids.

Methyl chavicol (or estragole), the main chemical component of the *O. selloi* essential oil, designed as sample A, is used in the perfume industry and it has an odor resembling that of fennel and anise. Interestingly, estragole is a key component of the essential oils from aromatic plants belonging to other families such as aniseed *(Pimpinella anisum* L., Apiaceae), star anise *(Illicium verum* Hook f., Magnoliaceae), bitter fennel *(Foeniculum vulgare* Mill., Apiaceae) and tarragon *(Artemisa dracunculus* L., Asteraceae) [18-21].

O. selloi essential oil from the other variety, chosen as sample B, showed a derivative from the allylphenolic eugenol. Eugenol is a major component of the essential oil from cloves *(Eugenia caryophyllus* Bullock & S.G. Harrison = *Syzygium aromaticum* Merr. & L.M. Perry, Myrtaceae) and cinnamon leaf *(Cinnamomum zeylanicum* Blume, Lauraceeae) and it has been used as a dental analgesic and disinfectant product and it has also shown antioxidant, antifungal, anti-inflammatory and antibacterial activities [22-24]. So, the differences in composition between the analyzed varieties can play an important role in the biological properties, mostly in the medicinal and toxicological activities. In addition, the use of secondary metabolites, in this case the essential oil composition, can add some information to the taxonomy, as well recognized.

Some papers have been published showing differences in the composition of *O. selloi* essential oil [1,6,8]. These works indicated the presence of estragole, methyl eugenol and *trans*-anethol as chemotypes found in Brazil. For different *O. selloi* specimens, collected in different seasons, these authors reported several other minor compounds with numerous variations. Sample A (estragole chemotype) showed similarities to the volatile oil evaluated by Vieira & Simon although the results differ in the relative percentage of the constituents [8]. Moraes *et al.* [6] founded, as major constituents, *trans*-anethol (58.59%) and methyl chavicol (29.96%) for the leaves collected in January 2001. In our results (Table 1), methyl chavicol and *trans*-anethol presented almost the opposite, 46.33% and 31.20%, respectively. Sample B (eugenol chemotype) has a composition similar to a specimen studied by Martins *et al.* [1]. Again, differences can be observed, such as the high relative percentage of *trans*-caryophyllene (Table 2) in the current investigation.

Furthermore, Amaral and Casali characterized the isoenzymatic variability of *O. selloi* specimens by electrophoresis and confirmed the occurrence of distinct biochemical varieties for the species [7]. Thus, particular attention to the *O. selloi* chemotype is recommended for its use as a traditional medicine.

In vivo evaluation of the mutagenic potential

Considering the co-existence of two different chemotypes of *O. selloi* in the region of Ponta Grossa - PR - Brazil, often used by the population as a folk medicine, the focus of this investigation was to perform a toxicity data evaluation of the species.

In the present study, the genotoxicity of *O. selloi* essential oil, of estragole and eugenol chemotypes, was examined through the micronucleus test, an *in vivo* evaluation for the measurement of structural chromosomal damage. The micronucleus test, one of the most frequently adopted sensitive assays for investigat-

Sample	mean number of MNRETs ± standard deviation		
	food-grade corn oil group (negative contol)	Mitomycin C group (positive control)	test group [c]
A (estragole chemotype)	1.42 ± 0.38	2.75 ± 0.41	0.75 ± 0.25
B (eugenol chemotype)	0.40 ± 0.31	2.81 ± 0.85	0.50 ± 0.35

Table 3. Number [a] of micronucleated reticulocytes (MNRETs) observed in the bone marrow cells of Wistar rats [b] treated with *O. selloi* essential oils. [a] 36 h after injection, 2,000 cells per animal. [b] 10 rats per group (5 male and 5 female); [c] significantly different from negative control at $P < 0.05$.

ing the toxicological profile of chemicals with mutagenic, clastogenic and aneugenic effects, is highly relevant in the human context [15,16].

The data obtained in the *in vivo* test system are summarized in Table 3. The numbers of micronucleated reticulocytes, as mean ± standard deviation, were 1.42 ± 0.38, 2.75 ± 0.41, 0.75 ± 0.25 and 0.40 ± 0.31, 2.81 ± 0.85, 0.50 ± 0.35, respectively for the negative, positive and test groups of samples A and B. A statistically significant difference between the experimental group and the positive control group was observed, with a *P* value of 0.001 and 0.002, respectively for the estragole and eugenol chemotypes.

These results obtained from the micronucleus test showed that none chemotypes of *O. selloi* essential oils presents statistically significant increase in chromosome aberrations in the mean number of MNRETs in the bone marrow of Wistar rats. The results from this study therefore suggest that the essential oils tested are not mutagenic. However, previous findings are somewhat controversial, when the major constituents of these essential oils, methyl chavicol and methyl eugenol, were analyzed as pure compounds. From a chemical point of view, these compounds are substituted allylbenzenes and are supposed to be carcinogenic [22-24].

While some authors reported that estragole was weakly mutagenic for *Salmonella typhimurium* strains TA100, TA1535 and TA1537, other studies have indicated no evidence of methyl chavicol-induced genotoxicity in the Ames test and in other tested systems [25]. Chronic exposures to high doses of estragole and also to its 1-hydroxy metabolites by oral, intraperitoneal or subcutaneous routes, have been shown to increase the incidence of tumors (mostly hepatomas) in CD-1 and B6C3F1 mice [25,26].

Methyl eugenol had a negative response in multiple tests applied in various strains of *Salmonella typhimurium* and *Saccharomyces cerevisae* with and without metabolic activation [27]. Curiously, other papers demonstrated that methyl eugenol is not strongly mutagenic in bacterial or yeast test systems with metabolic activation. However, high doses of methyl eugenol and other substituted allylbenzenes showed carcinogenic effect in rodents. This has been observed in several different studies on mice and rats, both newborn and adult [27]. In 2000, it was reported that chronic oral intake of high dose levels of methyl eugenol was associated with increased incidence of hepatotoxicity and liver and stomach neoplasms in F344/N rats and B6C3F1 mice [28]. Moreover, in humans, for a risk assessment, special attention should be devoted to interindividual differences in the bioactivation of methyl eugenol. In particular, smokers or patients who use barbiturates showed a higher methyl eugenol 1'-hydroxylation rate, with a higher risk of the adverse effects from exposure to methyl eugenol [22].

In the present work, the results from the micronucleus test indicated that the *O. selloi* volatile oils are not mutagenic, a finding that seems to confirm that their major constituents (estragole and methyl eugenol) are not genotoxic compounds. These findings, as an *in vivo* test, support information about the low toxicological risk in the use of the *O. selloi* as a traditional medicine by the population.

Additionally, the genotoxicity of an estragole chemotype of *O. selloi* essential oil was evaluated in the *Salmonella/*microsome assay with and without metabolic activation. The results showed no mutagenic responses to tester strains TA97a, TA98 and TA100 [9], which reinforce to the present data.

Nevertheless, further studies are needed to confirm both the nature and implications of the dose-response curve in rats at low levels of exposure to the estragole and methyl eugenol chemotypes of *O. selloi* essential oils.

Acknowledgements. The authors are thankful to Inês Janete Mattozo Takeda for the species identification and to the Institute of Technology for Development (LACTEC) for the GC/MS-MSD analyses.

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