Elionurus muticus as an Alternative Source of Citral from Pampa biome, Brazil

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Abstract: *Elionurus muticus* is a grass from Pampa biome and it is an excellent alternative as natural source of citral in southern Brazil. The essential oil has a high citral level (80%) and presents variability for other important chemical compounds. The present studies indicated its cytotoxic effect against *Artemia salina* and also suggested its application in the control of pathogenic fungi. *E. muticus* could become an alternative to synthetic fungicides for using in agro-industries and also to screen and develop novel types of selective and natural fungicides.

Key words: essential oil, chemical profile, biological activity

1 INTRODUCTION

Elionurus muticus is a grass from Pampa biome, Brazil, known as lemongrass. Its essential oil is rich in citral, which is widely used in the aroma, food, and cosmetic industries. The world market for essential oil is increasing at about 11% per year, where Brazil is among the 15 largest exporters, with 1% of market share¹⁾. One of the essential oils' major compound that is most commercialized is citral, which has a strong citrus odor and is used in perfumery, food, and cosmetics industries. Citral is a mixture of two geometric isomers known as geranial and neral²⁾. Although citral can be obtained synthetically, there is an increasing demand for its natural production.

The mixture of compounds typical in natural products or bioproducts is one of their many advantages, and this may be a major cause of their success on biological tests³⁾. Currently, *Cymbopogon citratus* is one of the natural sources of citral; however, its production per hectare is low in Brazil⁴⁾. *Elionurus muticus* is an aromatic grass that is characterized by the presence of citral as major compound of its essential oil⁵⁾. *E. muticus* belongs to the Poaceae family and is known as lemongrass. It is native from Pampa biome, in Rio Grande do Sul, Brazil. The essential oil yield of both species is similar⁶⁾. In addition, as *E. muticus* occurs in abundance in Pampa biome, it demands little or no management, especially when compared with *C. citratus*. All these reasons make *E. muticus* a potential natural

source for citral production in Brazil.

Another reason for exploitation of bioproducts is their function as new biopesticide agents in plant protection and postharvest protectant. Post-harvest diseases render heavy losses to perishables during transit and storage. Higher water content, nutrient composition, and pH of most perishables make them capable of supporting the growth of a number of microorganisms. Fruits, due to their low pH, are spoiled primarily by fungi, which in addition to causing rot, may also contaminate fruits by producing mycotoxins. Worldwide post-harvest loss of perishables due to fungi is between 10% and 50%. Essential oils have been proven to be active against several pathogenic fungi⁷⁻⁹⁾.

Among post-harvest fungal pathogens, *Botrytis allii*, *B. cinerea*, and *Penicillium expansum* are three of the most common causal agents of diseases. *B. allii* species are very important in onion crop; it causes the disease known as neck rot during storage, causing losses of more than 60% in production in a few years. Infected onions are often asymptomatic and may develop the disease in transit or during storage, resulting in poor quality, mainly affecting onions for export^{10, 11)}. *B. cinerea* has extensive worldwide distribution; this fungus causes the so-called gray mold and can infect almost all plants and plant parts. In addition, it can cause latent infections that damage fruits before ripening¹²⁾. *B. cinerea* produces a range of enzymes, toxins, and other low molecular weight compounds that degrade cell

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wall. Evidence suggests that the pathogen induces the host to trigger the process of programmed cell death as a highlighted strategy¹³. *P. expansum* affects most fruits and vegetables. In some fruits, such as citrus, infections can start in the field, but are essentially post-harvest diseases and can often cause up to 90% decomposition in transit, storage, and marketing¹⁰. *Penicillium* enters the tissues through wounds. However, it can also spread from infected fruits to healthy skin upon contact, even if the skin does not have open wound. In addition to the losses caused by rotting of fruits and vegetables, *Penicillium* also produces several mycotoxins that contaminate products made from healthy fruits and rotten parts¹⁰. For instance, almost 100% of *P. expansum* strains produces patulin as mycotoxin¹⁴.

In addition to fungicidal activity, several essential oils are known to show cytotoxic activities^{15, 16}. One of the most common tests to determine the toxicity of various plant extracts is the *Artemia salina*'s lethality assay. *A. salina* has purine metabolism similar to mammalian cells and has been demonstrated to have a good correlation with antitumor activity^{17, 18}.

The essential oil of *E. muticus* shows high potential as a bioproduct, but despite the demand, few studies have been conducted on this species^{5, 6, 19–22}. Thus, the aim of this study was to characterize chemically the essential oil of *E. muticus* collected in southern Brazil to compare the profile with *C. citratus* and to verify its biological activity.

2 EXPERIMENTAL

Plants were collected in São Borja, Brazil (S28° 47' 25.9" / W 56° 05' 52.2"), and assembled and catalogued in the Herbário ICN from Departamento de Botânica/UFRGS (152282), Brazil. The collected plants were transplanted to pots containing 5 L of substrate and maintained in the greenhouse of the Departamento de Horticultura of the Faculdade de Agronomia/UFRGS. Plants were collected in October 2010 and all the work was carried out in 2 years (2010-2012).

Fresh leaves were harvested from 18 individual plants and kept in the freezer for further extraction. The essential oil was extracted through hydrodistillation in a Clevenger apparatus. The aqueous phase was separated with anhydrous magnesium sulfate. The extracted oil was stored in glass containers protected from light at -20°C . Plant fresh weight vary from each plant in the range of 20 to 102 g. Essential oil yield was estimated according to Santos et al. (2004)²³.

Essential oil constituents were analyzed for each plant using gas chromatography with a Thermo Scientific Focus GC with flame ionization detector (FID) using DB-5 capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) with helium as

carrier gas (flow 1.0 mL/min) with an injection volume of 1 μL (2% solution in heptane); the initial pressure was 1.91 Pa. Column temperature was programmed as follows: 80°C for 5 min, 80°C to 170°C ($2^{\circ}\text{C}/\text{min}$), 170°C to 260°C ($10^{\circ}\text{C}/\text{min}$), and the end was 260°C for 2 min. The injector temperature was 250°C , with injection in "splitless" mode. The analyses lasted 61 min. Samples were analyzed in three replicates each.

The essential oils were also analyzed and identified using mass spectrometry in chromatography coupled to mass selective detector DSQ II with the same conditions described previously. The condition has an impact energy of 70 eV. Chemical constituents were identified through studies of mass spectra, supplemented by computerized comparison of library equipment and the literature.

A series of alkanes (C9 to C30) was injected under the same conditions of the samples. The linear regression analysis of retention time corresponding to the number of carbons in each alkane was performed to obtain the Kovats index (KI), together with the retention time of each compound. KIs were compared with the literature data^{24, 25}. Each sample was analyzed three times. Mean and standard error were estimated for each compound detected in the analysis. The means were further compared with a commercial *Cymbopogon citratus* oil sample with a t-test using R software²⁶.

The experiments designed to test the biological activity of the essential oil of *E. muticus* were also performed with the essential oil of *Cymbopogon citratus* and then compared with the pure compound citral. Citral was obtained from Alfa Aesar (Lot 10168526), and the essential oil of *C. citratus* was obtained from BIO EEC Madagascar (Lot 564N001-Ech. 839).

Fungal activity experiments tested strains of *Penicillium expansum* (03/02/10 - IP 1350.82), *Botrytis cinerea* (22/02/20 - IP 1854.89), and *Botrytis allii* (02/03/10 - IP 1405.82), all obtained from Institut Pasteur (Paris, France). The activity was determined by contact and fumigation. The strains were cultivated for 7 days in bacteriological-malt agar medium (BM) consisting of 2% malt agar and 2% bacteriological agar. Three replicates were evaluated for each strain, and they were maintained at 23°C . After cultivation, the strains were collected with the aid of a sterile pipette to obtain discs with 0.5 cm of diameter, and were then spread on a Petri dish containing medium BM for the tests.

The fungicidal activity by contact was determined using diffusion in agar²⁷ consisting of an *in vitro* test where the strain was submitted to a contact with the essential oil. The strains were cultured for 7 days on BM and subsequently maintained at 23°C . After culture period, five plates with 12 wells were filled with 2 mL of medium BM and essential oil or citral. Five concentrations were tested: 0%, 0.01%, 0.015%, 0.02%, and 0.03%. Each plate corre-

sponded to a concentration.

The fumigation activity consisted of an *in vitro* test where the strains were submitted to the essential oil vapor. The strains were cultured for seven days on BM medium and subsequently maintained at 23°C. After seven days of culture, strains were transferred into a new plate containing MB medium and the essential oil was added to a paper pulp with a diameter of 8 mm, fixed on the lid of the plate. Each plate contained three strains for evaluation, and there were three replicates for each treatment. Treatments were evaluated at seven doses: 0, 1, 2, 4, 6, 8, and 10 $\mu\text{L}\cdot\text{mL}^{-1}$ air; each plate corresponded to a dose.

The extent of fungal mycelium was measured at 24h interval until the mycelium of the negative control reached the side of the plate on the 4th day. Results were submitted to analysis of variance, considering the type of essential oil, doses, application mode (contact and fumigation), and species of fungus as factors. The percentage of minimum inhibitory concentration (MIC_{50}) was estimated using simple linear or quadratic regression adjusting the dependent variable to 50 and estimating the independent variable using the fitted model. All statistical analyses were performed with R⁽²⁶⁾.

Artemia's cysts were hatched in filtered seawater collected in Canet, France. To the cysts' eclosion, seawater was added to approximately 80% of a Petri dish. Half of the plate remained in the dark, and the opposite side with light was maintained for 48 h. Because they are phototropic, the artemias migrated toward the light side.

After eclosion (48 h), the solutions were prepared in acrylic tubes in seven different concentrations of essential oil or citral: 1, 2.5, 5, 7.5, 10, 20, and 30 $\mu\text{g}\cdot\text{mL}^{-1}$. The solutions were diluted in dimethyl sulfoxide (DMSO) 1%, and 5 mL of seawater was added in each tube. Control treatment consisted of seawater with DMSO 1%. As positive control, potassium dichromate was used in the same concentrations tested.

With a pipette, 10 artemias were counted for each tube. Each concentration was tested in triplicates. The tubes were left in contact with the light and, after 24 h, were analyzed. The larvae were considered dead when they did not show normal movement during 10 s observation. Toxicity was determined using a 50% lethal dose LC_{50} , which was estimated according to the Reed-Muench (1938)⁽²⁸⁾ method and the percentage of larvae mortality. The larvae mortality data were submitted to analysis of variance, and the means were separated using Tukey's test ($\alpha = 0.05$) and R⁽²⁶⁾.

3 RESULTS AND DISCUSSION

Elionurus muticus showed an essential oil yield of 0.7 $\pm$ 0.14%, which is high compared with values obtained by other authors, that reached values around 0.1% to 0.5%

(considering other species from the genus *Elionurus*)^(5, 6, 29). It is also high, considering that the essential oil was extracted from a wild species and during the winter season, which usually causes a decrease in essential oil yield⁽⁵⁾. The yield per plant ranged from 0.13% to 1.26%, the ones with higher plant fresh weight with the most productive.

Chemical profile showed the predominance of monoterpenes, representing about 90% of the essential oil, and it also revealed the presence of some sesquiterpenes for *E. muticus* and *C. citratus* (Table 1). The essential oils of both species exhibit the typical characteristics of oils rich in monoterpenes as clear coloration, low viscosity, and high volatility. Because of these features, the essential oils rich in monoterpenes are widely used as flavorings and repellents. In addition, being small molecules, it easily penetrates tissues and cells.

The major compound for both species is citral (neral + geranial), corresponding to approximately 80% of the essential oil. Citral is also the major compound of the essential oil from *Elionurus* species collected in Argentina, Uruguay, and Zimbabwe^(6, 7). Silou *et al.* (2006)⁽³⁰⁾, analyzing the composition of the essential oil from leaves of *E. hensii* from Congo, observed the predominance of the compounds *cis*- and *trans*-*p*-mint-2.8(9)-dien-1-ol, *cis*- and *trans*-*p*-mint-1(7)8-dien-2-ol, 2-undecanone and 2-tridecanone. Still in Africa, Mevy *et al.* (2002)⁽²⁹⁾ obtained the following major compounds from the aerial parts of *E. elegans*: campherenone (43.0%), caryophyllene oxide (4.9%), and bisabolone (4.9%). In Brazil, the essential oil composition varies according to region. In the central region, the presence of camphene (11.5%), (E)-caryophyllene (17.9%), and spathulenol (18.6%) as major components was verified⁽⁴⁹⁾. In southern Brazil, especially in the border regions with Argentina and Uruguay, geranial and neral are present as major constituents of the essential oil⁽²⁰⁾.

Usually, species from the genus *Cymbopogon* are used in the perfumery and pharmaceutical industries because of its citral content. However, in Brazilian climate conditions, its production per hectare is low, mostly because of its lower resistance to cold, slower growth, and susceptibility to leaf rust⁽⁴⁾. On the other hand, *E. muticus* is an excellent alternative as natural source of citral because it is native to Brazil, being adapted to the environment and, therefore, requiring little or no field management. *E. muticus* develops in poor soils, improving degraded areas⁽⁴⁾. In addition, *E. muticus* essential oil has higher diversity of compounds that can be present in low concentration (Table 1), such as limonene, which has an important commercial demand.

Analysis of fungicidal activity indicated that citral and essential oil from *E. muticus* and *C. citratus* are effective against the fungi tested, because all treatments are found to inhibit the growth of all fungi tested in a dose-dependent manner (Table 2). In comparing contact and fumigation tests, fumigation was observed to be more effective in in-

Table 1 Retention index (RI) in a DB-5 column and percentage of the compound in the essential oil of *Elionurus muticus* and *Cymbopogon citratus*.

Compound	<i>E. muticus</i>		<i>C. citratus</i>	
	RI	%	RI	%
Alpha-pinene	939	2.73 ± 0.902**	—	—
Myrcene	989	2.69 ± 0.741**	989	9.81
Limonene	1027	0.54 ± 0.126**	—	—
Eucaliptol	1032	0.44 ± 0.092*	1034	0.37
Ocimene-cis-beta	1041	0.40 ± 0.097	1044	0.55
Linanool	1101	1.98 ± 0.131**	1099	1.50
Cis-cis-photocitral	1162	0.59 ± 0.085	1162	0.11
Trans-trans-photocitral	1181	1.14 ± 0.120	1181	0.20
Citronellal	1228	0.14 ± 0.027	1228	0.27
Neral	1239	31.54 ± 0.856**	1244	32.22
Geraniol	1252	0.80 ± 0.395**	1253	4.34
Geranial	1275	47.33 ± 1.889	1267	42.55
Bornyl acetate	1284	0.17 ± 0.064*	1279	—
Alpha-ylangene	1365	0.02 ± 0.025	—	—
Geranyl acetate	1377	0.74 ± 0.194**	1383	1.54
Beta-caryophyllene	1413	0.34 ± 0.118**	—	—
Ar-curcumene	1477	0.27 ± 0.130*	—	—
Zingiberene	1491	0.15 ± 0.075*	—	—
Elemol	1532	0.04 ± 0.025	—	—
Trans-nerolidol	1559	0.28 ± 0.251	—	—
Cariophyllene oxide	1571	0.12 ± 0.041**	—	—
10-epi-gamma-Eudesmol	1602	0.02 ± 0.010*	—	—
Isospathulenol	1621	0.01 ± 0.006*	—	—

*, ** Mean significantly different from *Cymbopogon citratus* value at 5% and 1% of probability, respectively, according to t test.

hibiting mycelium growth. When oil is added in the culture medium, the absorption of the essential oil is slower, depending on the fungus growth and substrate consumption³¹⁾. On the other hand, the higher toxicity by fumigant treatment occurs more rapidly because of the direct effect of the essential oil vapors on the fungal mycelia. Some investigators reported that the lipophilic nature of essential oils render them more absorbable by the fungal mycelia than by agar due to the highly lipophilic nature of the fungal mycelia and the high water content of the agar media^{32, 33)}.

Among all the essential oils tested, volatile and contact phases of citral caused the largest inhibition of mycelium growth for all fungi (Table 2). These results could be comparable with MIC values of the most frequently used synthetic fungicides³⁴⁾. According to literature, the MIC values

obtained in this work indicate a notable fungicidal effect for all treatments. For example, literature shows a MIC₅₀ range from 1.6 to 500 µg.mL⁻¹ in contact phase^{9, 34–38)} and a range of 0.08 to 300 µg.mL⁻¹ air in volatile phase^{39–41)} for the fungus tested.

When the treatment was applied by contact, the pure compound citral had higher inhibition of mycelium of *Botrytis allii*, inhibiting 50% of the mycelial growth at a concentration of 2.15 µg.mL⁻¹ (Table 2). Against this fungus, *C. citratus* essential oil showed the poorest response, with an average of 11.38% inhibition, not reaching MIC₅₀ at any concentration tested (Table 2). Fumigation test was more effective for *B. allii* inhibition. All essential oil tested produced similar average mycelium inhibition. Minimum inhibitory concentration (MIC₅₀) was 3.48 µg.mL⁻¹ air for citral, whereas for the essential oil of *E. muticus*,

Table 2 Average percentage of mycelium growth inhibition and minimum inhibitory concentration (MIC₅₀) for *Elionurus muticus* and *Cymbopogon citratus* essential oil and citral by contact (µg.mL⁻¹) and fumigation (µg.mL⁻¹ air) against the fungi *Botrytis allii*, *B. cinerea* and *Penicillium expansum*.

Treatment	Inhibition (%)								MIC ₅₀		
	E		C		Citral		Mean		E	C	Citral
<i>B. allii</i>											
Contact	33.33	b*	11.38	c	66.67	a	38.12	D**	3.07	—	2.15
Fumigation	54.81	a	46.63	ab	59.45	a	53.77	C	3.96	4.94	3.48
<i>B. cinerea</i>											
Contact	33.33	b	66.67	a	66.67	a	55.56	C	3.07	2.32	2.15
Fumigation	83.57	a	82.24	a	85.71	a	83.84	A	0.75	0.68	0.46
<i>P. expansum</i>											
Contact	44.51	b	67.41	a	71.68	a	61.51	C	1.71	3.59	1.66
Fumigation	67.70	a	69.10	a	77.71	a	71.61	B	2.76	2.57	1.21

* Means followed by the same lower case letter in each fungus are not statistically different according to Tukey's test ($\alpha = 0.05$).

** Means followed by the same upper case letter are not statistically different according to Tukey's test ($\alpha = 0.05$).

MIC₅₀ was reached at 3.96 µg.mL⁻¹ air (Table 2). All treatments on volatile phase exhibited total control of *B. allii* (100%) at 8 µg.mL⁻¹ air.

Botrytis cinerea also responded to the treatments. The pure compound citral and the essential oil of *C. citratus* showed higher activity when used in contact, MIC₅₀ was reached at a concentration of 2.15 µg.mL⁻¹ and 2.32 µg.mL⁻¹ for citral and *C. citratus*, respectively. For the essential oil of *E. muticus*, a concentration of 3.07 µg.mL⁻¹ was necessary (Table 2). The fumigation test for *B. cinerea* also showed more efficiency than contact test, revealing inhibition of 50% of mycelial growth at 0.46 µg.mL⁻¹ air for citral, followed by the essential oil of *C. citratus* that inhibits growth at 0.68 µg.mL⁻¹ air, and the essential oil of *E. muticus* at 0.75 µg.mL⁻¹ air (Table 2).

Results for *P. expansum* were also similar for all oil treatments, except for *E. muticus* oil used in contact, which showed a lower mycelium inhibition, 44.51% (Table 2). For the contact treatment, citral inhibits 50% of the mycelium growth at a concentration of 1.66 µL.mg⁻¹ (Table 2). With the fumigation test, citral was highly toxic with 1.21 µL.mg⁻¹ air. Total inhibition was achieved at 2 µL.mg⁻¹ air for citral, 4 µL.mg⁻¹ air for *C. citrates*, and 6 µL.mg⁻¹ air for *E. muticus*, evidencing a strong fungicidal effect.

The activity of essential oil is related to its chemical composition, structural configuration of its compounds, and its functional groups, with synergistic antagonist interactions of its chemical components⁹. Essential oils abundant in citral are well known for its antibacterial and fungicidal activities^{9, 21, 22, 42}. Ben-Yehoshua *et al.* (1992)³¹ observed a correlation between the reduction of the con-

centration of citral in lemon and the increase of susceptibility to infection by *P. digitatum*. *E. muticus* essential oil was proven to be efficient against *Candida spp.*, *Candida albicans*, *Saccharomyces cerevisiae*, and some bacteria^{21, 22, 29}. Studies on *C. citratus* essential oil also showed fungicidal activities against *Colletotrichum coccodes*, *B. cinerea*, *Cladosporium herbarum*, *Rhizopus stolonifer*, *Aspergillus niger*, *A. ochraceus*, and *P. expansum*^{9, 42, 44}.

The results show that in general, citral was more efficient as or similar to a fungicide compared with the essential oils. Linde *et al.* (2010)⁴⁵ found a higher fungicidal activity of the pure compound in comparison with essential oils whose major component was citral. This can be explained by the complexity of compounds of essential oils that, even with citral as the major compound, exhibit small amounts of other compounds that can provide an association between its effects and the presence of citral in the essential oil. Working with *Orthosiphon stamineus* essential oil, against *B. cinerea*, Hossain *et al.* (2008)⁴⁶ attributed the fungicidal effect to the essential oil's major components, β-caryophyllene, caryophyllene oxide, α-humulene, β-pinene, limonene, and β-elemene, which were found in *E. muticus* essential oil in minor concentration.

Few studies have focused on the mechanism by which essential oils inhibit microorganisms. Generally, the essential oils are known to be lipophilic, and thus, pass through the cell wall and cell membrane, affecting the structure of different layers of polysaccharides, fatty acids, and phospholipids and causing its permeabilization. Therefore, the effect of cytotoxicity is believed to include damage to the membrane⁴⁷.

Table 3 Mortality and lethal dose (LD₅₀) of essential oil of *Elionurus muticus* (EM). *Cymbopogon citratus* (CC) and citral in *Artemia salina*.

Treatment	Mortality (%)	LD ₅₀ (µg.mL ⁻¹)
Control	0.00b*	
Elionurus	51.43a	7.64
Cymbopogon	21.43ab	20.00
Citral	50.00a	7.96

* Means followed by the same letter are not statistically different according to Tukey's test ($\alpha = 0.05$).

The toxic activity of citral may be related to its conformation⁴⁸. Being a class of aldehydes α , β -unsaturated, the position of carbonyl group and the carbons α and β generates a conjugation between them, which makes the β carbon more positively polarized and consequently, more reactive with nucleophiles. The toxic effect of these aldehydes is based on their ability to act as direct-alkylating agents capable of covalent binding to cellular nucleophilic groups, modifying cellular processes and being potentially toxic to pathogens. Thus, the fungal difference in susceptibility to the treatments may be related to the content of protein in the fungal cell wall.

The test with *Artemia salina* revealed that the essential oils of both species (*E. muticus* and *C. citratus*) and citral are highly cytotoxic (Table 3). However, the essential oil of *C. citratus* showed an intermediate behavior, being similar to the control (no treatment). This result suggests that the toxic effect is mainly due to the high content of citral found in the essential oil. Several studies have shown the toxic activity of citral in various organisms and cells^{49,50}. The lethality of *Artemia salina* may be used as an indicator of antitumor compound and insecticide activity⁵¹. These authors proposed a classification based on LD₅₀, where values between 1 (1 mg/mL) and 40 ppm (40 mg/mL) characterize an antitumor compound and values below 1 ppm characterize an insecticide product. Consequently, the oils studied in the present work could be exploited for antitumor activity but not insecticide activity (Table 3).

4 CONCLUSIONS

Elionurus muticus is an excellent alternative as natural source of citral in southern Brazil. Its essential oil is rich in citral and presents variability for other important chemical compounds. The present study indicated its cytotoxic effect against *A. salina* and suggested its application in the control of pathogenic fungi. *E. muticus* could become an alternative to synthetic fungicides for use in agro-industries, also for use in screening and developing novel types

of selective and natural fungicides.

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