ARTIGO ORIGINAL

Toxicological effect of essential oils of plants against *Artemia salina*

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ABSTRACT: The present study was carried out with the objective of assessing the acute toxicity of four essential plant oils against the brine shrimp lethality bioassay. The essential oils were dissolved in dimethyl sulfoxide (DMSO) at 1 % and concentrations at 0.01; 0.05; 0.25; 1.25; 6.25 and 31.25 µg/ml were prepared which were added in microcells of 96 dishes containing from 10 to 15 nauplii of *Artemia salina* in saline solution with a total volume of 150 µL. Each concentration was replicated 8 times. Two controls were used, one with saline water solution containing 38 g of coarse salt/l and the other with DMSO 1 % solvent dissolved in the saline solution. Samples were maintained with light and at environmental temperature. Larvae death on exposing them to contact for 24 h was counted. The Lethal Concentration 50 (LC_{50}) was determined by the Probit method using the statistical package SPSS, version 22. All essential oils tested manifested a high acute toxicity at low dosages. These showed high lethality and its results followed by order of toxicity were: *Cymbopogon citratus* ($LC_{50=}$ 1.212 µg/ml) >*Lippia rotundifolia* ($LC_{50=}$ 1.256 µg/ml) >*Lippia origanoides* ($LC_{50=}$ 1.267 µg/ml) >*Cymbopogon citratus* Lemon-Grass© ($LC_{50=}$ 1.284 µg/ml). It is concluded that given the lethality exhibited by the essential oils of *Cympogogon citratus* Lemon-Grass©, *C.citratus*, *Lippia origanoides* and *Lippia rotundifolia* against the *Artemia salina* micro-crustaceous can be considered promissory essential oils for its use for the control of harmful organisms. Other more specific studies are recommended for demonstrating its biological activity.
Key words: essential oils, toxicity, LC_{50}, *Artemia salina*

RESUMO - Efeito toxicológico de óleos essenciais das plantas contra *Artemia salina.* O presente estudo foi realizado com o objetivo de avaliar a toxicidade aguda de óleos essenciais frente á *Artemia salina*. Os óleos essenciais foram dissolvidos em dimetilsulfóxido (DMSO) a 1% e em concentrações de 0,01; 0,05; 0,25; 1,25; 6,25 e 31,25 µg/ml e foram adicionados em microplacas de 96 poços contendo de 10 a 15 náuplios de *Artemia salina* em solução salina totalizando um volume de 150 µl, sendo que o ensaio com cada concentração foi repetido 8 vezes. Os controles foram com solução salina (38 g/l) e o outro com DMSO (1%). As amostras foram mantidas com luz e à temperatura ambiente e a mortalidade das larvas avaliada após 24h. A concentração letal 50 (CL_{50}) foi determinada pelo método Probit utilizando o pacote estatístico SPSS, versão 22. Todos os óleos essenciais testados manifestaram uma toxicidade aguda elevada a baixas dosagens. Os óleos que apresentaram alta letalidade foram o *Cymbopogon citratus* ($CL_{50=}$ 1.212 µg/ml) >*Lippia rotundifolia* ($CL_{50=}$ 1.256 µg/ml) >*Lippia origanoides* ($CL_{50=}$ 1.267 µg/ml) >*Cymbopogon citratus* Lemon-Grass© ($CL_{50=}$ 1.284 µg/ml). Conclui-se que, dada a letalidade exibida pelos óleos essenciais de *Cympogogon citratus* Lemon-Grass©, *C. citratus*, *Lippia origanoides* e *Lippia rotundifolia* frente aos microcrustáceos, *Artemia salina*, *esses* óleos essenciais podem ser considerados eficientes para serem utilizados no controlo de organismos nocivos. Estudos mais específicos são recomendados para demonstrar a sua atividade biológica.
Palavras-chave: óleos essenciais, toxicidade, CL_{50}, *Artemia salina*

INTRODUCTION

The native flora of Brazil presents an important biological diversity, in its great endemic majority. It is known that plants naturally elaborate essential oils and produce chemical substances and secondary metabolites for its protection from diseases (Silva 2003), repel phytophagous insects or attract beneficial insects that contribute to pollinization. For that reason, such secondary metabolites are denominated ecologically effective

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substances. The recognition of its biological properties has encouraged the development of this field in the search for new drugs, antibiotics, insecticides and herbicides.

Artemia salina Leach constitutes a micro-crustaceous indicator of pesticide and antitumor activity and its use is important in programs for the discovery and development of new pesticides of natural origin (García-Ocón et al. 2009). It has the advantages of being fast, cheap and simple. A large number of organisms can be easily utilized for the statistical validation. No special equipment is required and small numbers of samples are employed.

Diverse studies have been carried out with the use of the brine shrimp *lethality* in medicinal plant of the Brazilian flora for assessing its cytotoxic activity (Montanher et al. 2002; Brasileiro et al. 2006; Arcanjo et al. 2012). However, there is no evidence of these results in other plant species of interest in the region such as: *Lippia origanoides* Kunth, *Lippia rotundifolia* Cham. and *Cymbopogon citratus* (DC.) Stapf.

Hence, the objective of the present study was assessing the acute toxicity of four essential oils of plants against the lethality bioassay of *Artemia salina.*

MATERIAL AND METHODS

Location and experimental area

The experiment was conducted at the Laboratory of Medicinal Plants from the Institute of Agricultural Sciences, Federal University Minas Gerais (ICA/UFMG), Montes Claros Campus, Minas Gerais State, Brazil, located in the S 16º 40'53.3" WO 43º 50'27.3" at 635 m.a.s.l. in the period September to December, 2014.

Experimental procedure

A bioassay was carried out with larvae of the micro-crustaceous *Artemia salina* Leach (Crustaceous: Anostraca), an organism indicator of toxicity. This was realized according to Meyer et al. (1982). The cysts of *A. salina* were used with shells from MARAMAR® 5 g, adding 2 g in a liter of water that became saline on adding 38 g of coarse sea salt. Eggs were submitted for 24 h to its hatching in a cylinder with illumination (20 W) and constant aeration. Later, the technique of darkness-illumination was used for attracting the hatched artemias by phototropism for facilitating its collection with the aid of a Pasteur pipette. Microcells of 96 dishes were used. To each one of them it was added successively, with an automatic micropippete, 100 µl of a suspension of saline water containing 10-15 nauplii of *A. salina* and an aliquot of 50 µl of the dissolved sample of each treatment, in a solvent: saline solution mixture.

The essential oils used were: Lemon-grass© [*C. citratus*, Poaceae family], lemon-grass [*C. citratus*, Poaceae family], alecrim-pepper [*L. origanoides*) (Verbenaceae family] and rosemary [*L. rotundifolia*) (Verbenaceae family]. The botanical material was deposited in the Herbarium of the EPAMIG registered with the voucher number PAMG 56526 and PAMG 58100 corresponding to *L. origanoides* and *L. rotundifolia*, respectively.

Lemon-Grass©, national commercial oil from the Bauru Ltda distillery, Catanduva, Sao Paulo. Lemon-grass, alecrim-pepper and rosemary extracted through the steam dragging technique by the Clevenger equipment. The components present in the essential oils were identified. For that samples of them were submitted to individual analysis in a 7890A chromatograph (Agilent Technologies) fit together to the mass spectrometer (MS 5975C) equipped of capillary column of molten silica DB5-MS (30 m x 0.25 mm x 0.25 µm) and helium (99.9999 % purity) as dragging gas with a flow of 1 ml/min. The injector was maintained at 220 ºC with flow division (split) at a rate of 1.5 following the temperature planning from 60–240 ºC (3 ºC/min) maintained for 10 min. The interface temperature was kept at 240 ºC. The system was operated by way of complete scanning with electron impact of 70 eV, in the strip of 45-550 (*m/z*). Data generated were analyzed using the MSD ChemStation software organized according to the elution order.

The relative abundance (%) of total ions was calculated from the peak area of the chromatograph (GC-MS) and organized according to the elution order. The percentage of each component was calculated from the normalized mean of the chromatogram area. The identification of the components was realized by comparing the mass spectrum to that of the NIST 2.0, 2009 library and to data from the literature (Adams 2007).

Stock solutions were prepared from the four essential oils used and from them the serial dilutions were made. The final concentrations were of 0.01; 0.05; 0.25; 1.25; 6.25 and 31.25. All LC_{50}< 30 µg/ml were considered active according to the criteria of Meyer et al. (1982). A total of 8 dishes (replications) were used for each concentration following the same methodology of Sánchez et al. (2011).

Treatments consisted of the 6 formulated concentrations of the four essential oils previously described and two negative controls: only one provided with saline water and the other with the mixture of the solvent employed. The concentrations were dissolved with dimethyl sulfoxide (DMSO) at 1 %. The test was considered valid if the mortality percentage in the control did not exceeded 10%.

Once established all treatments in the plate of 96 dishes it was covered with Parafilm paper. Samples were maintained with constant illumination and environmental temperature. The experiment was replicated 5 times.

Larvae mortality was determined on contact exposing for 24 h. Death was established by the total lack of movements for 10 seconds of observation using a stereoscopic microscope. The mortality was corrected using Abbott's formula (Abbot 1925).

$$\text{Corrected mortality} = \frac{(\text{Treatment mortality} - \text{Control mortality})}{100 - \text{Control mortality}} \times 100$$

Statistical analysis

Database were prepared by Excel and the PROBIT method was utilized which regression analysis provided in the statistical package SPSS (2013) (version 22.0) made possible the calculation of the medium lethal concentration (LC_{50}) of each one of the essential oils used with a confidence interval of 95%.

RESULTS AND DISCUSSION

A directly proportional relationship between mortality and the dosages or concentrations tested was observed (figure 1). All essential oils manifested a high acute toxicity to low dosages. The figure shows sigmoid curves of the different oils tested. In spite that Lemon-grass© has a higher threshold regarding the rest of the oils tested and thus, exhibits a more pronounced slope attaining a toxicity of more than 50 % with lower concentrations, it is considered less toxic than the rest of the oils and in this way is demonstrated by its LC_{50} value (table 1).

According to their order of toxicity they were arranged in the *A. salina* bioassay: *C. citratus* ($LC_{50=}$ 1.21 µg/ml) > *L. rotundifolia* ($LC_{50=}$ 1.25 µg/ml) > *L. origanoides* ($LC_{50=}$ 1.26 µg/ml) > Lemon-grass© *C. citratus* ($LC_{50=}$ 1.28 µg/ml). Toxicological studies realized by Olivero-Verbel et al. (2009) in Colombia to 43 medicinal plant species of the region against *A. franciscana* obtained LC_{50} values between 4.43 to 34.09 µg/ml for species of *Lippia* and *Piper* genera.

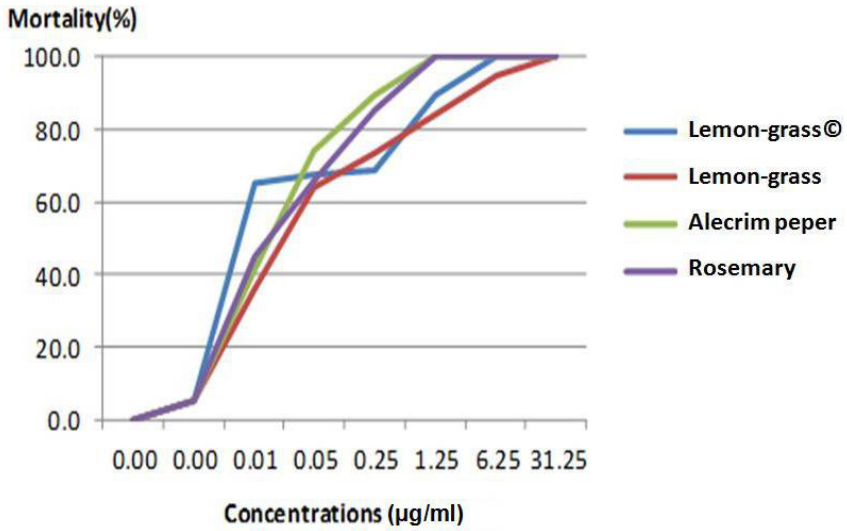

FIGURE 1. Dosage-response curve of the different essential oils against *Artemia salina*

TABLE 1. Values of the medium lethal concentration (LC_{50}) exhibited by the formulations of the essential oils used against the *Artemia salina* bioassay.

ESSENTIAL OILS	LC_{50} (µg/ml)	CONFIDENCE INTERVAL (95 %)	
		LOWER LIMIT	UPPER LIMIT
Lemon-grass© (*C. citratus*)	1.28	1.10	2.90
Lemon-grass (*C. citratus*)	1.21	0.37	2.05
Alecrim-pepper (*L. origanoides*)	1.26	1.16	1.37
Rosemary (*L. rotundifolia*)	1.25	0.26	2.24

According to studies of Sánchez et al. (2011) the biological activity of each plant species can be associated with the sensibility demonstrated with the organism used as well as to the essential oil composition.

The components present in the essential oils showing higher toxicity, lemon-grass (Figure 2, Table 2), alecrim-pepper (Figure 3, Table 3) and rosemary (Figure 4, Table 4) were analyzed.

Twelve components are observed in lemon-grass and rosemary oils while in alecrim-pepper 24 compounds, being the majority in this study: geranial (33.4 %) and β-citral (26.2 %) in lemon-grass, myrcene (15.5 %), 2,4-dimethyl 2,6 octadiene (13.6 %) and tagetone (11.8 %) in rosemary, carvacrol (57.5 %) and ρ-cymene (12.3 %) in alecrim-pepper.

Studies carried out in these same plants have shown similar performances to those of this work and others very contrasting. Such as in the case of the essential oil from *C. citratus* that the major components in this study were geranial (33.4%) and β-citral (26.2 %). However, researches by Rezende

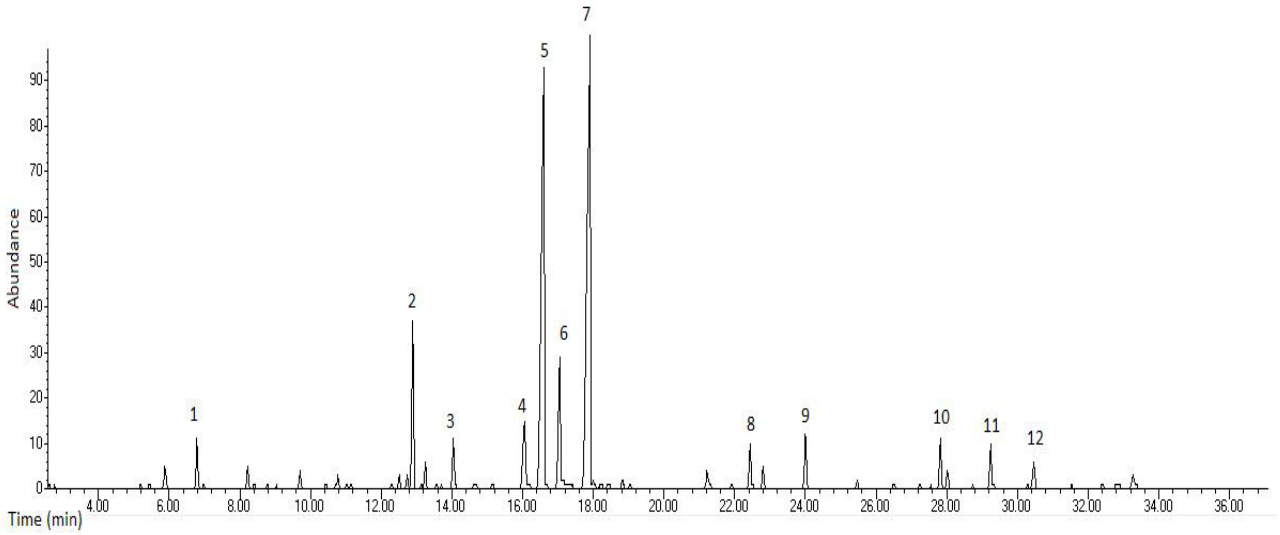

FIGURE 2. Total ion chromatogram obtained through the GC-MS analysis of the lemon-grass essential oil. Numbers as indicated by table 2.

TABLE 2. Components identified by gas chromatography fitted together to a mass spectrometer (GC-MS) of the lemon-grass essential oil.

Number of peak	Retention Time (minutes)	Component	% Total ions chromatogram[1]
1	6.7	6-metil-5-hepten-2-one	1.5
2	12.8	citronellal	5.8
3	14.0	unknown	1.9
4	16.0	β-citronellal	3.9
5	16.6	β-citral	26.3
6	17.0	geraniol	5.7
7	17.9	geranial	33.5
8	22.4	Geraniol acetate	1.7
9	24.0	β-cariophyllene	2.0
10	27.8	γ-cadineno	1.9
11	29.2	elemol	1.8
12	30.4	Cariophyllene oxide	1.2
	Total		*87,2*
	Traces		*12,8*

Traces: Trace components correspond to those lower to 1 % of the peak area in the chromatogram of total ions.
[1] Relative area obtained from the chromatogram of total ions (GC-MS).

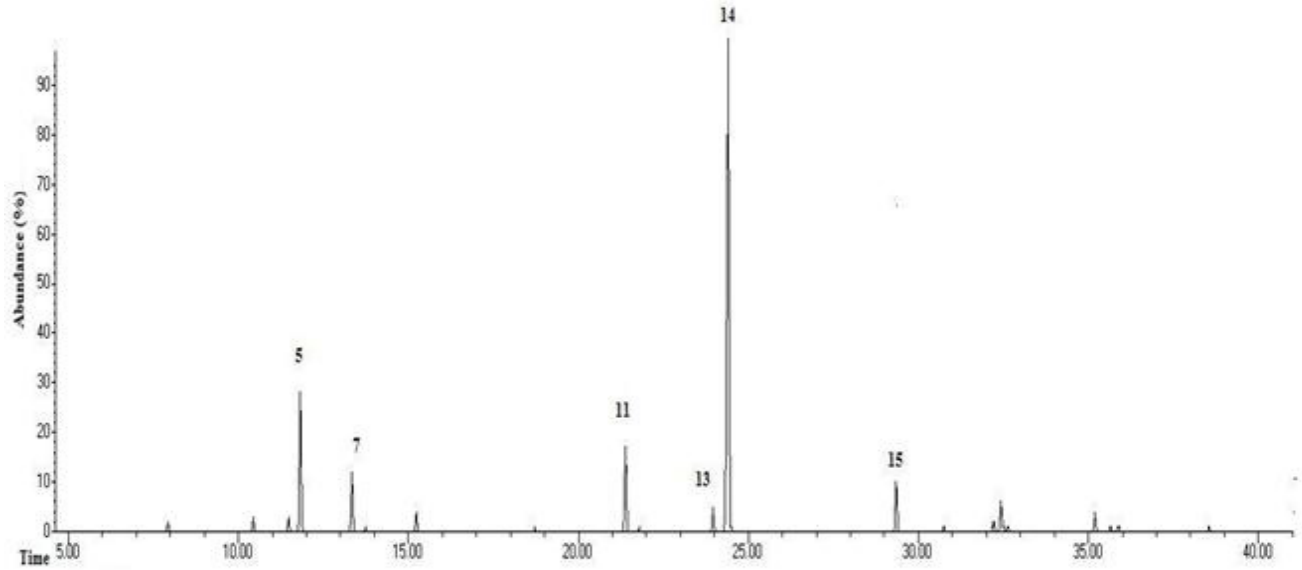

FIGURE 3. Chromatogram of total ions obtained by the GC-MS analysis of the alecrim-pepper essential oil.

TABLE 3. Components identified by gas chromatography fitted together to a mass spectrometry (GC-MS) of the alecrim-pepper essential oil.

Number of peak	Retention Time (minutes)	Component	% Total ions chromatogram[1]
1	7.9	unknown	0.8
2	8.1	α-Pinene	0.1
3	10.4	unknown	1.1
4	11.4	α-Terpinen	1.1
5	11.8	ρ-Cymene	12.4
6	12.0	D-Limonene	0.2
7	13.3	γ-Terpinen	5.3
8	13.7	5-Isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol,	0.1
9	15.2	β-Linalool	1.5
10	18.7	4-Terpineol	0.2
11	21.4	Thymol methyl ether	7.8
12	21.7	unknown	0.2
13	24.0	Thymol	2.1
14	24.3	Carvacrol	57.6
15	29.3	Caryophyllene	4.5
16	30.1	Aromadendrene	0.2
17	30.7	α-Humulene	0.3
18	32.0	unknown	0.1
19	32.2	unknown	0.9
20	32.6	unknown	0.6
21	35.1	unknown	1.7
22	35.6	unknown	0.3
23	35.8	Caryophyllene oxide	0.6
24	38.5	unknown	0.3
		TOTAL	100

[1] Relative area obtained from the chromatogram of total ions (GC-MS).

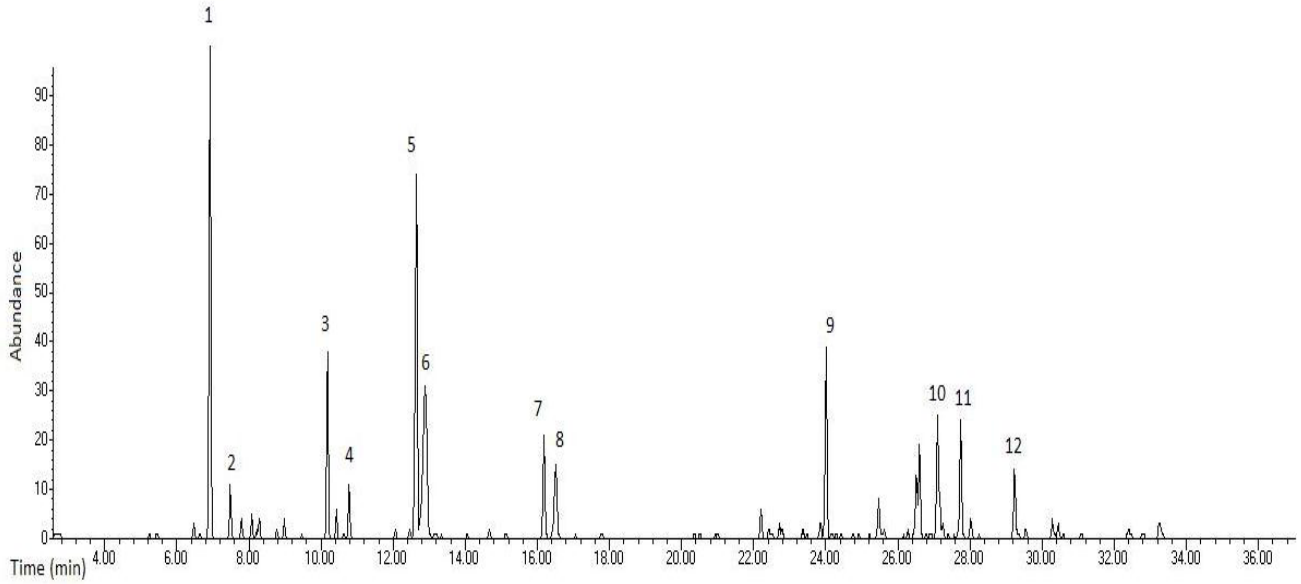

FIGURE 4. Chromatogram of total ions obtained by the GC-MS analysis of the rosemary essential oil.

TABLE 4. Components identified by gas chromatography fitted together to a mass spectrometry (GC-MS) of the rosemary essential oil.

Number of peak	Retention Time (minutes)	Component	% Total ions chromatogram[1]
1	6.9	myrcene	15.52
2	7.4	α-phellandrene	1.65
3	10.1	unKnown	5.58
4	10.7	β-linalool	1.68
5	12.6	2,4-dimethyl 2,6-octadiene	13.70
6	12.8	tagetone	11.86
7	16.1	verbenone	3.51
8	16.5	berbenone	3.83
9	24.0	caryophyllene	6.54
10	27.1	γ-elemene	5.61
11	27.7	α-cedrene	4.20
12	29.2	elemol	2.82
	Total		76.50
	Traces		23.50

Traces: Trace components correspond to those lower to 1 % of the peak area in the chromatogram of total ions.
[1] Relative area obtained from the chromatogram of total ions (GC-MS).

et al. (2017) in Lavras, Minas Gerais state, Brazil detected geranial, neral and myrcene. Those authors found different proportions of geranial (47.74%), neral (35.43%) and myrcene (8.46%).

Gomide et al. (2013) in the chemical characterization of the essential oils extracted from five *Lippia* species in Brazil detected for *L. rotundifolia* that the compounds with the highest percentages were β-myrcene (18.48%), and (E,Z)-farnesol (16.47%), (E,E)-farnesol (10.91%), and (Z,Z)-farnesol (10.60%).

Investigations explain that composition of essential oils from a particular species of plant can differ among harvesting seasons, geographical sources, maturity stages at harvest, cultivation techniques and drying methods (Blank et al. 2007; de Melo et al. 2011; Hanaa et al. 2012; Tajidin et al. 2012; Meira et al. 2021).

On the other hand, Coy and Acosta (2013) attribute the observed toxicity against the *A. salina* assay to the presence of strong cytotoxic components such as: monoterpens and sesquiterpens which are

majorly in the evaluated essential oil. There are evidences that such components can exert biological effect against pests and diseases (Saavedra et al. 2002; Stefanazzi et al. 2006; Negrelle and Gomes, 2007; Sonker at al 2014; Hamad et al. 2017; Govindarajan et al. 2018).

CONCLUSION

It is concluded that in view of the lethality exhibited by the essential oils of *C. citratus* Lemon-Grass©, *C. citratus*, *L. origanoides*, and *L. rotundifolia* against the *A. salina* micro-crustaceous can be considered promissory essential oils for its use for the control of harmful organisms. Other more specific studies are recommended for demonstrating its biological activity.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design. The first draft of the manuscript was written by NVV and subsequently ERM and FSADF commented and contributed to previous versions of the manuscript. Finally all authors read and approved the final manuscript.

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