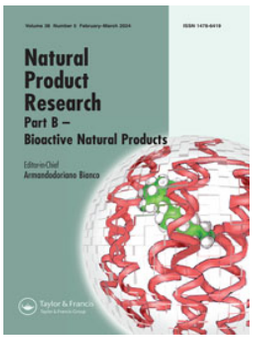

Natural Product Research
Formerly Natural Product Letters

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/gnpl20

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To cite this article: Ana Maria Henrique Moniz, Francisco Humberto Xavier Junior, Giovanna Melo Martins Silva, Ana Cristina Gonçalves Reis de Melo, Maylla Maria Correia Leite Silva, Weslley Souza Paiva, Hugo Alexandre Oliveira Rocha, Luiz Antônio Mendonça Alves da Costa, Antônio Alves de Melo Filho & Riva de Paula Oliveira (02 Dec 2023): *Lippia origanoides* essential oil increases longevity and ameliorates β-amyloid peptide-induced toxicity in *Caenorhabditis elegans*, Natural Product Research, DOI: 10.1080/14786419.2023.2287183

To link to this article: https://doi.org/10.1080/14786419.2023.2287183

NATURAL PRODUCT RESEARCH
https://doi.org/10.1080/14786419.2023.2287183

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Lippia origanoides essential oil increases longevity and ameliorates β-amyloid peptide-induced toxicity in *Caenorhabditis elegans*

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ABSTRACT

Lippia origanoides essential oil (LOEO) is extensively utilised as food preservative due to its antioxidant and antibacterial activities. In this study, the antioxidant and anti-ageing effects of LOEO was investigated *in vivo* using the nematode *Caenorhabditis elegans*. The gas chromatography-mass spectrometry analysis indicated that the main components of LOEO are carvacrol and thymol. LOEO treatment improved physiological parameters such as pharyngeal pumping, locomotion and body size indicating that is not toxic to *C. elegans*. LOEO treatment showed antioxidant effect in *C. elegans* by reducing endogenous ROS (Reactive Oxygen Species) production and increasing their survival under oxidative stress. Finally, LOEO treatment significantly extended *C. elegans* lifespan and alleviated the paralysis induced by β-amyloid peptide overexpression in the muscle. This work demonstrates for the first time LOEO antioxidant and anti-ageing properties on an organism level providing a valuable proof of principle to support further studies in the development of nutraceuticals or antioxidant phytotherapy.

ARTICLE HISTORY
Received 1 July 2023
Accepted 18 November 2023

KEYWORDS
Antibacterial; carvacrol; thymol; antioxidant; anti-ageing; Alzheimer disease

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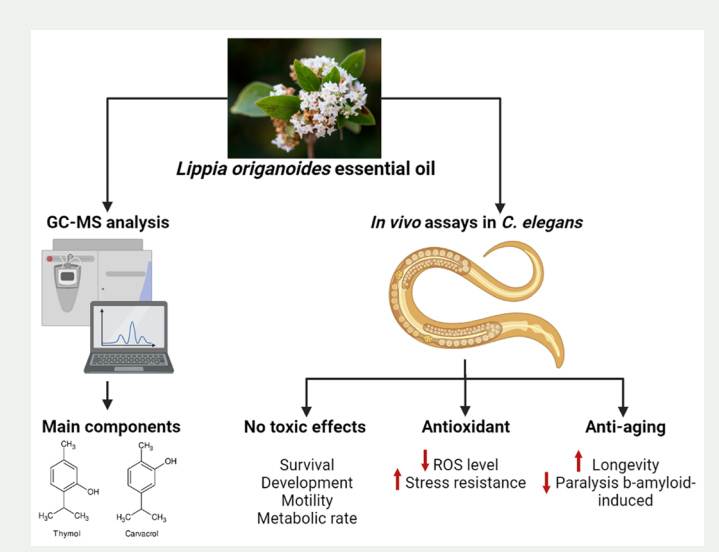
Supplemental data for this article can be accessed online at https://doi.org/10.1080/14786419.2023.2287183.



1. Introduction

Essential oils are aromatic liquids extracted from different plant parts consisting of a complex mixture of volatile and non-volatile compounds such as terpenes, phenolics, alcohols, aldehydes and ketones (Aziz et al. 2018). These phytochemicals present a wide range of pharmacological properties which have been used for therapeutic use as well in cosmetic and food industries (Lanzerstorfer et al. 2021).

Lippia origanoides Kunt (syn *Lippia sidoides* Cham), also known as 'alecrim-pimenta' or 'salva do campo', is an aromatic plant naturally found in North Brazil. Because of its use in popular medicine as antiseptic and analgesic (Oliveira et al. 2014), this plant has been included in the phytotherapy program implemented by the Brazilian Ministry of Health (Almeida et al. 2018). Essential oil from *L. origanoides* is rich in terpenes, mainly carvacrol and thymol, which provide several biological activities such as antimicrobial (Monteiro et al. 2021), antioxidant (Sarrazin et al. 2015; Teixeira et al. 2022), anti-inflammatory (Figueiredo et al. 2014; Ruff et al. 2021), and larvicidal (Braga de Oliveira et al. 2021; Tiwari et al. 2022). Because of their antimicrobial and antioxidant activities, these components have been proposed as natural preservatives for a wide variety of food products, and embryo preservation (Hernandes et al. 2017; Pandey et al. 2017; Sollecito et al. 2019; Kachur and Suntres 2020; Al-Maqtari et al. 2022). However, to the best of our knowledge, the *in vivo* antioxidant effect of *L. origanoides* essential oil (LOEO) treatment has not yet been properly explored.

The free-living nematode *Caenorhabditis elegans* has been widely used in many research areas such as developmental biology, ageing, neurobiology, and toxicology (Leung et al. 2008; Ma et al. 2018). Its numerous advantageous traits include small body size, short life cycle, fast reproduction, and easy and inexpensive maintenance. Most importantly, many cellular and molecular processes in *C. elegans* are conserved

given the high degree homology with human genome (Kaletta and Hengartner 2006). Studies based on *C. elegans* treated with essential oils or its isolated components have shown to be very promising for toxicological, anti-ageing and neurodegenerative diseases research (Chen et al. 2020; Lanzerstorfer et al. 2021; Zhang et al. 2021; Fuentes et al. 2022). In this study, we used *C. elegans* as an *in vivo* model to evaluate the antioxidant and anti-ageing capacity of LOEO. Moreover, we explored LOEO capacity to ameliorate the toxicity induced by β-amyloid peptide by using transgenic *C. elegans* model for Alzheimer Disease (AD). Our results demonstrated that LOEO treatment reduces ROS production, extends the lifespan and delays Aβ-mediated paralysis.

2. Results and discussion

2.1. Chemical analysis of Lippia origanoides essencial oil (LOEO)

The substances present in the essential oil we extracted are monoterpene (46%), sesquiterpene (8%) and aromatic hydrocarbons (4%). The remaining substances are oxygenated hydrocarbons (21%) and oxygenated aromatic (21%). Twenty-four main substances were identified being carvacrol (22.64%), thymol (16.77%), para-cymene (14.85%) the most abundant compounds (supplemental Table S1, Figure S1).

Based on their principal component, *L. origanoides* is classified in at least five chemotypes (A, B, C, D and E) (Stashenko et al. 2010; Ribeiro et al. 2014; Moreno et al. 2022). The chemical characterization of our LOEO indicates to be chemotype B (carvacrol + thymol + p-cymene). It is noteworthy that the percentage of carvacrol in the LOEO we obtained is lower compared to others LOEO also classified as chemotype B. For example, the presence of carvacrol varied from 23% to 47% in their oils (Brito et al. 2018; Ribeiro et al. 2021). This result endorses that, in addition to the variation in the composition of the LOEO, there is also modification in the quantity of each compound present.

2.2. Antioxidant and antibacterial activities of LOEO

Next, we evaluated the LOEO *in vitro* antioxidant capacity using three different assays. Each gram of LOEO had an activity in the TAC test equivalent to that of 510.64 mg AAE/mL. For all concentrations tested, LOEO showed a radical scavenging potential superior of 80% with no statistical difference between the different concentrations tested ($p = 0.754$; supplemental Figure S2A). For the reducing power test, LOEO exhibited a dose-dependent effect from 0.05 to 1 mg/mL ($p < 0.0001$). LOEO showed an activity of 60% at 0.05 mg/mL, 75% at 0.1 mg/mL, 100% at 0.25 mg/mL, 104% at 0.5 mg/mL and reached 109% at 1 mg/mL (supplemental Figure S2B). These results indicate that LOEO exhibited strong antioxidant capacity *in vitro* similar to other LOEO chemotypes B (Teles et al. 2014; Sarrazin et al. 2015; Damasceno et al. 2019; Teixeira et al. 2022).

Since carvacrol and thymol are isomers having well documented antibacterial activity, we tested whether LOEO we produced would inhibit bacterial growth. We monitored the growth of *E. coli* OP50 over 5 h in the presence of LOEO (supplemental Figure S3). Except for the lower concentration tested (0.1 mg/mL), our LOEO was able to inhibit bacterial growth of *E. coli* OP50. The antibacterial mechanism of carvacrol and thymol involves the disruption of the outer membrane of gram-negative bacteria,

release of lipopolysaccharides and subsequent increase of cell membrane permeability (Kachur and Suntres 2020).

2.3. Evaluation of cytotoxic effect of LOEO in 3T3 cells

Despite LOEO being considered safe as a food additive, evaluation of its toxicity is extremely important in face of its increasing use in different pharmacological applications and drug development. Cytotoxicity studies on LOEO are rare in the literature, and the few available are discordant. Previous studies have shown that LOEO is cytotoxic to different mammalian cells with C50 values < 100 µg/mL (Guimarães et al. 2021; Perera et al. 2022), while others did not observe any cytotoxic effect (Borges et al. 2012; Teixeira et al. 2022). These differences could be attributed to the LOEO's chemical composition and therefore the importance of cytotoxicity evaluation.

Here, we evaluated whether our LOEO has a cytotoxic effect on 3T3 fibroblast cells (supplemental Figure S4). Our results indicated that the LOEO had no toxic impact on 3T3 cells' viability at concentration up to 1 mg/mL. However, the percentage of viable cells treated with 0.1% DMSO was significantly reduced. Even DMSO has been reported to be cytotoxic at certain concentrations, and this effect varies between cell types, this result was unexpected for this concentration. Even with these results, we considered our LOEO not cytotoxic.

2.4. Evaluation of toxicological effects of LOEO in C. elegans

Toxicity assays based on *C. elegans* are considered as an intermediate approach between cell culture and mammalian testing (Lanzerstorfer et al. 2021). Here, we evaluated the toxicological properties of LOEO exposure on *C. elegans* survival, growth, movement, and pharyngeal pumping.

Exposure to LOEO for 72 h did not affect nematode survival. No significant differences were observed between the survival rates of the control and treated worms at any of the tested concentrations (supplemental Figure S5A). Exposure to LOEO resulted in a significant increase in all these three parameters. Compared to 0.1% DMSO control, body size increased 14.9%, 15.4%, 17.4% and 19.0% for 0.1, 0.25, 0.5 and 1 mg/mL LOEO treatment respectively (supplemental Figure S5B). Worms treated with 0.1, 0.25, 0.5 and 1 mg/mL LOEO exhibited a locomotion dose-dependent response at 149.1, 168.6, 185.6, 190.6 bends per minute compared to DMSO-treated group 126.0 bends per minute (supplemental Figure S5C). Pharyngeal pumping also showed dose-dependent response to LOEO treatment (supplemental Figure S5D). The rate was 68.83, 71.89, 76.11, 83.91 contractions per minutes for 0.1, 0.25, 0.5 and 1.0 mg/mL LOEO treatment respectively compared to DMSO-treated group 59.27 contractions per minutes. Taken together, our results indicate that LOEO was not toxic since it did not affect the nematode's survival. Instead, body size, motility and pharyngeal pumping rate on the worms treated with LOEO were significantly increased compared to control.

Similarly, Fuentes et al. (Fuentes et al. 2022) observed that carvacrol and thymol administered in sublethal doses improved *C. elegans* motility and oxidative stress resistance. A possible explanation for the improvement on these endpoint assays is related to LOEO antibacterial effect. Even though bacteria are a food source, it may cause

pathogenic infection influencing longevity and other physiological traits in *C. elegans* (Kim 2013). For instance, cultivation of *C. elegans* on heat-killed *E. coli* OP50 or on *E. coli* OP50 that has been previously treated with antibiotics to inhibit bacterial proliferation results in an extension of life span compared to propagation on standard live *E. coli* OP50. Therefore, we believe that the improvement of toxicity endpoints in *C. elegans* being due to attenuation of bacterial pathogenicity and toxicity induced by LOEO.

2.5. LOEO treatment reduces ROS production and increases stress resistance independently to its antibacterial effect

In order to determine the antioxidant potential of LOEO *in vivo*, we evaluated the endogenous ROS production and stress resistance in the nematode *C. elegans.* ROS production was significantly reduced in the nematodes treated with LOEO (supplemental Figure S6A). LOEO treatment at any concentration tested also increased the nematode's survival under oxidative stress conditions (supplemental Figure S6B). Since LOEO has an antibacterial activity (Ribeiro et al. 2021) and cultivation of *C. elegans* on *E. coli* OP50 treated with antibiotics results in extended life span and stress resistance (Garigan et al. 2002; Khan et al. 2018; Schumacker et al. 2019), we investigated whether the protective effect of LOEO was a consequence of bacterial growth inhibition. We repeated the oxidative stress resistance assay using heat-killed bacteria and observed that the wild-type animals treated with 0.1 and 0.25 mg/mL LOEO no longer showed increased survival under stress condition (supplemental Figure S6C). However, 0.5 and 1.0 mg/mL LOEO treatment still increased the worm's survival, indicating that the LOEO antimicrobial propriety is not the only factor affecting oxidative stress resistance in these animals (supplemental Figure S5C). Worth mentioning, the mean survival variation observed for the LOEO-treated animals on heat-killed bacteria was substantially lower compared to the, respectively, LOEO-treated animals on live bacteria. The mean survival variation was 47% and 91% for the animals treated with 0.5 mg/mL LOEO on live and heat-killed bacteria, respectively. Likewise, the mean survival variation was 58% and 89% for the animals treated with 1.0 mg/mL LOEO on live and heat-killed bacteria, respectively. These findings strongly support that in *C. elegans*, LOEO treatment with the highest concentrations provides an antioxidant effect for itself independently of its antimicrobial effect.

2.6. LOEO treatment increases longevity and delays β-amyloid-induced paralysis in C. elegans

To further explore LOEO antioxidant properties *in vivo*, we tested whether 0.5 and 1.0 mg/mL LOEO treatment would affect *C. elegans* longevity under standard laboratory conditions. The lifespan of wild-type worms treated with LOEO was significantly increased compared to control worms (supplemental Figure S6A; Table S2). The mean lifespan of worms treated with 0.1% DMSO was 13.54 ± 0.17 days, while for the worms treated with 0.5 and 1.0 mg/mL LOEO the mean lifespan was 19.07 ± 0.12 and 18.47 ± 0.12 days, which represents a 41% and 36% increase, respectively (supplemental Table S2).

Considering that ageing plays an important role in late-onset neurodegeneration (Dillin and Cohen 2011) and that ROS accumulation is accelerated in transgenics

worms expressing constitutive Aβ1-42 peptide compared to wild-type animals (Link 1995; Smith and Luo 2003), we investigated whether LOEO treatment would be effective in protecting *C. elegans* against Aβ-induced paralysis. Our results show that the onset of Aβ-induced paralysis was significantly delayed with LOEO treatment compared to the 0.1% DMSO (supplemental Figure S6B; Table S2). The mean paralysis time for worms treated with 0.5 and 1.0 mg/mL LOEO was increased by 39% and 43% respectively (supplemental Table S2). This result could be associated with the presence of carvacrol and thymol in LOEO. Azizi et al. (Azizi et al. 2022) observed that thymol and carvacrol improve cognitive abilities in AD rat models *via* activation of Protein Kinase C and antioxidant pathways. Thus, we could suggest that our LOEO, which is rich in carvacrol and thymol, exerts its protection against Aβ-induced paralysis partly by reducing the level of ROS *in vivo*. Possibly other mechanisms could be also involved in the attenuation of Aβ1-42 paralysis such as autophagy and proteostasis (Ga et al. 2018; Chen et al. 2020) which it would be very interesting to further explore.

3. Experimental

See supplemental material.

4. Conclusions

Taken together, our work demonstrates that LOEO is not toxic and possesses *in vivo* antioxidant and anti-ageing activities in the *C. elegans* model. Furthermore, LOEO showed a protective effect against abnormal protein aggregation and provided a valuable contribution to support further studies for mammals and humans. According to the results presented, we can suggest that LOEO may become a potent pharmacological agent against dementia, promoting antioxidant action, consequently, improving the life and health of *C. elegans*.

Acknowledgements

We would like to thank Dr. Regildo Max Gomes Martins, Dr. Caio Rodrigo Dias de Assis (in memorian) for the technical support and Dra. Amelia Tuller, for the taxonomic identification of *L. origanoides*. We also would like to thank the Faculdade Cathedral, located Boa Vista, RR and Secretaria de educação do estado de Roraima, for had given permission to carry out this work.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This study was supported by Universidade Federal do Rio Grande do Norte (UFRN), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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