BIOACTIVE VOLATILE COMPOUNDS OF GINGER LEAVES: A NOVEL PERSPECTIVE FOR AN UNDER-INVESTIGATED MATERIAL

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ABSTRACT: For centuries, the rhizome of ginger (*Zingiber officinale* Roscoe) has been used for therapeutic purposes. In contrast, the aerial parts of this plant have received comparatively little attention. This study aimed to evaluate the essential oil of ginger leaves (EOL), with respect to both chemical composition and *in vitro* antioxidant, antibacterial, and cytotoxic activities. GC-MS analysis identified the presence of 14 compounds, among which the major compounds were beta- pinene (67.9%), eucalyptol (9.9%), and beta-phellandrene (9.5%). In vitro antioxidant assays revealed IC50 values of 2702.7 and 2512.6 μg/mL for DPPH and ABTS radicals, respectively. The EOL also showed good cytotoxicity against colorectal adenocarcinoma cell line, and at 600 μL/mL inhibited 39.33% of the cells. Furthermore, in disc diffusion assays, the EOL showed best performance against Pseudomonas aeruginosa. Collectively, the results indicate that ginger EOL has considerable bioactivity and may have application potential as a functional ingredient, providing health benefits.
KEYWORDS: Essential oil; *Zingiber officinale*; Leaves; Cytotoxicity activity; Functional ingredient.

1 | INTRODUCTION

Ginger essential oil (EO), extracted from the rhizomes of *Zingiber officinale* Roscoe, has been extensively investigated by researchers worldwide, with the antibacterial, antifungal, anti-inflammatory, anti-ulcer, and immunomodulatory effects of this oil being confirmed in both experimental and preclinical studies (1–3). The safety issues regarding the use ginger rhizome oils are well documented, and these oils are generally considered to be safe (4). In contrast, however, there has been comparatively limited research on the EO derived from the aerial parts of ginger, notably leaves, although studies on leaf extracts have yielded promising findings. Nonetheless, at present, following harvest processing, the leaves of ginger are typically used for little more than composting.

In numerous spheres of everyday life, there is a growing challenge to use natural resources more rationally, thereby minimizing negative impacts on the environment and reducing waste production. Research has shown that a considerable fraction of the waste generated by humans can be recycled, and that organic materials, such as the leaves, peels, and stems of vegetables, can be used to varying extents in human food (5,6). In this

regard, the complete or partial use of the discarded parts of food to obtain colorings and flavorings, has been adopted as a sustainable practice. Given these considerations and the relative paucity of relevant studies, it would be of considerable interest, both academically and practically, to discover whether ginger leaf EO (EOL) contains bioactive compound.

To the best of our knowledge, there have been no studies that have investigated the application of ginger EOL as a potential source of functional constituents. In this study, we accordingly sought to evaluate the *in vitro* biological potential of ginger EOL in conjunction with an elucidation of its phytochemical profile, and based on our findings, propose the use of a ginger by-product as a novel source for the extraction of bioactive constituents.

2 | MATERIAL AND METHODS

2.1 Chemical and reagents

All reagents were analytical grade. The substances ABTS, DPPH, MTT, and alkane standard solution (C8-C20) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ultrapure water was obtained from a Milli-$Q_{®}$ reverse osmosis system (Merck Millipore, Darmstadt, Germany). The cell culture reagents, McCoy's 5A medium, HBSS (Hank's balanced salt solution), fetal bovine serum (FBS), trypsin, and penicillin/streptomycin (100 U/mL, 100 μg/mL) were purchased from Gibco (Grand Island, USA). For the disc diffusion assay were used: Muller Hinton agar (MHA) from Kasvi (Paraná, Brazil) and sodium chloride from Synth (São Paulo, Brazil).

2.2 Plant material and isolation of essential oil

EO was obtained from the fresh and organic leaves of *Zingiber officinale* Roscoe, grown in southern Brazil (Rio Grande do Sul, 29.5821 S, 51.0944 W). A voucher specimen has been deposited under the registration number PACA-AGP 95364 at Herbarium Anchieta PACA of Universidade do Vale do Rio dos Sinos, Brazil. The scientific name was checked in The Plant List Database (http://www.theplantlist.org). The plant material was submitted to hydrodistillation by a continuous process using Clevenger apparatus for 3 h, as described by the Brazilian Pharmacopoeia (7). The EO was stored in amber glass vial and maintained at -18 °C until analysis.

2.3 GC-MS analysis

The EO was analyzed using a Shimadzu GC-2010 Plus and a Shimadzu GCMS-QP2010 Ultra equipped with a Shimadzu autoinjector AOC-20i. The compounds were separated using a Rtx- 5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm, Thames Restek, UK). Injector and detector temperatures were set at 220 and 250 °C,

respectively. Column temperature was programmed at 80°C for 3 min, from 80 °C to 220 °C at a rate of 4 °C/min, and kept at 220 °C for 10 min. Helium (99.999%) was used as carrier gas with a constant flow rate of 1.5 mL/min. For MS detection, an electron ionization mode with ionization energy of 70 eV was used. Injector and MS transfer line temperatures were set at 220 and 290 °C, respectively. A sample of 1.0 μL (EOL diluted in ethyl ether) was injected, using split mode (split ratio, 1:40). The composition was reported as a relative percentage of the total peak area.

2.4 Identification of essential oil components by GC-MS

To perform the Relative Index (RI), the identification of ginger EOL components was based on a comparison of their retention indices relative a homologous series of *n*-alkanes (C8-C20). The calculated RIs were compared with published data and spectra of authentic compounds. Further, all compounds were identified and authenticated using their MS data compared to the NIST11 mass spectral library and published mass spectra (8).

2.5 In vitro radical scavenging

To evaluate the antioxidant activity of ginger EOL, we performed two assays based on scavenging of the radicals $ABTS^{\cdot+}$ and $DPPH^{\cdot}$. The ABTS assay was performed in ethanolic medium according to the protocol described by Re et al. (1999). The concentration of EO assayed ranged from 0.00 to 4000.0 μg/mL, with the antioxidant activity being detected after a 30-min reaction at 734 nm using a SpectraMax M5 spectrophotometer (Molecular Devices, CA). DPPH scavenging activity was determined by assaying EO in a methanolic medium at concentrations ranging from 0.0 to 3000.0 μg/mL, using the method described by Brand-Williams et al. (1995). The absorbance of samples was measured at 517 nm after a 30-min reaction (9,10).

Both tests were performed in triplicate and the respective antioxidant concentrations required to scavenge 50% of the radicals (IC_{50}) were determined from linear curves obtained from plots of percentage radical inhibition against EO concentration (µg/mL).

2.6 Cell culture and MTT cytotoxicity assay

Cells of the HT-29 colorectal adenocarcinoma cell line (ATCC® HTB38™) were cultivated in McCoy's medium supplemented with 10.0% v/v fetal bovine serum and 1.0% v/v antibiotics (100 U/mL penicillin/100 μg/mL streptomycin). The cells were maintained at 37 °C in a humidified atmosphere containing 5.0% CO_2.

The *in vitro* cytotoxic potential of the ginger EOL was determined using the MTT assay (11) with minor modifications. Briefly, 96-well plates were seeded with 100 μL of medium containing 1.5×10^5 cells/well. After 24 h, the medium was replaced with 100 μL

of serially diluted EOL (100.0 to 600.0 μL/mL) and incubated for a further 24 h. Thereafter, 10 μL of MTT solution (5.0 mg/mL) was added to each well and the plates were incubated at 37 °C for a further 4 h. The medium was then removed and 100 μL of dimethyl sulfoxide (DMSO) was added, and the absorbance of each well was determined using a SpectraMax M5 spectrophotometer at 570 nm. A solution of medium containing 3% v/v DMSO served as a positive control, and cells cultured in the absence of EOL were used as negative controls. Percentage cytotoxicity was calculated, and the results were expressed as the concentration of EOL capable of inhibiting cell growth by 50% (IC_{50}). Each experimental condition was analyzed in triplicate, with three separate experiments performed for each EO.

2.7 Disc diffusion assay

The antibacterial effect of ginger EOL against a panel of selected pathogenic bacteria was examined using the agar disc diffusion method described by Bauer et al. (1966) and adapted by the Clinical and Laboratory Standards Institute (12,13). The panel comprised the following seven clinical or commercial bacterial strains: *Bacillus cereus* (ATCC® 11778™), *Enterococcus faecalis* (ATCC® 19433™), *Staphylococcus aureus* subsp. *aureus* (ATCC® 25923™), *Clostridium perfringens* (ATCC® 13124™), *Escherichia coli* (ATCC® 8739™), *Pseudomonas aeruginosa* (ATCC® 27853™), and *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* (ATCC® 14028™). The bacteria were cultured for 24 h at 37 °C in Mueller–Hinton aga (MHA) medium. The working inoculum was prepared in 0.85% w/v sodium chloride solution, with turbidity being adjusted to 0.5 on the McFarland scale and confirmed spectrophotometrically at 600 nm with absorbance values of 0.1 (± 0.01).

One-hundred microliters of the incubated bacterial suspensions was spread on plates containing MHA medium, onto which was placed a 6-mm-diameter paper disc infused with 5 µL of ginger EOL. After incubation at 37 °C for 24 h, the diameters of the zones of inhibition (IZD; mm) around the discs were measured using a ruler.

2.8 Statistical analysis

Statistical analyses were performed by GraphPad Prism software (version 6.0), with ANOVA-one way with post-hoc Tukey test for *in vitro* cytotoxicity assay. The significance level was set at 5.00%.

3 I RESULTS AND DISCUSSION

In this study, we detected 14 compounds in the examined samples of ginger leaf EOL, which were predominantly monoterpene hydrocarbons (83.61%) and oxygenated monoterpenes (11.67%) (Table 1). Among these, the major compounds identified were beta-

pinene (67.9%), eucalyptol (9.9%), and beta-phellandrene (9.5%), with smaller amounts of beta-caryophyllene (4.3%), limonene (2.7%), and beta-myrcene (2.0%). Other constituents were detected at only trace levels <1.00%. However, we were unable to detect the presence of the zingiberene marker compound that is commonly found in the EO of ginger rhizomes (1).

Peak	Compound	RT	RI	MW (g/mol)	Area (%)
1	beta-phellandrene	4.128	-	136	9.5
2	beta-pinene	4.229	-	136	67.9
3	beta-myrcene	4.375	-	136	2.0
4	delta-3-carene	4.987	1023	136	0.31
5	limonene	5.267	1035	136	2.7
6	eucalyptol	5.356	1038	154	9.9
7	trans-beta-ocimene	5.652	1050	136	0.32
8	gamma-terpinene	5.982	1064	136	0.70
9	terpinolene	6.768	1086	136	0.18
10	borneol	9.032	1172	154	0.40
11	terpinen-4-ol	9.364	1183	154	0.94
12	alpha-terpineol	9.757	1196	154	0.43
13	beta-caryophyllene	16.951	1375	204	4.3
14	ledol	17.995	1459	222	0.43
				Total identified	**100.01**
				Monoterpene hydrocarbons	83.61
				Oxygenated monoterpens	11.67
				Sesquiterpenes hydrocarbons	4.3
				Oxygenated sesquiterpenes	0.43

RT: retention time; RI: relative index (experimental values); MW: molecular weight.

Table 1. Chemical composition of *Z. officinale* leaves essential oil.

Mostafa et al. (2018) have reported the presence of 90 constituents in ginger EOL, the most prominent of which are methyl cinnamate (29.21%), beta-pinene (8.59%), and terpinolene (7.46%), whereas the content of eucalyptol, which we found to be relatively high, was only 1.8%. Sivasothy et al. (2011) reported the presence of 46 constituents in ginger EOL, among which beta-caryophylene (31.7%), geranial (13.1%), and alpha-pinene (4.1%) predominated. However, in contrast to the findings of the present study, these authors detected relatively lower levels of beta-pinene (2.0%), eucalyptol (0.7%), and beta-phellandrene (2.6%) (14). Notably, the content of beta-pinene detected in the present study is considerable higher than that reported in all previous studies on ginger EO, which have tended to indicate that this substance is not commonly found among the major constituents of ginger EO (1).

In the present study, we found that the extracted ginger EOL showed considerable antioxidant activity, with and IC_{50} values of 2702.7 μg/mL and 2512.6 μg/mL for DPPH and ABTS, respectively. Apparently, ginger EOL has a lower antioxidant capacity, in both techniques (4). These results are consistent, given that in the present and previous studies, no molecules containing hydroxyl groups were detected among the major compounds.

The *in vitro* cytotoxicity of ginger EOL was evaluated in a colorectal adenocarcinoma cell line (HT-29). As indicated in Figure 1, all treatment groups showed equivalent cell viability, with no statistical differences detected among cells exposed to EOL in the applied concentration range (100.0 to 600.0 μL/mL). The estimated IC_{50} value was 590.1 μL/mL. In terms of mortality, only cells treated with 100.0 μL/mL EOL differed significantly from the 3% v/v DMSO positive control ($p<0.05$). The results of our disc diffusion assay indicated that the ginger EOL may have some antibacterial potential. Whereas the EOL did not show antibacterial activity against *S. aureus*, *C. perfringes*, *E. coli*, *E. faecalis*, or *S. enterica* activity (IZD = 6.0 mm), we did detect some activity against *B. cereus* (IZD = 8.0 mm) and more notably against *P. aeruginosa* (IZD = 17.0 mm). In this regard, it is probable that the action of this EO is dose-dependent, and it is also conceivable that the therapeutic target is not the cell wall. Further investigations of the activities of these oils against other food-borne pathogens should accordingly be conducted.

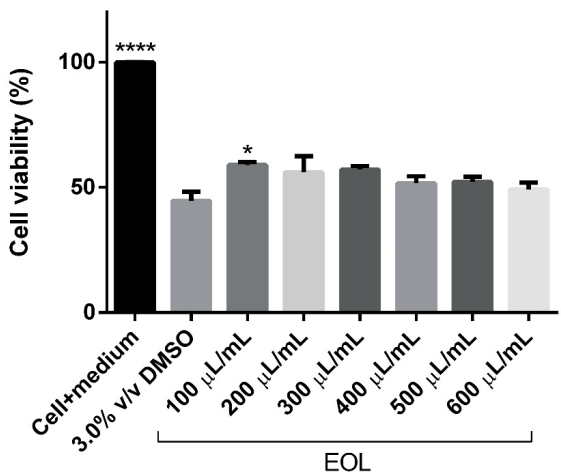

Figure 1. *In vitro* cytotoxicity data in HT-29 cell line (n=6/group, in triplicate). Groups: Control (cell + medium), 3.0% v/v DMSO (positive death control); Treatments (EOL μL/mL). Statistical analysis: ANOVA, post hoc Tukey test (****) $p<0.0001$, (*) $p<0.05$ compared to positive death control.

In an *in vitro* assay using *Streptococcus mutans*, Mostafa et al. (2018) reported a minimum inhibitory concentration value of 62.5 μL/mL for a nano-emulsion of ginger EOL, which is equivalent to 0.61 μL/mL of the pure EO. Although the vehicle control (blank

nano-emulsion) did not show antibacterial activity, it should be noted that nanostructured materials facilitate permeation. Consistent with our observations, on the basis of evaluations of antibacterial activities using the micro-dilution technique, Sivasothy et al. (2011) observed no pronounced antibacterial activity, with both leaf and rhizome oils showing only moderate activity against the gram-positive *Bacillus licheniformis*, *Bacillus spizizenii,* and *S. aureus*, and gram-negative *E. coli*, *Klebsiella pneumoniae,* and *Pseudomonas stutzeri*.

However, these findings tend to contrast with those reported elsewhere, which have indicated that the ginger rhizome EO may exhibit higher antibacterial activity (1). In the present study, we observed the selective action of ginger EOL against specific bacterial strains, which may be of relevance with respect to potential practical applications. For example, ginger EOL could be used as a preservative in beverages derived from fermented grains, which can become contaminated with *B. cereus*. These findings may thus be of interest to the scientific community that is dedicated to the development of functional foods, for which a primary objective is to identify functional constituents that can be used replace synthetic products, which are also safe (as confirmed by *in vitro* and *in vivo* toxicological tests) and can be sourced from waste by-products of the food industry.

4 | CONCLUSIONS

In this pioneering study, we demonstrated the *in vitro* radical scavenging ability, cytotoxicity, and antibacterial activity of an essential oil isolated from the leaves of ginger. However, to establish whether this oil has chemoprotective activity, further studies, including those using animal models, will be necessary. Nevertheless, our findings indicate that ginger leaf essential oil has potential utility as a functional constituent of a range of commercial products, as well as contributing to the exploitation of a by-product material that is currently discarded as waste.

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