Ocimum gratissimum L.: A natural alternative against fungi associated with bean and maize seeds during storage

Ocimum gratissimum L.: una alternativa natural contra hongos asociados con semillas de frijol y maíz durante el almacenamiento

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ABSTRACT

The aim of the study was to evaluate *in vitro* antioxidant and antifungal activities of the ethanolic extract and its fractions from *Ocimum gratissimum* leaves. The ethanolic extract was obtained by maceration in ethanol and subsequent fractionation with solvents of increasing polarity (hexane, dichloromethane, ethyl acetate and butanol). The Minimum Inhibitory Concentration (MIC) was determined for the ethanol extract and dichloromethane fraction. The antioxidant capacity was evaluated by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) free radical scavenging methods, and by FRAP (Ferric Reducing Antioxidant Power). The *in vitro* antifungal effect was determined by the agar diffusion method on *Aspergillus* sp. and *Rhizopus* sp. fungi associated with corn and bean seeds during storage. The best samples with antifungal effect were determined by gas chromatography-mass spectrometry (GC/MS). The ethanolic extract had strong antioxidant capacity for all tested methods (DPPH 371.10±2.98 µg ml^{-1}, ABTS 182.43±1.10 µg ml^{-1}, FRAP 262.39±3.61 TEAC). Regarding the antifungal activity, the ethanolic extract and dichloromethane fraction resulted in total suppression (100%) of fungal growth and MIC ranged from 0.625 to 1.25 mg ml^{-1}. In the GC/MS analysis, 22 substances were detected in all samples evaluated, with predominance of eugenol. These results indicated high biological potential of this plant as a biofungicide.

Key words: antifungal activity, antioxidant capacity, *Aspergillus*, *Rhizopus*.

RESUMEN

El objetivo del estudio fue evaluar *in vitro* las actividades antioxidantes y antifúngicas del extracto etanólico y sus fracciones a partir de las hojas de *Ocimum gratissimum*. El extracto etanólico se obtuvo por maceración en etanol y posterior fraccionamiento con disolventes de polaridad creciente (hexano, diclorometano, acetato de etilo y butanol). La Concentración Mínima Inhibitoria (CMI) se determinó para el extracto de etanol y la fracción de diclorometano. La capacidad antioxidante se evaluó mediante los métodos de eliminación de radicales libres DPPH (2,2-difenil-1-picril-hidrazil-hidrato) y ABTS (ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfónico)) por PARF (Poder Antioxidante Reductor Férrico). El efecto antifúngico *in vitro* se determinó mediante el método de difusión de agar sobre hongos *Aspergillus* sp. y *Rhizopus* sp. asociados con semillas de frijol y maíz durante el almacenamiento. Las mejores muestras con efecto antifúngico se determinaron por cromatografía de gases acoplada a espectrometría de masas (CG/EM). El extracto etanólico presentó fuerte capacidad antioxidante para todos los métodos probados (DPPH 371.10±2.98 µg ml^{-1}, ABTS 182.43±1.10 µg ml^{-1}, PARF 262.39±3.61 TEAC). En cuanto a la actividad antifúngica, el extracto etanólico y la fracción de diclorometano mostraron supresión total (100%) del crecimiento fúngico y la CMI varió de 0.625 a 1.25 mg ml^{-1}. En el análisis CG/EM se detectaron 22 sustancias en todas las muestras evaluadas, con predominio de eugenol. Estos resultados indicaron un alto potencial biológico de esta planta como biofungicida.

Palabras clave: actividad antifúngica, capacidad antioxidante, *Aspergillus*, *Rhizopus*.

Introduction

It is estimated that at least 15% of losses during seed storage occur due to product contamination by insects and fungi (Silva *et al*., 2021). Extracts and essential oils from plants have demonstrated ability to inhibit the action of phytopathogens or the production of mycotoxins; these promising biofungicides could mitigate the use of chemical pesticides (Mohr *et al*., 2017). This is due to the presence of compounds from the secondary metabolism of plants. These chemical compounds, such as terpenes, phenolics, and alkaloids are important biological agents that can play

Received for publication: November 16, 2022. Accepted for publication: December 31, 2023 Doi: 10.15446/agron.colomb.v40n3.105851

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antifungal roles against species of the genus *Aspergillus*, *Rhizopus*, *Penicillium*, *Colletotrichum* as well as antioxidant roles (Elisée *et al*., 2020).

Natural plant-based products are a more appropriate strategy in agricultural management when compared to commercial fungicides, as they are biodegradable, prevent pathogen resistance, and are less harmful to human health (Chowdhary *et al*., 2018). However, the composition and activity of the bioactive components of plants vary according to genotype, geographic location, and vegetative stage, as well as method, solvent and temperature used in the extraction process (Onyebuchi & Kavaz, 2020). The extraction of secondary metabolites can be performed using a fractionation technique with solvents of different polarities to concentrate chemical groups in distinct fractions with completely different chemical characteristics in a single plant (Mann, 2012).

Ocimum gratissimum L., popularly known as clove basil, is an aromatic herb that belongs to the Lamiaceae family and is found in South America, Africa, and Asia (Mohr *et al*., 2017). It is used as a food condiment and in folk medicine; the infusion of leaves is prepared for the treatment of fever, flu, and kidney problems (Matos, 2007; Penido *et al*., 2016). It has a diversity of chemical groups with proven biological activities due to its anti-inflammatory (Dzoyem *et al*., 2021), insecticidal (Benelli *et al*., 2019), antibacterial (Hamma *et al*., 2020), antioxidant (Onyebuchi & Kavaz, 2020) and antifungal properties against several species of fungi, among them *Aspergillus niger, Botryodiplodia theobromae, Rhizopus stolonifer, Fusarium oxysporium, Penicillium expansum,* and *Colletotrichum* spp. (Uchegbu *et al*., 2019).

The chemical profile of these compounds includes the presence of flavonoids, tannins, sterols, terpenoids, saponins, and alkaloids (Hamma *et al*., 2020). Previous studies have shown that the ethanolic extracts and essential oils obtained from *O. gratissimum* leaves are rich in phenolic compounds with antioxidant and antimicrobial action against various pathogens (Dambolena *et al*., 2010; Elisée *et al*., 2020; Onyebuchi & Kavaz, 2020). The leaves are rich in essential oils, whose main components contain eugenol, thymol, and linalool, with antimicrobial activities already documented in the literature (Faria *et al*., 2006; Mohr *et al*., 2017).

Most studies on the control of phytopathogens have focused on the biological properties of essential oils; there are few data based on extracts and their fractionation from *O. gratissimum* leaves against phytopathogens (Dambolena *et al*., 2010; Mohr *et al*., 2017; Elisée *et al*., 2020). Therefore, the aim of the present study was to evaluate the *in vitro* antifungal and antioxidant capacity and the chemical profile of the most active samples from *O. gratissimum* leaves.

Materials and methods

Collection and botanical identification of the plant species

Collection and botanical identification of *O. gratissimum* L. leaves were done at the Engenheiro Agrônomo Reginaldo Conde (FERC) Experimental Farm of the Institute for Research, Technical Assistance and Rural Extension of Espírito Santo (INCAPER), Viana, Espírito Santo, Brazil, in October 2019.

Preparation of the ethanolic extract and its fractions

The plant extract was prepared at the Laboratory of the Federal Institute of Espírito Santo (Ifes), Vila Velha campus (Brazil). Leaves were dried in an oven with air circulation at 40°C for 24 h and then crushed. To obtain the ethanolic extract (EEtOH), leaves were macerated in 96% ethanol at 1:10 w/v ratio (dryer plant: ethanol), at room temperature and protected from light. Subsequently, the extract was filtered, and the solvent removed on a rotary evaporator (Buchi Rotavapor R-3 CH 9230 Flawil 1, Switzerland). The recovered solvent was added to the leaf residue and crushed again until the plant drug was depleted. The concentrated residue (EEtOH) obtained was stored in amber glass under refrigeration at 4°C. To obtain fractions of different polarities, part of the EEtOH was resuspended in the ethanol-water mixture v/v (8:2) and submitted to successive liquid-liquid partitions with organic solvents of increasing polarities; after total removal of solvents, the following fractions were obtained: hexane (FHex), dichloromethane (FDCM), ethyl acetate (FAce), *n*-butanol (FBuOH), and the aqueous residual (FAq).

Antifungal activity

Mycelial growth inhibition percentage

The experimental design was completely randomized, in a 6x3 +2 factorial scheme, with three replicates. Factor A was composed of six different extracts (EEtOH; FHex; FDCM; FAce; FBuOH and FAq) and factor B was composed of three concentrations (0.1; 5.0, and 10.0 mg ml^{-1}), plus two additional treatments, negative control, and positive control. The negative control did not contain EEtOH or fraction and the positive control was Cercobin® commercial fungicide

based on thiophanate methyl (Dimethyl 4,4'-(o-phenylene) (3-thioallophanate) at 0.8 mg ml^{-1}.

For the *in vitro* antifungal activity evaluation, the agar diffusion method according to Barros *et al*. (1995) was used. The ethanolic extract and fractions were tested on *Aspergillus* sp. and *Rhizopus* sp., isolated from traditional bean and corn seeds and identified at the Laboratory of Diagnosis of Plant Diseases of Federal Institute of Espírito Santo (Ifes), Santa Teresa campus.

Sterilized stock solutions (15 mg ml^{-1}) were diluted in sterilized Potato-Dextrose-Agar (PDA) medium, in a melting state, to obtain final concentrations of 0.1, 5.0, or 10.0 mg ml^{-1} for each sample. Then, the final solutions were poured into 5 cm diameter Petri dishes. The fungi were spiked into the center of each Petri dish. Plates were incubated in a growth chamber (BOD) at 25°C, 12 h photoperiod, until the mycelial growth of the respective fungi in the negative control treatment reached the edge of the plate. Mycelial growth was evaluated by daily measurement of the diameter (in cm) of colonies. The mycelial growth inhibition percentage (GIP) was estimated using the equation (Kordali *et al*., 2003):

$$GIP(\%) = \frac{D_c - D_t}{D_c} \times 100$$

where D_c is average mycelial diameter of the negative control (cm) and D_t is average mycelial diameter of treatments (cm).

Determination of the Minimum Inhibitory Concentration (MIC)

The most promising (due to preview antifungal action in susceptibility tests) samples from *O. gratissimum* leaves were selected for the determination of the Minimum Inhibitory Concentration (MIC). The MIC of the main component of both samples, eugenol, was also evaluated.

The EEtOH and the FDCM were added to PDA to make a 5 mg ml^{-1} stock solution. Serial dilution was performed to obtain concentrations of 5.0, 2.5, 1.25, 0.625, 0.3125, and 0.1 mg ml^{-1}; then the agar diffusion method was performed, as previously mentioned. For evaluation of pure eugenol, a stock solution of 1.0 mg ml^{-1} of pure eugenol in 0.5% DMSO (dimethyl sulfoxide) was prepared; subsequently serial dilutions were performed with final concentrations of 1.0, 0.75, 0.5, 0.25, and 0.125 mg ml^{-1}. They were then incubated for 48 h at 25°C and the fungal growth was observed. The lowest concentration, which inhibits the visible growth of the tested organism after macroscopic evaluation, was determined as Minimum Inhibitory Concentration (MIC) (Talibi *et al*., 2012). All determinations were tested in triplicate.

In vitro antioxidant capacity

DPPH test

The antioxidant capacity of the EEtOH and fractions using the 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging was determined according to Casagrande *et al*. (2007), with modifications. Five hundred µl of 250 µM DPPH ethanolic solution was mixed to 30 µl of solutions containing decreasing concentrations of the extracts in ethanol (1000, 500, 250, 125, and 62.5 µg ml^{-1}) plus 1,000 µl of 0.1 M acetate buffer and 1,000 µl of absolute ethanol. The so-called "blank" solution was prepared with ethanol-DPPH mixture. After 30 min of incubation at room temperature, the absorbance was read against a blank at 517 nm in a spectrophotometer. The DPPH radical scavenging activity was expressed as inhibition percentage:

$$IC\%_{(DPPH)} = 100 - \frac{A_{SAMPLE}}{A_{CONTROL}} \times 100$$

where A_{SAMPLE} is the absorbance of treatments, and $A_{CONTROL}$ is the absorbance of control (containing all reagents, except treatment sample).

The antioxidant activity was evaluated based on the IC_{50} value (µg ml^{-1}) extract concentration necessary to scavenge 50% of the DPPH free radical by linear regression using concentration values (µg ml^{-1}) versus inhibition percentage (IC%).

ABTS test

The antioxidant capacity of the EEtOH and fractions was determined using the 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical according to Sánchez-González *et al*. (2005). $ABTS^{+\bullet}$ cation was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate. The mixture was stored in a dark bottle at room temperature for 16 h. The ABTS solution was diluted in phosphate buffer (pH 7.4) to an absorbance of 0.7 at 730 nm. The samples were resuspended in ethanol, generating solutions with concentrations ranging from 62.5 to 1,000.0 µg ml^{-1}. After adding 10 µL of each sample standard to 4 ml of diluted $ABTS^{+\bullet}$ solution, absorbance readings at 730 nm were performed after 6 min of reaction in the spectrophotometer. The antioxidant capacity was

calculated by the inhibition percentage of the ABTS radical activity (IC% (ABTS)), according to the following equation:

$$IC\%_{(ABTS)} = 100 - \frac{A_{SAMPLE}}{A_{CONTROL}} \times 100$$

where A_{SAMPLE} is the absorbance of treatments and $A_{CONTROL}$ is the absorbance of control (containing all reagents except the treatment sample).

The antioxidant activity was evaluated based on the IC_{50} value (μg ml^{-1}) of the extract concentration necessary to scavenge 50% of the ABTS free radical by linear regression using concentration values (μg ml^{-1}) versus percentage inhibition (IC%).

Iron reduction assay (FRAP)

The antioxidant capacity of the EEtOH and fractions was evaluated by reducing iron (FRAP) based on the method of Sánchez-González *et al*. (2005), with some modifications. This method is based on the reduction of the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) by antioxidant molecules present in the extracts and subsequent formation of a colored complex of the Fe^{2+} and 4,6-tripyridyl-s-triazine (TPTZ). The FRAP reagent was prepared as follows: 2.5 ml of a 10 mM solution of TPTZ in 40 mM HCl were added to 2.5 mL of $FeCl_3.6H_2O$ and 25 mL of 0.3 mM acetate buffer pH 3.6. The solution was incubated at 37°C for 30 min in a water bath. For the evaluation of the antioxidant capacity, 900 μl of the previously prepared FRAP reagent was mixed with 90 μl of distilled water and 10 μl of the sample or standard. The samples were incubated at 37°C for 30 min and the reading was performed at 595 nm in a UV-Visible spectrophotometer (Biospectro SP-220). Standard solutions with different concentrations of Trolox (0.5, 1.0, 2.5, 5.0, 10.0, 15.0, and 20.0 μmol) were used for calibration. Results were expressed as μmol Trolox equivalent/g sample (TEAC - Trolox equivalent antioxidant capacity).

Analysis by gas chromatography coupled to a mass spectrometer

The analyses by gas chromatography coupled with mass spectrometry (GC/MS) were performed at the Laboratory of Chromatography of LabPetro, Federal University of Espírito Santo. The nonpolar fractions that showed the greatest biological effects were analyzed by a gas chromatograph coupled to an Agilent 7890B mass spectrometer (Agilent, CA, USA) and a model 5977A MSD mass detector with electronic ionization of 70 eV. The column used was a 30 m x 250 μm x 0.25 μm HP-5 column. The injector was set to a temperature of 290°C and the detector to 310°C. Elution was done on a heating ramp, starting of 40°C with a heating rate of 5°C/min to 280°C, followed by a heating rate of 15°C/min to 310°C, remaining at that temperature for 10 min.

For characterization, a C10 to C40 alkane standard was used and submitted to the same chromatographic conditions. Compounds were identified through comparison with the National Institute of Standards and Technology (NIST) database library followed by comparison of literature retention rates (NIST, 2018).

Statistical analysis

Mycelial growth inhibition percentage data were submitted to analysis of variance (ANOVA). The interaction between factors was analyzed; later the Tukey test ($P<0.05$) was applied for the factorial group, and the Dunnett test ($P<0.05$) was applied for the comparison of means of the factorial group with additional treatments (positive and negative control), using Assistat 7.6 software.

The antioxidant activity was evaluated using ANOVA followed by the Tukey test ($P<0.05$) using Assistat 7.6 software. All experiments were completed in triplicate to ensure reproducibility.

Results and discussion

The mycelial growth inhibition percentage

Antifungal activity of the samples of *O. gratissimum* at different concentrations against *Aspergillus* sp. and *Rhizopus* sp. fungi is shown in Table 1. For the variable growth inhibition percentage (GIP), there was a significant interaction between the concentrations of the plant extracts tested. The results indicate that EEtOH and FDCM at 5 and 10 mg ml^{-1} had high antifungal activity, with complete suppression (100%) of the mycelial growth of *Aspergillus* sp. and *Rhizopus* sp. These were the only treatments against *Aspergillus* sp. and *Rhizopus* sp. with antifungal activity higher than treatment synthetic fungicide, in 0.8 mg ml^{-1}. Among the polar fractions, FAce at 10 mg ml^{-1} was the most active with GIP of 37.4 and 61% for *Aspergillus* sp. and *Rhizopus* sp., respectively.

Minimum Inhibitory Concentration (MIC)

The MIC results of the most activity samples of *O. gratissimum* and eugenol are found in Table 2. The MIC ranged from 0.625 to 1.25 mg ml^{-1}. The samples FDCM exhibited the lowest MIC with a value of 0.625 mg ml^{-1} for *Aspergillus* sp. and *Rhizopus* sp., while for EEtOH, the MIC for

TABLE 1. Mycelial growth inhibition percentage (GIP) of *Aspergillus* sp. and *Rhizopus* sp. submitted to different ethanolic extract concentrations (mg ml^{-1}) and fractions from *Ocimum gratissimum* leaves.

Treatments	*Aspergillus* sp.			*Rhizopus* sp.		
	0.1	5	10	0.1	5	10
EEtOH	8.6 b	100 a	100 a	5.2 a	100 a	100 a
FHex	7.6 bc	40.7 b	52.5+ b	4.9 a	36 c	47.7 c
FDCM	11.9 a	100 a	100 a	6.1 a	100 a	100 a
FAce	3.7 d	19.3 c	37.4 c	4.7 a	44.5 b	61 b
FBuOH	8.3 b	11.3 d	21.8 d	0* b	3.1 d	8.6+ d
FAq	5.5 cd	10.5 d	19.6 d	0* b	1.5* d	2.8 e
DMSO	0	0	0	0	0	0
Thiophanate methyl	54	54	54	9.8	9.8	9.8

EEtOH: ethanolic extract; FHex: hexane fraction; FDCM: dichloromethane fraction; FAce: ethyl acetate fraction; FBuOH: butanol fraction; FAq: aqueous fraction; DMSO: negative control. Means followed by different lowercase letters in the column differ statistically from each other by the Tukey's test ($P<0.05$). Means followed by * and + do not differ statistically from DMSO and fungicide treatments, respectively (Dunnett; $P>0.05$).

Aspergillus sp. was 1.25 mg ml^{-1} and for *Rhizopus* sp. it was 0.625 mg ml^{-1}. Preliminary chemical analysis showed eugenol as major compounds in the sample of the EEtOH and FDCM, MIC was done with eugenol pure, and the result was 0.125 mg ml^{-1}.

TABLE 2. Minimum Inhibitory Concentration (MIC, mg ml^{-1}) of samples from *Ocimum gratissimum* leaves and eugenol pure solutions.

Samples	*Aspergillus* sp.	*Rhizopus* sp.
EEtOH	1.25	0.625
FDCM	0.625	0.625
Eugenol	0.125	0.125

EEtOH: ethanolic extract; FDCM: dichloromethane fraction.

Antioxidant capacity

The antioxidant potential of *O. gratissimum* extracts was studied by means of synthetic radical tests, DPPH and ABTS, and by the FRAP assay, as with the results shown in Table 3. The antioxidant potential of extracts by DPPH and ABTS consisted of the ability to eliminate free radicals by donating a hydrogen atom or an electron. The antioxidant capacity is related to the degree of discoloration of the reaction solution with the synthetic free radical (Re *et al.,* 1999; Sousa *et al.,* 2007).

The EEtOH partition exhibited the highest antioxidant capacity for both DPPH and ABTS assays, followed by nonpolar fractions, FHex and FDCM, respectively. Low potential was identified in polar fractions, with FAce significantly lower among all extracts evaluated in the DPPH and ABTS assays. FAq was the only extract that did not show significant potential for scavenging the ABTS radical. The FRAP assay defines antioxidant as any substance in the reaction medium with reducing power by donating a hydrogen atom (Duh *et al.,* 1999). Thus, EEtOH presented the highest reducing power and as the polarity is increased, the reduction capacity of samples is smaller, with FAq showing the lowest value.

TABLE 3. Antioxidant activity by DPPH, ABTS, and FRAP of the ethanolic extract and fractions from *Ocimum gratissimum* leaves.

Treatments	DPPH IC_{50} (μg ml^{-1})	ABTS IC_{50} (μg ml^{-1})	FRAP (TEAC)
EEtOH	371.1±2.98 e	182.43±1.1 e	262.39±3.61 a
FHex	405.60±3.21 e	325.86±3.49 d	229.88±1.65 b
FDCM	707.11±2.75 d	370.00±1.76 c	111.51±5.03 c
FAce	2088.33±13.52 a	641.06±8.05 a	39.62±1.63 e
FBuOH	905.96±5.64 c	495.66±5.63 b	64.13±2.29 d
FAq	1748.52±8.36 b	4816.50±20.35 ns	27.32±1.9 f

EEtOH: ethanolic extract; FHex: hexane fraction; FDCM: dichloromethane fraction; FAce: ethyl acetate fraction; FBuOH: butanol fraction; FAq: aqueous fraction; ns: not significant. Means followed by different lowercase letters in the column differ statistically from each other by the Tukey's test ($P<0.05$).

Identification by GC/MS

In the GC/MS chromatographic profile EEtOH samples and their nonpolar fractions from *O. gratissimum* leaves, 22 substances were identified, shown in Table 4. From chromatograms, the presence of eugenol as the major substance in all samples was observed, with the highest relative percentage (61.26%) in the FDCM fraction, followed by the EEtOH extract (59.61%) and FHex fraction (37.65%).

The *Aspergillus* sp. and *Rhizopus* sp. fungi can accelerate the deterioration process of stored seeds (Silva *et al*., 2021). The present study demonstrated *in vitro* antifungal activity of the EEtOH and FDCM samples from *O. gratissimum* leaves at 5 mg ml^{-1} against *Aspergillus* sp. and *Rhizopus* sp. fungi associated with seeds in the storage phase, which indicates biological potential of extracts obtained from this plant. Onaebi *et al.* (2020), using the EEtOH at 100 mg ml^{-1} from *O. gratissimum* leaves, found reductions in the growth of *Aspergillus flavus* (51.93%) and *Aspergillus niger*

TABLE 4. Compounds identified by gas chromatography coupled to mass spectrometer from the ethanolic extract and the hexane and dichloromethane fractions from *Ocimum gratissimum* leaves.

Ocimum gratissimum samples with % of chromatogram areas						
Number	**Retention time (min)**	**Identified compounds**	**KI[a]**	**EEtOH**	**FHex**	**FDCM**
1	17.499	Eugenol	1357	59.61	37.65	61.26
2	17.945	a-Copaene	1374	-	0.83	-
3	18.168	(-)-β-Bourbonene	1382	-	0.51	-
4	18.531	Vanillin	1396	-	-	1.22
5	19.045	Caryophyllene	1416	1.32	2.28	-
6	20.617	Germacrene D	1478	1.91	1.5	-
7	21.686	δ-Cadinene	1522	-	1.21	-
8	23.061	Caryophyllene oxide	1580	-	1.42	-
9	25.432	Germacra-4(15),5,10(14)-trien-1α-ol	1683	-	0.74	-
10	26.537	Coniferyl alcohol	1734	-	-	0.86
11	28.758	Phytol derivative	1839	1.52	0.94	-
12	31.207	n-Hexadecanoic acid	1963	-	0.63	-
13	31.861	Ethyl hexadecanoate	1994	0.85	1.72	-
14	34.045	Phytol	2112	3.08	6.97	-
15	34.528	Methyl α-linolenate	2139	-	2.03	1.11
16	34.938	Linoleic acid ethyl ester	2162	-	0.65	-
17	35.052	Ethyl linoleate	2169	1.7	3.14	-
18	35.545	Octadecanoic acid, 17-methyl-, methyl ester	2196	-	0.45	-
19	43.255	Squalene	2832	4.72	10.24	-
20	44.682	Vitamin E	3151	0.81	2.88	-
21	45.445	Chondrillasterol	3297	-	3.18	-
22	45.803	γ-Sitosterol	3356	-	3.05	-
Total (%)				75.52	82.02	64.45

[a] Retention index obtained as a standard reference of ν-alkanes using HP-5MS column. EEtOH = Ethanolic extract, FHex = Hexane fraction, and FDCM = Dichloromethane fraction.

(23.7%), but the same did not occur for *Rhizopus delemar* (0%); the difference from the present study may be due the extract preparation.

The fraction FDCM and the EEtOH extract showed the best antifungal activities, and the GC/MS analysis indicates eugenol as the major compound. Eugenol is a phenylpropanoid commonly found in essential oils extracted from *O. gratissimum* leaves and, due to its nonpolar characteristic, it was easily identified in the less polar fractions and in the ethanolic extract in this study. Eugenol is related to several biological activities, mainly against phytopathogens (Faria *et al.,* 2006; Dambolena *et al.,* 2010).

There are numerous studies with essential oils of *O. gratissimum*; however, research for ethanolic extracts and fractions of different polarities is scarce (Zareiyan & Khajehsharif, 2022). In our experiment, the MIC results showed that eugenol has higher activity than EEtOH and FDCM samples. Ethanol is a non-selective solvent in the process of extracting compounds from plants, thus allowing for a greater chemical diversity of constituents in the extracts obtained, but at low concentrations. On the other hand, fractions obtained from the fractionation process with selective solvents of different polarities have higher concentrations and lower diversity of phytochemicals and are more selective in terms of polarity, thus the DCM sample had higher concentrations of the compounds with antifungal activity. Nwofor *et al.* (2021) presented the MIC of 100 mg ml^{-1} for methanolic extracts of *O. gratissimum* against *Penicillium citrinum, Aspergillus aculeatus, Aspergillus fumigatus, Curvularia kusanol*, *and Absidia* spp.

The EEtOH showed the highest antioxidant capacity among samples used in the DPPH, ABTS and FRAP methods. Ouyang *et al*. (2013) showed antioxidant activities of methanolic extracts and ethyl acetate fraction from *O. gratissimum* leaves using the same methods as in this study.

In our study, the non-polar fractions demonstrated more antioxidant capacity than polar fractions. This could be explained by the presence of eugenol substance in all samples and other antioxidants like squalene and vitamin E.

The results of this study confirmed that *O. gratissimum* extracts have antifungal and antioxidant activity, but the type of solvent can interfere with the chemical composition and biological properties. The mechanism of action is likely related to the presence of eugenol. In addition, extracts can scavenge free radicals, which could reduce seed deterioration during storage. However, it is still necessary to verify the *in vivo* efficacy and improve the activity with the release of the active ingredient at the specific site of action.

Acknowledgments

The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for the scholarship – Financial Code 001, the Espírito Santo Research and Innovation Support Foundation (FAPES) APES/SEAG (Process 76449130/16) and the Federal Institute of Espirito Santo (IFES) for financial support from PRODIF.

Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

JTL carried out the field and laboratory experiments and contributed to writing of the manuscript. AFS designed the experiments and revised the manuscript. HSF designed the methodology, obtained the financial support for the project leading to this publication and reviewed the final edition and style. All authors have read and approved the final version of the manuscript.

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