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Original Research Article

Essential Oil of *Eucalyptus polybractea* (L.): Chemical Composition, Antifungal, Insect Repellent and Insecticidal Activities

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ABSTRACT

Eucalyptus essential oil has a wide range of bioactivity, but research on the activity of *Eucalyptus polybractea* is limited. Due to the great need for sustainable pesticides, this study was carried out to assess the antifungal and insecticidal activities of essential oil (EO) of *E. polybractea*. EO was extracted from the leaves by hydrodistillation and the composition of the essential oil was determined by gas chromatography-mass spectrometry (GC-MS). The antifungal activity was tested against the following organisms; *Aspergilus niger*, *Fusarium graminearum*, *Penicillium sp1*, *Penicillium expansum*, *Cladosporium herbarum*, *Penicillium sp2*, *Fusarium sp*, *Aspergilus flavus*, *Alternaria alternata* and *Trichoderma viride* which were isolated from wheat grain. The insecticidal and repellant activity was tested against stored product pests; *Tribolium castaneum*. GC-MS analysis revealed a significant number of monoterpenes in the essential oil with Eucalyptol (34.87%) being the major component. The highest antifungal activity was observed against *Fusarium graminearum*, *Penicillium sp2*, *Aspergilus flavus* and *Trichoderma viride*. EO showed repellent activity to *T. castaneum* (PR = 65%, after 45 min) and highly toxic with 100% mortality after 72 hours of exposure. The study therefore revealed significant intra-specific changes in EO quality, which is reflected in the different rates of antifungal and insecticidal activity.

Keywords: Essential oil, *Tribolium castaneum*, *Eucalyptus polybractea*, Antifungal activity.

Introduction

Wheat (*Triticum durum* L.) is one of the most important staple foods and cereal crops in the world. Its proper storage is essential, and more importantly, the storage conditions should be such that it is unfavorable for pests and moulds. During storage, wheat is affected by fungal, bacterial and viral pathogens, which cause diseases of varying severity. Various insect pests attack wheat and cause significant loss of grain yield. About thirty-nine (39) pest species have be found to attack stored grain and grain products.[1] Amongst the different insect pests that affect wheat, *Tribolium* species are known to infest stored wheat. As a secondary pest, *T. castaneum (Herbst) (Coleoptera: Tenebrionidae)* is the species that feeds mostly on processed or damaged stored products.[2] The major fungi associated with stored wheat grain, are those belonging to the genera of *Aspergillus*, *Penicillium*, *Alternaria*, and *Fusarium* and are responsible for production of mycotoxins that are harmful to animals and humans.[3,4] The establishment and spread of these pests on stored wheat has two consequences: changes in grain quality, which affects the nutritional value of the derived products, and mycotoxins production. As a result, grain stock pests can obstruct any production attempt if no protective measures are applied.[5] Due to their high concentration of bioactive components which readily breakdown into harmless products, plant essential oils (EOs) may be an alternative source of pest control agents and are therefore suited for use in integrated management programs.

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The *Myrtaceae* family is a potentially attractive choice for biocontrol agents in this context.[6] The volatile chemical components present in Eucalyptus essential oil are abundant in the plant's flowers, bark, seeds, roots, fruits, and wood. The genus Eucalyptus has over 900 species, 300 of which have volatile essential oils in their leaves.[7] Eucalyptus essential oils have several commercial and medicinal applications. Insecticidal, herbicidal, anti-microbial, antiviral, and fungicidal effects are found in the oils.[8] The most abundant component of eucalyptus leaf essential oils are monoterpenes and sesquiterpenes. However, depending on the species and variation, their relative numbers or ratios may differ. Even within the same variety, the chemical composition might vary depending on its geographical origin.[7] There is little research on the antimicrobial activity of *E. polybractea* essential oil against grain pests and moulds. The present study therefore seek to determine the chemical composition of Algerian *Eucalyptus polybractea* essential oils, their antifungal activity against ten stored wheat moulds as well as their repellent and insecticidal properties against *T. castaneum*.

Materials and Methods

Plant materials and EO extraction

The leaves of *E. polybractea* were collected in January 2021 in Algiers forest (Northern Algeria). The essential oil was obtained by hydrodistillation of 100 g of dry leaves using Clevenger-type apparatus for 3 hours. The water vapour laden with essential oils condensed in a refrigerant were collected in a separatory funnel, the less dense oil were collected by simple decantation and dried on anhydrous sodium sulphate (Na_2SO_4) before analysis. The essential oil was stored in a refrigerator at 4°C.

Gas Chromatography-Mass Spectrometric (GC-MS) analysis

GC-MS analysis was performed on a Hewlett Packard 5890 II/MSD 5973 system outfitted with a DB-5MS column (30 m 0.25 mm, film thickness 0.5 m; J&W) using the above operative column and conditions; helium flow was set to 1 mL/minute. It was in electron

impact (EI) mode at 70eV, 300A, with a 220°C ion-source temperature and a 250°C quadrupole temperature. In splitless mode, samples were injected. The mass spectra were scanned in the 33-500 m/z range.

Insect Breeding
Strain of *Tribolium castaneum* (Coleoptera: Tenebrionidae), originated from stored durum wheat was used in the study. A group of 20 adult insects of indeterminate sex was placed in 500 mL glass jars with mesh lid filled with 250 g of healthy durum wheat for the breeding of *T. castaneum*. The assembly was placed in a laboratory chamber with appropriate temperature and humidity conditions of 27 ± 1°C and 70 ± 5%, respectively. After two or three weeks of infestation, the adult insects were removed from the breeding environment.

Fungal isolates
Ten fungal isolates: *Aspergilus niger, Fusarium graminearum, Penicillium sp1, Penicillium expansum, Cladosporium herbarum, Penicillium sp2, Fusarium sp, Aspergilus flavus, Alternaria alternate*, and *Trichoderma viride* were isolated directly from stored durum wheat grain for one year on Potato Dextrose Agar (PDA) medium.

In vitro antifungal activity assay
The antifungal activity of essential oils was tested using the radial growth technique.[9] The concentrations prepared were 0.089, 0.133, 0.178, 0.222 and 0.267 mg/L. To accomplish this, appropriate volumes of essential oils were dissolved in Dimethyl sulfoxide (DMSO) and immediately added to PDA medium before being poured into 9.0 cm diameter Petri dishes. The controls were made with DMSO mixed with PDA (no essential oil was used). The isolated fungi were loaded into mycelial, then transferred aseptically to the center of the Petri dishes and incubated. This procedure was carried out three times. The percentage inhibition of mycelial growth was expressed by the antifungal index according to the following formula:

IA (%) = [1-(D-test /D-control)] x 100

Where, IA(%) = Inhibition rate expressed as a percentage
D-test = test colony diameter in mm
D-control = diameter of control colonies in mm

Insect Repellent assay
Insect repellent assay of *E. polybractea* EO was performed on filter paper using the preferred area method, as described by Jemˆaa *et al*. (2012).[10] The 9 cm diameter filter paper discs used for this purpose were cut in halves. The EO was diluted in acetone to make four doses (2, 4, 6, and 8 µL/L). Then, each dose was uniformly spread over one half of the disc, while the other half received only acetone, the two halves of the discs were re-welded using adhesive tape. In a Petri dish, a reconstituted filter paper disc was placed, and 10 adult non-sexed insects were placed in the center of each disc. Each dose was subjected to three repetitions. The number of insects present on the part of the filter paper treated with the EO (Nt) and those present on the untreated area (Nc) were counted after 15, 30 and 45 minutes. The following formula was used to calculate the percentage repellency (PR):

PR = [Nc – Nt / (Nc + Nt)] x 100
The average repellency rate for essential oil was calculated and assigned according to ranking of McDonald *et al*. (1970),[11] to one of several repellent classes ranging from 0 to 5. Results were presented as the mean of percentage repellency ± the standard error.

Insecticidal activity assay
To assess the insecticidal activity of *E. polybractea* essential oils, 10 insects were placed in a glass jar with a capacity of 100 mL as an exposure chamber to test the toxicity of the essential oil against adults of *T. castaneum*, in which a single load of EO was spread on a Whatman paper disc 5 cm in diameter and then attached to the inner face of the lid. Thereafter, the device was sealed and left at room temperature. Seven doses of essential oil (0.5, 1, 2, 4, 6, 8 and 10 µL/L) were tested. Control insects were kept in the same conditions as the experimental insects but were not given any essential oil. The number of dead insects in each jar was counted every 24, 48, and 72 hours of exposure. Each concentration was replicated four times. Insects were considered dead when no leg or antennal movement was observed.[10] The percentage insect mortality was calculated using Abbott's (1925) correction formula.[12,13]

Mc = Mo – Mt/ 100 – Mt [(M0 - Mt) / (100 - Mt)] x 100
Where, Mc = percentage corrected mortality;
Mt = mortality of the tested sample;
Mo = mortality in the untreated control.

Statistical analysis
Statistical analysis of the test results of insecticidal fumigant toxicity was carried out using Graph Prism software version 9, data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. The differences between treatments were considered significant if p-value was less than 0.05.

Results and Discussion

Chemical composition of *E. polybractea* Essential oil
The chemical composition of the EO of *E. polybractea* analyzed by GC-MS revealed 45 compounds (Table 1, Figure 1). The most prominent compounds were Eucalyptol (34.87%), alpha-phellandrene (13.10%), alpha-Terpineol (12.17%), (1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (5.49%) and Terpinen-4-ol (5.18%). The chemical composition analysis showed that *E. polybractea* EO is dominated by monoterpenoids. Previous studies on *E. polybractea* EO revealed the presence of different principal constituents. The results from the present study are different from that of Poli *et al* (2018)[14] who showed the domination of p-cymene (25.5%) and cryptone (11.42%) in *E. polybractea* EO. In some works, other chemotypes have been found with the predominant compound being 1,8-Cineole.[15-17]

In vitro antifungal activity
The majority of the detected fungi in stored wheat grain such as *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* are common mycotoxigenic fungi. The growth of test fungi treated with essential oil varied between 9 mm and 88 mm. The high concentration of EO (0.267 mg/L) showed higher antifungal activity compared to the control. *Fusarium graminearum, Penicillium sp2, Aspergilus flavus* and *Trichoderma viride* were significantly more sensitive. The inhibition zone diameter (IZD) of mycelium growth by the EO at low concentration (0.089 mg/L) was 87 mm and at high concentration (0.267 mg/L) was 38 mm. *Penicillium sp2* had IZD of 71 mm at low concentrations and 9 mm at high concentrations of the EO (Table 2). *Aspergilus flavus* at low concentrations of EO had IZD of 86 mm and at high concentrations had IZD of 38 mm. *Trichoderma viride* had IZD of 87 mm and 17 mm at low and high concentrations of EO, respectively.

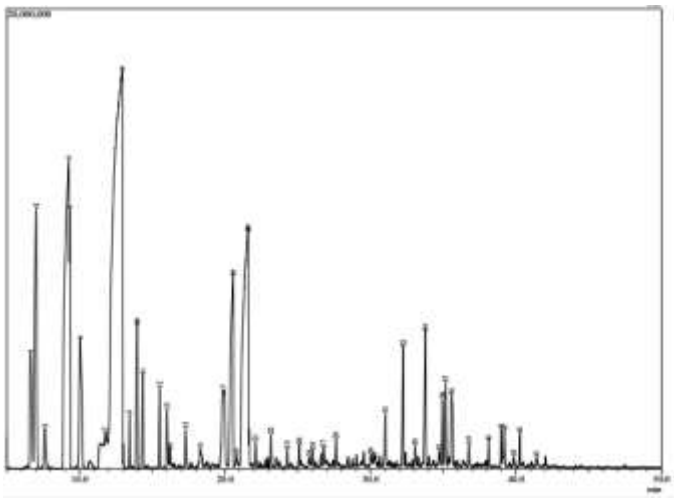

Figure 1: GC Chromatogram of leaf essential oil of *E. polybractea*

Table 1: Chemical composition of *E. polybractea* Essential oil

Peak	RT (min)	Area%	Compound Name
1	6.664	2.33	alpha.-phellandrene (only name in Wiley6)
2	7.033	5.49	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
3	7.619	0.61	Camphene
4	9.268	13.10	alpha.-phellandrene (only name in Wiley6)
5	9.344	1.83	2(10)-Pinene
6	10.052	3.11	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-
7	11.784	0.29	Benzene, 1-methyl-4-(1-methylethyl)-
8	12.943	34.87	Eucalyptol
9	13.440	0.42	1,3,6-Octatriene, 3,7-dimethyl-, (E)-
10	13.952	1.69	gamma.-Terpinene
11	14.350	1.00	5-Isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol
12	15.510	0.80	4-Isopropylidene-1-cyclohexene
13	16.016	0.78	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1.alpha.,2.alpha.,5.alpha.)-
14	16.240	0.23	Linalool
15	17.291	0.49	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-
16	18.305	0.45	p-Menth-2-en-1-ol
17	19.888	2.75	alpha.-Terpineol
18	20.550	5.18	Terpinen-4-ol
19	20.808	0.21	2-Cyclohexen-1-one, 4-(1-methylethyl)-
20	21.568	12.17	.alpha.-Terpineol
21	22.105	0.27	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, trans-
22	23.154	0.33	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-
23	24.278	0.20	p-Menthane-1,2,3-triol
24	25.105	0.32	trans-Ascaridol glycol
25	25.799	0.18	Bicyclo[2.2.1]hept-2-ene, 2,3-dimethyl-
26	26.012	0.22	trans-Ascaridol glycol
27	26.782	0.29	2H-Pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl-
28	27.635	0.29	Cyclohexene, 1-(1,1-dimethylethoxy)-2-methyl-
29	30.032	0.20	Ylangene
30	30.265	0.27	Copaene
31	31.020	0.63	1-Methyl-1-ethenyl-2,4-bis(1'-methylethenyl)cyclohexane
32	32.246	1.45	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-
33	33.082	0.24	Neoalloocimene
34	33.776	1.96	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
35	34.727	0.28	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4aR
36	34.954	0.73	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]
37	35.178	0.94	beta.-Selinene
38	35.583	1.42	1.beta.,4.beta.H,10.beta.H-Guaia-5,11-diene
39	36.768	0.27	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-
40	38.132	0.24	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-
41	38.991	0.39	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.
42	39.203	0.45	5-Oxatricyclo[8.2.0.04,6]dodecane, 4,12,12-trimethyl-9-methylene-, (1R,4R,6R,
43	39.835	0.17	Guaiol
44	40.266	0.31	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene

45	41.432	0.17	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.

RT = Retention time

Table 2: Antifungal activity of *E. polybractea* EO

Strains	Concentrations (mg/L)					
	0.089	**0.133**	**0.178**	**0.222**	**0.267**	**Control**
Aspergilus niger	$87^{a} \pm 2.51$	$84^{a} \pm 6.11$	$88^{a} \pm 2.86$	$87^{a} \pm 3.00$	$84^{ad} \pm 4.00$	$90^{a} \pm 00$
Fusarium graminearum	$87^{a} \pm 2.51$	$60^{b} \pm 3.05$	$54^{b} \pm 2.51$	$34^{b} \pm 25.42$	$38^{b} \pm 3.51$	$90^{a} \pm 00$
Penicillium sp 1	$87^{a} \pm 2.51$	$87^{a} \pm 4.61$	$85^{a} \pm 5.13$	$79^{a} \pm 9.64$	$65^{c} \pm 4.00$	$90^{a} \pm 00$
Penicillium expansum	$85^{a} \pm 4.04$	$78^{ac} \pm 2.30$	$77^{a} \pm 2.51$	$72^{c} \pm 3.51$	$72^{ac} \pm 3.00$	$90^{a} \pm 00$
Cladosporium herbarum	$90^{a} \pm 0.00$	$88^{a} \pm 1.52$	$89^{a} \pm 0.57$	$86^{a} \pm 3.05$	$85^{d} \pm 4.04$	$90^{a} \pm 00$
Penicillium sp2	$71^{b} \pm 3.21$	$66^{bc} \pm 8.50$	$15^{c} \pm 3.05$	$12^{d} \pm 2.00$	$9^{e} \pm 1.15$	$90^{a} \pm 00$
Fusarium sp	$90^{a} \pm 0.00$	$87^{a} \pm 3.00$	$86^{a} \pm 3.60$	$83^{ac} \pm 5.68$	$56^{f} \pm 7.76$	$90^{a} \pm 00$
Aspergilus flavus	$86^{a} \pm 3.51$	$83^{a} \pm 6.24$	$77^{a} \pm 12.05$	$55^{b} \pm 5.00$	$38^{b} \pm 3.60$	$90^{a} \pm 00$
Alternaria alternata	$87^{a} \pm 2.51$	$83^{a} \pm 6.02$	$79^{a} \pm 9.0$	$78^{ac} \pm 10.81$	$59^{cf} \pm 5.50$	$90^{a} \pm 00$
Trichoderma viride	$87^{a} \pm 2.51$	$84^{a} \pm 5.03$	$55^{b} \pm 4.04$	$30^{e} \pm 7.00$	$17^{e} \pm 4.16$	$90^{a} \pm 00$

Data are Mean ± SD. Values followed by the same letter within each column are not significantly different

The essential oil of *E. polybractea* showed antifungal activity against *Fusarium graminearum, Penicilium sp2, Aspergilus flavus* and *Trichoderma viride*. However, for *Aspergilus niger, Penicillium sp1, Penicillium expansum, Cladosporium herbarum, Fusarium sp, Alternaria alternate*, it showed the lowest activity (Table 2). The strains studied showed different responses to eucalyptus essential oils. With inhibition rates of 57%, 57%, 81%, and 90% for *F. graminearum, Aspergillus flavus, Trichoderma viride*, and *Penicillium sp2*, respectively appear to be by far the most susceptible strains to different doses of eucalyptus oil. In the presence of EO, the inhibition rates of *Penicillium expansum, Penicillium sp1, Alternaria alternata*, and *Fusarium sp* were less than 50%. The lowest inhibition rates were shown by *Aspergillus niger* and *Cladosporium herbarum* with 6% and 5% inhibition, respectively (Table 3). Currently, very little data are available in the literature on *E. polybractea* EO antifungal activity. The present study showed the effect of the essential oil of this plant on ten species of fungi, six of which (*Aspergilus niger, Penicillium sp1, Penicillium expansum, Cladosporium herbarum, Fusarium sp* and *Alternaria alternate*) proved insensitive, while four strains (*Fusarium graminearum, Penicillium sp2, Aspergillus flavus* and *Trichoderma viride*) were susceptible. The efficacy of *E. polybractea* EOs against microorganisms could in part be attributed to their volatile bioactive components.[18] Previous research on the antifungal activity of eucalyptus oil indicated the resistance of *Penicillium sp* and *Aspergillus sp* against eucalyptus oil.[19] In contrast to our study, the results of Pedrotti *et al*. (2022)[20] showed that *Eucalyptus staigeriana* oil was effective in reducing the incidence and severity of black rot caused by *Alternaria alternata* in preventive and curative treatments at different concentrations, indicating susceptibility of *Alternaria alternata*. In general, the composition of the fungal microbiota of wheat grains found in the current study agree with previous studies.[3,21] The antifungal activity of *E. polybractea* essential oil may be attributed to Eucalyptol a major bioactive terpenoid constituent of the plant whose antifungal properties have been explored previously.[22]

Insect repellent activity

The repellent effect of *E. polybractea* essential oil was tested on adult *T. castaneum*. Table 4 shows the repellent capacity of *E. polybractea* EO at concentrations of 2, 4, 6 and 8 µL/L of air at different times after treatment. The results showed that the essential oil repels *T. castaneum*. The repellent effect of the essential oil against *T. castaneum* showed that the higher the concentration, the longer the repulsion time. *E. polybractea*'s EO repulsion time was 15 min, 30 min, 45 min, and 65 min at concentrations of 2, 4, 6 and 8 µl/L air, respectively. This finding agrees with the work of Mangang and Manickam (2022)[23] who demonstrated that the active components of eucalyptus oil formulated as insect repellent pellets (IRPs) showed repellent effect against adult *T. castaneum*, and the work of Alsudani *et al*. (2021)[24] who showed that the rellepent effect of Eucalyptus EO against this pest is about 46.66% 12 h post treatment.

Insecticidal activity

After 24 h of exposure to EO, insect mortality was 43% and 93% at 0.5 and 10 µL/L air, respectively. After 48 h, the mortality was 100% at 10 µL/L air (Table 5). This shows that essential oil of *E. polybractea* demonstrated significant insecticidal activity against adult *T. castaneum* after 48 h exposure. The mortality of adults *T. castaneum* was less than 50% after 48 h of exposure to the essential oil at 0.5 and 1 µL/L air. Insecticidal activity tests of *E. polybractea* essential oil showed significant insecticidal activity after 72 h of treatment (Table 5). The results showed that the insecticidal activity of *E. polybractea* EO against *T. castaneum* varies with the concentration and duration of treatment, allowing for maximum mortality after 72 h of exposure to a dose of 10 µl/L air. The insecticidal properties of essential oils are most likely due to the main constituent of the essential oil. Some reports have indicated that *E. polybractea* essential oil has insecticidal activity against *Haematobia irritans* adults,[25] insecticidal and larvicidal activities against *Aedes aegypti*.[26] The study of Yeom *et al*. (2013)[27] showed that *E. polybractea* oil at 15 mg/L air and 7.5 mg/L air demonstrated 100% mortality against adult male German cockroaches. The calculated 50% lethal concentration (LC_{50}) of 0.00014 µL/L air after 72 h of exposure indicated that adults *T. castaneum* are extremely sensitive to this oil.

Conclusion

In the present study, the antifungal potential of *Eucalyptus polybractea* EO was investigated against ten (10) fungal strains of stored wheat grains and the insecticidal and repellent activity against *Tribolium castaneum* was also tested. The results showed that *Eucalyptus polybractea* EO has enhanced activity against *Fusarium graminearum, Aspergillus flavus, Trichoderma viride, Penicillium sp2.* The insecticidal and repellent activities reveal a strong effect of eucalyptus oil against *T. castaneum.* From this, it is clear that *E. polybractea* EO possess a wide spectrum of activity against stored wheat grain moulds and insect pest. However, the leaves essential oil, composed predominantly of eucalyptol, may be an excellent choice as a natural pesticide. These findings will serve as a basis for further research into this plant species with a view to the discovery of the bioactive compounds.

Table 3: Inhibition of fungal mycelial growth at different concentrations of EO

Concentration (mg/L)	**0.089**	**0.133**	**0.178**	**0.222**	**0.267**
Strains	**Inhibition (%)**				
Aspergillus niger	3.33	6.66	2.22	3.33	6.66
Fusarium graminearum	3.33	33.33	40	62.22	57.77
Penicillium sp 1	3.33	3.33	5.55	12.22	27.77
Penicillium expansum	5.55	13.33	14.44	20	20
Cladosporium herbarum	0.00	2.22	1.11	4.44	5.55
Penicillium sp2	21.11	26.66	83.33	86.66	90
Fusarium sp	0.00	3.33	4.44	7.77	37.77
Aspergillus flavus	4.44	7.77	14.44	38.88	57.77
Alternaria alternata	3.33	7.77	12.22	13.33	34.44
Trichoderma viride	3.33	6.66	38.88	66.66	81.11

Table 4: Mean repellence time (min) against *T. castaneum* at different concentrations of *E. polybractea* EO

Essential oil	**Concentrations (µL/L air)**			
	2	**4**	**6**	**8**
E. polybractea	14.00 ± 6.92	30.00 ± 11.94	45.00 ± 19.14	65.00 ± 34.15

Data are Mean ± SD

Table 5: Mortality (%) of *T. castaneum* on exposure to *E. polybractea* EO

Concentration	**Time (h)**		
(µl L air)	**24**	**48**	**72**
0.5	13 ± 5.77	26 ± 5.77	43 ± 5.77
1	*20* ± 0.00	36 ± 5.77	56 ± 5.77
2	26 ± 5.77	50 ± 10.00	73 ± 5.77
4	56 ± 5.77	63 ± 5.77	65 ± 5.77
6	56 ± 11.54	63 ± 5.77	73 ± 11.54
8	70 ± 10.00	76 ± 5.77	83 ± 11.54
10	93 ± 5.77	100 ± 0.00	100 ± 0.00

Data are Mean ± SD

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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