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Original Research Article

Antifungal Activities of Black Seed, Castor and Lemon Oils Against Pathogenic Plant Fungi

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ABSTRACT

Pathogenic plant fungi are of great concern due to their effects in reducing crop yield. Plant oils are environmentally friendly alternatives to non-biodegradable synthetic fungicides. This study investigated the potency of some plant oils *in vitro* against pathogenic plant fungi. Fungi were isolated from infected leaves of soybean, cowpea, groundnut and maize cultivated on the same farm land. These fungi were identified as *Rhizoctonia solani*, *Mucor racemosus*, *Fusarium verticillioides*, *Flavodon flavus* and *Aspergillus flavus*. Preliminary testing of the antifungal effects of black seed, castor, and lemon oilwas carried out against the isolated fungi using standard procedures. The results identified which of the tested plant oil demonstrated antifungal potential against the fungi species. Lemon oil and black seed oil showed the most promising effect due to their broad-spectrum mycelia inhibitory effects on the pathogenic fungi. Lemon oil was fungicidal against *F. verticillioides* and *F. flavus* in this study. Sporulation in *F. flavus* were also inhibited by the two oils. Lemon and black seed oils are suggested as promising candidates for further research *in vivo* and are major components for fungicides. This work has demonstrated the potency of plant oils as an important economical and environmentally friendly alternatives to non-biodegradable synthetic fungicides.

***Keywords*:**Antifungal, Black seed oil, Castor oil, Lemon oil, Pathogenic fungi, Legumes.

Introduction

Fungi infect plants due to their presence in the air, soils or their introduction by invertebrate hosts around plants. The infectious abilities of fungi are due to their active spores which confer high reproductive and survival potency on the organism under various environmental conditions. Pathogenic fungi can reduce crop yield on farm land by infecting different plant parts. This not only reduces crop yield but also causes pre-harvest and post-harvest losses.[1]

Several crops of global economic importance are infected by fungi in the tropics, most of these crops are not produced in the temperate parts of the world.[2] The loss if controlled or reduced will help increase world production of these important crops. Plant diseases such as *Fusarium* blight are reported in areas where soybeans are grown.[3.4] The incidence of the diseases caused by *Fusarium oxysporum*, due to yearly cultivation of soybean on the same land is associated with foliar part of the plant.[6] Other fungi diseases such as leaf spots are caused by *Aspergillus flavus* and *A. parasiticus* in groundnuts,[7.8] and *Fusarium spp.* infections in maize.[7] Most of these infections are usually difficult to control due to spore dispersal and the ability to contaminate different plant species.[9]Synthetic chemicals are usually used as fungicides on farm land, most of which produce residues toxic to the plant, soil and for human consumption.[10] In addition to the toxic nature, microorganisms also develop resistance to synthetic fungicide over time. Therefore, there is a continuous quest for the use of natural products as fungicides. Plant extracts are effective as antifungal agents as they have little or non-toxic effects when used as fungicides.

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Different plant parts have been extracted with different solvents such as water, ethanol, methanol, etc., and used as fungicides. Other forms such as oils are also in use *in vitro* and *in vivo* as an antifungal against plant pathogens. [11.12]Adjou et al.[13] reported the fungicidal and inhibitory potential of *Ocimum canum* oils against aflatoxins-producing toxigenic fungi isolated from seeds of peanuts. Gakuubi [14] highlighted the ability of essential oils of *Eucalyptus camaldulensis* to inhibit the growth of test *Fusarium* spp. infecting maize. Additionally, inhibition of *Aspergillus flavus* infection in maize plant by oil extracted from spices,[15] and the inhibition of *Fusarium sp*. in maize by plant oils have been well documented.[16] The presence of biologically active monoterpenes in plant oil has been reported to confer ability to control infections in plants (Mssilou et al., 2021).[17-19] Previous studies on plant oils selected for the purpose of this research revealed the antifungal effects of lemon oil on *Aspergillus flavus*, *A. niger*, and *Penicillium spp.* [18] and on food-borne mold.[20] Castor oil has been shown to have low inhibitory effect on *A. niger* and *Penicillum sp*,[21] while the antifungal activity of black seed oil on *Aspergillus spp.* and *Fusarium oxysporum* have equally been documented.[22.23]

Therefore, this research was aimed at evaluating the efficacy of the selected plant oils in controlling pathogenic fungi isolated from the leaves of grain legumes and maize on farm land. The objective of this study was to isolate and identify fungi isolated from infected leaves of soybean, cowpea, groundnut, and maize and also, to evaluate the antifungal activity of lemon (*Citrus limon*), castor (*Ricinus communis*) and black seed (*Nigella sativa*) oils on the isolated pathogenic fungi.

Materials and Methods

Leaf collection

Infected leaves of cowpea, soybean, groundnut, and maize were collected between January and March, 2021 on a local farm in Iwo, Osun State, Nigeria. Average temperature of 32 ± 3°C / 26± 3°C (day/night). The leaves collected were surface sterilized with 70% ethanol for one minute, and in 5% sodium hypochlorite (NaClO) solution. The leaves were immediately rinsed in distilled water thrice, and were cultured on a prepared Potato Dextrose Agar (PDA) media in

Petri dishes and incubated for 7 days at 27°C.Five distinct monocultures were ultimately separated from the PDA media containing plant leaf samples that were infected. A plug of a single colony was taken from the PDA and placed in the middle of a brand-new PDA plate to isolate the fungal culture. At 25°C, each colony was subcultured in triplicates, wrapped in parafilm, and incubated for seven days.[24]

Molecular identification of fungi isolates
DNA was extracted from each of the five fungal isolates using standard procedures.[25] For the PCR analysis, PCR reaction cocktail consisted of 10 µl of 5x GoTaq colourless reaction; 3 µl of 25Mm MgCl2; 1 µl of 10 mM of dNTPs mix; 1 µl of 10 pmol each forward primer (ITS 1)- 5' TCC GTA GGT GAA CCT GCG G 3' and reverse primer (ITS 4)- 5' TCC TCC GCT TAT TGA TAT GC 3'; 0.24µl of 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water and 8µl working DNA template. Amplification was carried out using PCR system thermal cycler (Applied Biosystem Inc., USA) with PCR profile of an initial denaturation, 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30s and 72°C for 1 minute 30 seconds; and a final extension at 72°C for 10 mins. The PCR products were purified and sequenced by the Inqaba Biotechnical Industries, Pretoria, South Africa. Sequences generated were compared using the BLAST tool on NCBI database to identify the five fungi to species level.

Plant oils collection
Plant oils used for this antifungal study were black seed oil, castor oil and lemon oil (100 % organic cold pressed oil). These were purchased from a local retailer in Iwo, Osun State and a product of Al-Hussan Food Products Factory, Ridayh, Saudi Arabia.

Antifungal assay (Direct method)
Antifungal assay was performed using the modified agar medium assay [13] modified. Approximately 20 ml of PDA media was poured in petri dish and separate volumes of plant oils (1 ml, 2 ml and 4 ml) mixed with *Tween 20* added and the Petri dish, appropriately labelled. The plant oil used were black seed oil, castor oil and lemon oil. Each of the labelled Petri dishes was inoculated at the center with a mycelial disc (4 mm) picked at the outmost part of each of a test fungal-colony grown on PDA for 5 days. The control experiment (without plant oil) was prepared using the same procedure. The plates were incubated at 27 °C and colony diameter was observed and recorded at day 3, day 5 and day 7 in the both the oil-treated and control experiments. This time course differences were conducted to observe differences in the colony growth rate and inhibitory efficacy of each oil over time. This experiment was conducted in triplicates. The percentage inhibition of fungal mycelia growth was calculated using Philippe et al. [26] equation:

$$\%\ Mycelia\ growth\ inhibition = \left(dc - \frac{dt}{dc}\right)\ X\ 100$$

Where: dc – mean diameter of colony in the control sample
dt – mean diameter of colony in the treated sample.

Determination of Minimum Fungicidal concentration
The strains fungal growth inhibited by a plant oil from the assay were re-inoculated into a fresh PDA media after day 7 of the experiment, and possible regrowth of fungal mycelia observed for another 3 days.[13] The Minimal fungicidal concentration (MFC) is the lowest oil concentration at which no regrowth of fungi occurred on the petri dishes. This was to establish if the plant oil possesses biocidal effect on the tested fungal isolates.

Disk diffusion Assay
Disc diffusion method was further used to identify the antifungal properties of the three plant oils.[27] A spore suspension was prepared by introducing 4 ml of sterile distilled water into each fungi PDA plates and were evenly spread using a sterilized glass spreader. Two hundred microlitres of spore suspension was transferred into a fresh PDA media and allowed to dry, while 1 ml of each plant oil each was separately added into a 20 mm diameter sterile filter paper and the oil allowed to soak. The impregnated paper was placed at the center of the PDA plates containing spores suspension and incubated at 27 °C for 5 days.

Data Analysis
Data collected were subjected to One-way ANOVA to determine the significant differences between percentage inhibition over time for each fungal species and plant oil using SPSS v20.0 Using Tukey's (HSD) at 5 % probability level.

Results and Discussion

On the basis of molecular screening, the fungal species isolated from the infected cowpea, soybean, groundnut and maize leaves were identified as; *Rhizoctonia solani* strain COUFPI204, *Mucor racemosus* strain GZ20190123, *Fusarium verticillioides* isolate FM14, *Flavodon flavus* strain E8864A and *Aspergillus flavus* strain AF051. The GenBank accession numbers for the nucleotide sequences assigned as OM714805, OM714806, OM714807, OM714808 and OM714809 for *Fusarium verticillioides* isolate FM14, *Mucor racemosus* strain GZ20190123, *Rhizoctonia solani* strain COUFPI204, *Flavodon flavus* strain E8864A and *Aspergillus flavus* strain AF051 respectively

Antifungal assay (Direct method)
The plant oil used in this experiment inhibited fungal mycelia growth in all the five pathogenic fungi (Table 1, 2 and 3). Lemon oil exhibited a broad-spectrum inhibitory effect on the mycelia growth across all the fungal species isolated from the plant leaves (Table 1). Although, *F. verticilloides* percentage mycelia inhibition growths were observed to decrease at 1 ml concentration at day 5 (94.11±10.18) and at day 7 (91.01±16.74) the decrease in percentage inhibition were insignificant at the lowest concentration ($p \leq 0.05$) of lemon oil treatment.
Castor oil exbihited a maximal percentage inhibition on mycelia growth (100±0.00) of *R. solani* at all oil concentrations and for the entire 7 days. The effect of castor oil on mycelia growth of *M. racemosus* was not pronounced at all concentrations, as the highest rate of inbition were observed at day 5 and day 7 at 4 ml concentrations (59.95±7.63 and68.95±7.63respectively). Mycelia growth inhibition with 4 ml oil concentration were significantly higher ($p \leq 0.05$) than mycelia inhibition with 1 ml and 2 ml oil concentrations on *F. verticillioides*, *F. flavus* and *A. flavus* at day 3 to day 7 (Table 2).
Black seed oil exhibited broad-spectrum antifungal activity at all oil concentrations (1, 2, and 4 ml), on the mycelia growth of all the fungi isolated from the plant leaves from day 3 to day 7 (Table 3).

Determination of Minimum Fungicidal concentration
Lemon oil was fungicidal against *F. verticillioides* and *F. flavus* for the three oil concentration, as there were no regrowth of organisms. Mycelia regrowth of *R. solani*, *M. racemosus* and *A. flavus* pretreated with lemon oil were observed after day 3 of re-inoculation (Fig. 1A).
Castor oil concentrations of 2ml and 4ml were fungicidal against *R. solani*, while 4ml concentration of castor oil was fungicidal against *F. verticillioides* and *F. flavus*. There were mycelia re-growth for *R. solani* pretreated with 1 ml oil, as well as mycelia re-growth for *F. verticillioides* and *F. flavus* pre-treated with 1 ml and 2 ml oil respectively. Mycelia re-growth of *M. racemosus* and *A. flavus* pre-treated with castor oil were observed after day 3 of re-inoculation (Fig. 1B).At concentration of 4ml, black seed oil was fungicidal against all the test isolates except *A. flavus* as there were no mycelia re-growth on the re-inoculated organisms. Mycelia re-growths were observed for all the five fungi pre-treated with 1 ml and 2 ml of black seed oil respectively (Fig. 3C).

Disk diffusion Assay
Fungal sporulation was partly inhibited in all fungi species except in *A. flavus* up to day 5. Sporulation inhibition by black seed oil (Fig. 2A) and lemon oil (Fig. 2B)were observed in *F. flavus* up to day five.

Table 1: Percentage of mycelia growth inhibition (mm) by Lemon oil

	Oil Conc. (mL)	*R. solani*	*M. racemosus*	*F. verticillioides*	*F. flavus*	*A.flavus*
Day 3						
	1	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
	2	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
	4	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Day 5						
	1	100 ± 0.00^{a}	100 ± 0.00^{a}	94.11±10.18^{ab}	100 ± 0.00^{a}	100 ± 0.00^{a}
	2	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
	4	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Day 7						
	1	100 ± 0.00^{a}	100 ± 0.00^{a}	91.01±16.74^{ab}	100 ± 0.00^{a}	100 ± 0.00^{a}
	2	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
	4	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}

Values are presented as means ± SD (n = 3). Means with the same letter in the same column are not significantly different at $p \leq 0.05$

Table 2: Percentage of mycelia growth inhibition (mm) by Castor oil

	Oil Conc. (ml)	*R. solani*	*M. racemosus*	*F. verticillioides*	*F. flavus*	*A.flavus*
Day 3						
	1	100 ± 0.00^{a}	38.61± 5.83^{b}	78.39± 10.63^{b}	10.69 ± 0.00^{b}	68.96±13.38^{b}
	2	100 ± 0.00^{a}	41.45± 6.22^{b}	83.34± 7.37^{ab}	23.97 ± 0.00^{b}	55.31±5.56^{b}
	4	100 ± 0.00^{a}	47.20±2.75^{b}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Day 5						
	1	100 ± 0.00^{a}	47.74±6.37^{b}	85.09 ±2.22^{ab}	25.25 ±0.8^{b}	70.19 ±6.47^{b}
	2	100 ± 0.00^{a}	52.48±11.44^{ab}	86.80±8.83^{ab}	34.48±1.05^{b}	67.64±9.56^{b}
	4	100 ± 0.00^{a}	59.95±7.63^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Day 7						
	1	100 ± 0.00^{a}	47.74±6.37^{b}	85.09±2.22^{ab}	29.12±6.42^{b}	71.37±25.35^{b}
	2	100 ± 0.00^{a}	52.48±3.44^{ab}	89.90±8.83^{a}	44.48±1.05^{b}	77.04± 6.55^{b}
	4	100 ± 0.00^{a}	68.95±7.63^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}

Values are presented as means ± SD (n = 3). Means with the same letter in the same column are not significantly different at $p \leq 0.05$

Table 3: Percentage of mycelia growth inhibition (mm) by Black seed oil

	Oil Conc. (ml)	*R. solani*	*M. racemosus*	*F. verticillioides*	*F. flavus*	*A.flavus*
Day 3						
	1	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
	2	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
	4	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Day 5						
	1	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
	2	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
	4	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Day 7						
	1	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
	2	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
	4	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}

Values are presented as means ± SD (n = 3). Means with the same letter in the same column are not significantly different at $p \leq 0.05$

Rhizoctonia solani was reported as fungi infesting soybean seedlings in North America reducing its yield up to about 48 % yearly.[28] The infection is associated with unfavourable weather conditions such as elevated temperature which increases the susceptibility of soybean seedlings.[23]*R. solani* can withstand wide range of soil temperature and moisture,[29] therefore its adaptability in different weather condition as can be found in Nigeria. Pathogenicity of *R. solani* in soybeans were previously reported in eastern Nigeria,[30] in northern Nigeria,[31] fungi also were documented to cause web blight disease in cowpea in the southern Nigeria.[32.33] Other fungi isolated from infected leaves in this research such as *Mucor racemosus* and *Aspergillus flavus* were previously documented as fungi which contaminate maize during storage.[34] The incidence of *Fusarium verticillioides* in reducing maize crop yield has been earlier reported.[35-37]*Aspergillus flavus* has also been documented to be associated with several other crops such groundnut and maize.[38]*Flavodon flavus* have not been documented to be associated with any crop in Nigeria, although it is reported to be usually found in the marine environment[39] or inhabiting woods.[40] This work is the first report of *F. flavus* infecting legumes and maize crops in Nigeria.

Mycelium is the vegetative stage of fungi used for foraging and absorbing nutrients consisting of organic matter from substrates. It is made up of several compounds such as cutin, cellulose, lignin, tannins, etc.[41] A way of restricting the growth of pathogenic fungi is by inhibiting the growth of mycelia using environmentally friendly compounds such as plant oils. This can cause shrinkages in the mycelia structure.The three plant oils used in this research possess antifungal activities, with lemon oil and black seed oil exhibiting broad spectrum antifungal activities against all the tested fungal species. Limonene, a monoterpene is reported to be the major compound in lemon oil.[42-44] This compound provides good defense against plant pathogens as a result of their great antimicrobial benefits,[45.46] therefore its beneficial applications in inhibiting microorganisms in stored foods and prevention against crop pest attacks.[47] Thymoquinones are the major compounds in black seed oil,[48.49] which is also reported to be an effective antifungal compound.[50.51] Castor oil, in this research work, has shown minimal antifungal potential against all tested species, although, it significantly inhibited *R. solani* mycelia growth at all concentrations. Castor oil mycelia growth inhibition is concentration depedent i.e. mycelia growth inhibition was significantly higher with 4 ml treatment than lower concentration of 1ml and 2 ml for *R. solani*, *M. racemosus*, *F. verticillioides*, *F. flavus* and *A.flavus.*Therefore, castor oil as an antifungal is dose dependent.

Lemon oil was fungicidal against *F. verticillioides* and *F. flavus*, but fungistatic against *R. solani*, *M. racemosus*,and *A.flavus* although was fungicidal against *R. solani* at a higher concentration (4 ml). This makes lemon oil a promising environmentally friendly fungicide for the agricultural sector in the country.Castor and Black seed oil were also fungicidal against *R. solani*, *M. racemosus*, *F. verticillioides* and *F. flavus* at the highest concentration (4ml) oil used in this study. The treatments of pathogenic fungi with lower concentrations of the plant oils in this study only prevents mycelia growth (i.e., fungistatic). Therefore, higher concentration is required for maximum inhibition of the mycelia. The functional roles of spores in the dispersal and as a resting phase in fungi,[52] confers the strong propagation ability on pathogenic fungi. Therefore, inhibiting the dispersal of fungal spores is good way to reduce infections caused by pathogenic fungi. Sporulation was inhibited in *F. flavus* by lemon oil and black seed oil in this study, which further confirms the potential of these oils as fungicides and in reducing the spread of the pathogenic plant fungi. Further studies on antifungal potential of more plant oils need to be carried out in combating the pathogenic fungi affecting crop yield identified in this study. Increasing the concentrations of lemon and black seed oils in fungicide formulations might also increase their fungicidal effects. This work demonstrates the potency of plant oils as an economical and environmentally friendly alternatives to non-biodegradable synthetic fungicides.

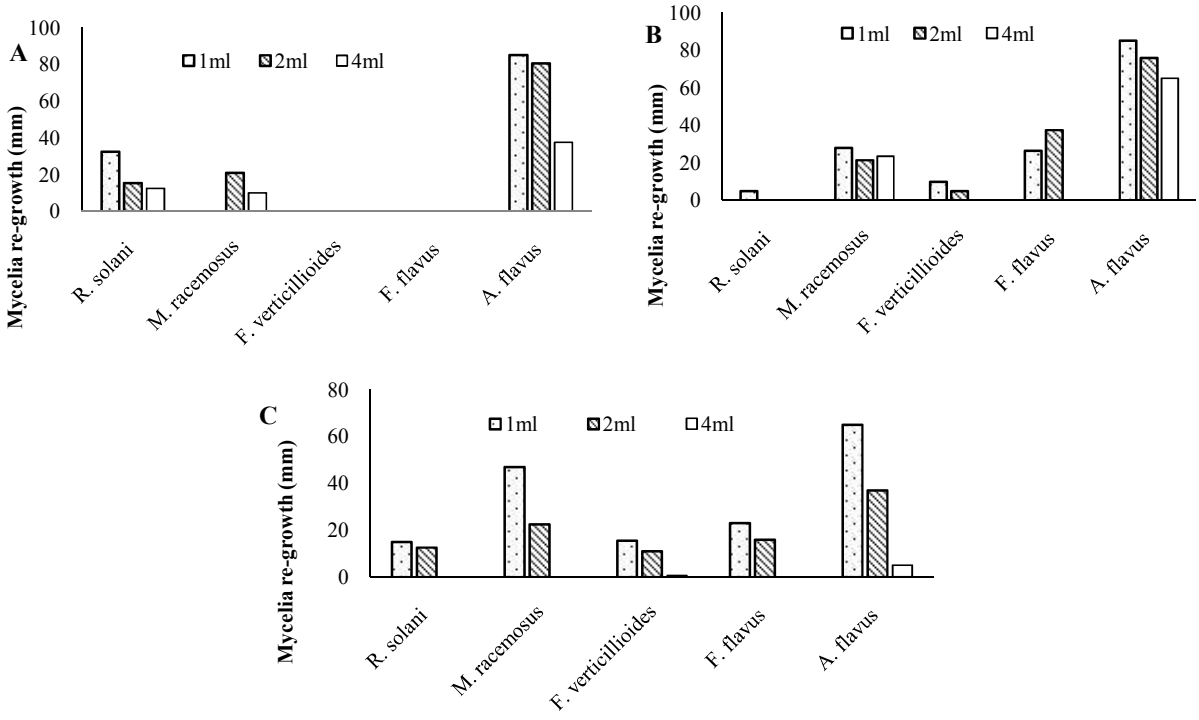

Figure 1: Caption: Mycelia Regrowth experiment after day 3
A: Minimum Fungicidal concentration of Lemon oil on pathogenic plant fungi
B: Minimum Fungicidal concentration of Castor oil on pathogenic plant fungi

C: Minimum Fungicidal concentration Black seed oil on pathogenic plant fungi

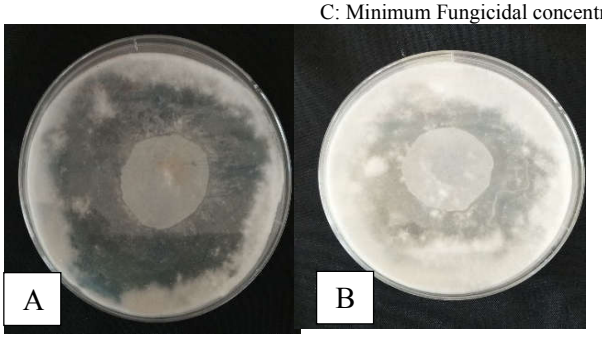

Figure 2: Caption: Disk diffusion assay for *Flavodon flavus* treated with Black seed oil (A) and Lemon oil (B), Showing clear zone (X), sporulation inhibition (Y).

Conclusion

Lemon oil and black seed oil are promising antifungal agents as a result of their broad-spectrum activities against the pathogenic fungi. Lemon oil was fungicidal against two of the pathogenic plant fungi.

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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