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Antifungal activity of *Leonotis nepetifolia* extracts against *Colletotrichum* species causing bean anthracnose and their phytochemical analysis using LC-MS

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ABSTRACT

Bean anthracnose results in significant yield losses, and the present primary management strategy of using synthetic fungicides is hampered by a number of factors. This study assessed the antifungal effects of aqueous, methanolic, and ethyl acetate extracts of *Leonotis nepetifolia (Lamiaceae)* against *Colletotrichum* species that cause bean anthracnose. The poisoned food technique was used to evaluate extracts in-vitro, and trials were carried out on farmer's plots. On the other hand, phytochemical analysis was done using LC-MS. Results obtained in-vitro show that the ethyl acetate extract was the most effective since it completely inhibited mycelial development at concentrations of 5 mg/mL and 2.5 mg/mL. On the other hand, the aqueous and methanolic extracts exhibited a comparable inhibitory effect, and at the higher concentration (5.0 mg/mL), a remarkable percentage of inhibition of 73.3% and 83.1% was recorded. According to field trials, bean anthracnose incidence was low and there were fewer infected pods per plant in plots treated with *L. nepetifolia* extracts than in the negative control. In comparison to the negative control plots, extracts-treated plots had larger weights of 100 seeds but no discernible negative effect in seed germination. LC-MS analysis indicated the presence of Linoleoyl ethanolamide, Curcumol, Deguelin, 9,10-dihydroxy-12Z-octadecenoic acid, Naringenin-7-O-glucoside, and 9S-hydroxy-10e 12z 15z-octadecatrienoic acid in the ethyl acetate extract whereas 6-Methoxyluteolin, Genistein, 5,7,3,4-tetramethoxyisoflavone, and 3,4-Dihydroxymandelic acid were identified in the aqueous extract. Antifungal activity and the presence of bioactive components revealed by extracts in this study demonstrate the potential of *L. nepetifolia* as a source of botanical fungicide for the management of bean anthracnose.

Introduction

Bean anthracnose, one of the most harmful diseases of the common bean (*Phaseolus vulgaris* L.), is caused by the fungus *Colletotrichum lindemuthianum (Sacc. and Magn) Scrib. C. lindemuthianum* exists in different forms called races that differ in virulence between regions (Padder et al., 2017). In humid and cool settings, bean anthracnose is more prevalent (Mwesigwa, 2009). Although it is also known to spread via wind and rainwater splashes that carry conidia from infected plants, this fungus is primarily transmitted through infected seeds (Yesuf, 2005; Sangchote, S., 2005).

Symptoms of bean anthracnose may include small, circular spots on leaves that may become larger and develop dark centers, dark-colored lesions on stems, brown sunken lesions on pods, and discolored or shriveled seeds. In severe cases, the disease can cause significant yield losses and reduce the quality of harvested beans. Bean anthracnose causes great losses, according to studies in various Sub-Saharan African countries, necessitating quick and coordinated control measures. According to Mohammed (2013), Tanzania might experience a yield loss of up to 80%, or US$304 million yearly. Yield loss trends of a similar nature have been noted in Kenya (Mogita et al., 2017), Uganda (Mwesigwa, 2009), and Ethiopia (Hirpa & Selvaraj, 2016; Mohammed, 2013).

The main management methods for bean anthracnose include synthetic fungicides and use of resistant varieties. But a number of factors, such as unfavorable effects on non-target species (Cristina et al., 2017), contaminating water sources (Hussain & Asi, 2008), and health

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problems for farmers and applicators (Kumar et al., 2012; Mwabulambo et al., 2018), make the use of synthetic fungicides challenging. If these fungicides are used regularly, the pathogen develops resistance to them in addition to their harmful effects (Staub, 1991). Because the pathogen exists in different races, this diversity threatens host resistance as a cultivar that is resistant to one race may become susceptible to a different race (Padder et al., 2017; Shenkalwa et al., 2013).

Leonotis nepetifolia (L.) R. Br. has historically been used to treat a variety of human diseases in different parts of the world, including bronchial asthma, malaria, influenza, epilepsy, womb prolapse, diarrhea, and rheumatism (Pushpan et al., 2012). In Tanzania, research has also been conducted on the therapeutic potential of *L. nepetifolia*. While *L. nepetifolia* was listed as one of the main medicinal plants used to treat asthma by Kingo & Maregesi, (2020), Qwarse et al., (2017) listed this plant species as helpful in treating a number of fungal skin diseases. Several studies have been conducted to evaluate the antibacterial, antioxidant, antifungal, and anti-parasitic properties of extracts and essential oils of *L. nepetifolia* in order to support the plant's well-documented medicinal values (Dhawan & Khan, 2020; Gang & Kang, 2022; Qwarse et al., 2017). The ability of *L. nepetifolia* to control agricultural diseases has also been researched, in addition to its potential for healing human diseases. For instance, according to Sylvanus, (2015), *L. nepetofilia* extracts have the potential to suppress the ascochyta blight of French beans. Due to its capacity to effectively inhibit phytopathogens, *L. nepetifolia* products have been proposed to serve as an alternative to or complement to synthetic fungicides currently employed in agriculture.

L. nepetifolia's medicinal properties are linked to the presence of a number of bioactive substances. As a result of *L. nepetifolia's* medicinal properties, a variety of classes of physiologically active compounds have been reported. Tanning, flavonoids, sterols, saponins, triterpenoids, phenols, coumarins, and glycosides are a few of classes of these compounds (Qwarse et al., 2017). However, previous studies have shown that environmental elements like soil type, light, altitude, the presence of toxic compounds, and geographic location have a significant impact on the biosynthesis and accumulation of secondary metabolites in medicinal plants. Despite its richness and potential, there is little information on the usage of *L. nepetifolia* that grows in Tanzania to control plant diseases including bean anthracnose and its bioactive content. This study therefore was undertaken to assess the antifungal activity of *Leonotis nepetifolia* extracts against *Colletotrichum* species causing bean anthracnose and their phytochemical analysis using LC-MS.

Materials and methods

Collection of plant material and crude extract preparation

The plant leaves were gathered, washed under running water, and allowed to dry for 14 days in the shade before being ground into a fine powder. In flasks, the powdered plant material was immersed in water, ethyl acetate and methanol for 48 h while being continuously shaken at 150 rpm. The mixtures were passed through sterile Whatman filter paper number 1 after being filtered through a piece of cheesecloth with two layers. The aqueous extract was concentrated using a freeze-drier, and the extracts of ethyl acetate and methanol were concentrated by rotavapor at 45 °C and 50 °C, respectively. Concentrates were stored in the fume hood for 48 h at room temperature to drain off excess solvents. To prepare the stock solutions of each extract, the known amounts of the aqueous, methanolic, and ethyl acetate extracts were dissolved in sterile distilled water and DMSO respectively.

Isolation and identification of the pathogen

Pods displaying typical symptoms of bean anthracnose were gathered from farmer's fields. Small pieces were sliced between the healthier and necrotic tissue. The sliced tissues were cleaned three times in sterile distilled water before being soaked for three minutes in a solution of 1% sodium hypochlorite. After being blotted to dry, tissues were put on PDA and incubated for seven days at room temperature. On freshly prepared PDA, the pathogen's typical growths were sub-cultured and incubated for four days at room temperature. Pathogenicity tests and a method published in Mathur & Kongsdal, (2003) were used to identify the pathogen. Pure pathogenic cultures were kept in the refrigerator at 4 °C for later use.

In-vitro testing of antifungal efficacy of extracts

A poisoned food technique was used to evaluate the efficacy of plant extracts against *Colletotrichum* species. The approach was adapted from Kritzinger et al., (2005), with minor changes, including adding the appropriate quantity of each plant extract to PDA before pouring it into Petri plates at approximately 45 °C to make 5, 2.5, 1.25, and 0.5 mg/mL of each extract. The PDA and extracts were thoroughly mixed before being allowed to harden.

An actively growing culture on the 7th day was sliced into 4 mm pieces and the sliced pieces were placed in the middle of Petri dishes with poisoned media. Snow power 45% WP was used as a positive control, whereas untreated plates were used as a negative control. The PDA plates were incubated at a temperature of between 20 and 24 °C and treatments were entirely randomized in four replications. On the eighth day following inoculation, observations on the mycelial growth were conducted, and the diameter of the fungal colony (mm) was measured, noted, and averaged. The following formula was used to compute the percentage inhibition as suggested by Tegegne et al., (2008):

$$percentage \quad inhibition = \frac{DC - DT}{DC} x100$$

The letters DT and DC stand for the mean fungal colony diameter in the treatment and the negative control, respectively.

Evaluation of efficacy of extracts on bean anthracnose under field conditions

Field trials were carried out to assess the effectiveness of plant extracts at two locations, Karagwe and Mwanga, during the 2022 and 2021 growing seasons, respectively. The choice of the experimental locations was made based on the disease infestation recorded in earlier surveys carried out during 2020 and 2021. The experiments were carried out in farmers' plots. According to an interview with the farmers who owned these plots, they used their own seeds from these plots across several seasons. Therefore, these farmers voluntarily contributed seeds from the same plots from previous growing seasons for the experiments. Six months before the onset of the rains, the plots were hand-hoed and plant residues burnt.

The trials at both sites were designed using a Randomized Complete Block Design and included eight treatments that were duplicated three times. One positive control, one negative control, three concentrations of ethyl acetate extracts (1.25 mg/mL, 2.5 mg/mL, and 5.0 mg/mL), and three concentrations of aqueous extracts (2.5 mg/mL, 5.0 mg/mL, and 10.0 mg/mL) were used as treatments. Before being sown in plots at a rate of 40 seeds per plot, 5 centimeters deep, at a distance of 20 centimeters between plants, and 45 centimeters between rows, the seeds were first soaked in distilled water for 30 min and then in the appropriate treatment for six hours.

Assessment of the disease incidence and yield components

The parameters assessed included seed germination, disease incidence, the number of diseased pods, and the weight of 100 seeds. The percentage of seeds that germinated was determined fourteen (14) days after sowing and was calculated as the proportion of seeds that sprouted per total number of seeds sown in a plot. Brown sunken lesions on pods

were used to evaluate the disease because leaf symptoms were not very obvious. The disease incidence was assessed while the pods were still green but had fully formed seeds. The incidence was thus determined as the proportion of infected plants in each plot using the formula below:

$$DI = \frac{\sum n}{\sum N} \quad x100$$

whereby: DI = disease incidence, $\sum n$ = sum of all infected plants in a plot, $\sum N$ = total number of plants in a plot.

When the plants were ready for harvest, 10 plants were chosen at random and pods were closely checked for the typical anthracnose symptoms. In a similar way, the number of infected pods per plant was counted, averaged and recorded in each plot. Plants were taken from each plot after all other factors had been considered and properly dried for the weight of 100 seeds. Then, 100 seeds were randomly chosen from a total of 10 plants, and the weight of the seeds was measured in grams using an analytical balance.

Phytochemical analysis using LC-MS

10 mL of the appropriate solvents (ethyl acetate and water) were added to a centrifuge tube along with one gram of *L. neperifolia* leaf powder. The tube was sonicated for 30 min after being warmed on a water bath for five minutes at 100 °C. The resulting extracts were concentrated under nitrogen to dryness and purified. 1 mL of a 1:1 acetonitrile-water solution was added to each extract before being centrifuged at 13000 rpm for 10 min.

The phytochemical content of the sample was examined using liquid chromatography (LC) (Ultimate 3000) coupled with tandem mass spectrometer (Q Exactive Orbitrap Mass Spectrometer). This is due to the fact that LC-MS/MS has a number of advantages, including the capacity to identify and quantify a wider variety of compounds and the ability to perform extraction methods that are speedier and less time-consuming (Perez et al., 2016). A C18 column (Hypersil GOLD aQ, Thermo Scientific) with 1.9-micron-sized particles was filled with 5 μL of each of the produced extracts. The mobile phase consisted of solution A, which had 0.1% formic acid in water, solution B, which contained 0.1% acetonitrile, and solution C, which contained 1:1 methanol to mil Q water. A 100 × 2.1 mm column was used in the analysis. The oven was set to 40 °C for 30 min to run each sample. Ionization source was electron spray ionization with a collision energy of 45 v.,The mass scan range was 150–2000 *m/z*, and the resolution was 140000. Compounds in the extracts were identified by comparing their mass spectra to those in the NIST library.

Data analysis

One-Way ANOVA was used to compare the means of percentage inhibition of pathogen mycelial growth, seed germination, disease incidence, number of infected pods per plant and weight of 100 seeds. Tukey's HSD test was used to separate between significantly different means. These tests were performed using jamovi statistical software version 2.2.5. The detected compounds from LC-MS data were compared with reference spectra from the NIST libraries for identification of compounds.

Results

Effect of L. nepetifolia extracts on mycelial growth

The study evaluated the antifungal activity of *L. nepetifolia* extracts as a potential source of antifungal agents against *Colletotrichum* species that cause bean anthracnose. The results demonstrate that the extracts have a remarkable inhibitory effect against the fungus though at varying degrees. The ethyl acetate extract was the most effective even than the positive control and methanolic and aqueous counterparts as it inhibited the mycelial growth by 100% at higher concentrations i.e 5.0 and 2.5 mg/mL (Fig. 1(a).

The aqueous extract had only significant inhibition at the highest doses, 2.5 and 5.0 mg/mL where the extract inhibited the mycelial growth of the fungus by 73.3% and 58.4% respectively. At the lower concentrations, the extract was less effective as it reduced mycelial growth by 23.6% and 44.6% at 0.5 mg/mL and 1.25 mg/mL respectively (Fig. 1(b). The study further revealed that the methanolic extract was not very effective compared with its ethyl acetate counterpart. The highest percentage inhibition of mycelial growth was at 5.0 mg/mL and 2.5 mg/mL, whereas at the lowest concentration, the extract was less effective as it only reduced mycelial growth by only 12.4% (Fig. 1(c).

Efficacy of L. nepetifolia extracts under field conditions

Field experiments demonstrated that the ethyl acetate and aqueous extracts had no significant negative effect on germination of bean seeds as compared with the negative control. However, the fungicide-treated seeds had lower germination percentage relative to the negative control and the extracts-treated seeds. The study also revealed slight variations in percentage germination across experimental sites (Fig. 2).

The highest incidence of bean anthracnose was recorded in the water-treated plots (negative control) compared with the extracts-treated and fungicide-treated plots. The lowest disease incidence was recorded in the plots whose seeds were treated with the aqueous extract at the highest dose of 10 mg/mL and the fungicide-treated plots at the recommended dose at both the Karagwe and Mwanga sites. The study also noted variation in disease incidence between sites, though such variation had no consistent trend (Fig. 3).

The plots treated with aqueous extract at the highest concentration (10 mg/mL) recorded the least percentage of infected pods per plant at both sites followed by the same extract at 5 mg/mL. Negative control on the other hand recorded the highest percentage of infected pods at both sites and slight variations were observed among treatments across the sites (Fig. 4).

All plots treated with plant extracts and the synthetic fungicide had a relatively higher weight of 100 seeds compared with those treated with water (negative control), except for ethyl acetate extract at 1.25 mg/mL. The plots treated with aqueous and ethyl acetate extracts at the highest doses had comparable weights of 100 seeds as those treated with synthetic fungicide (Fig. 5).

Phytochemical constituents of L. nepetifolia

The *L. nepetifolia* aqueous and ethyl acetate extracts contained a number of bioactive compounds that were identified using LC-MS. Four specific compounds, namely 6-Methoxyluteolin, Genistein, 5,7,3,4-tetramethoxyisoflavone, and 3,4-Dihydroxymandelic acid, were found in the aqueous extract. Contrarily, the ethyl acetate extract included the compounds 9S-hydroxy-10e 12z 15z-octadecatrienoic acid, Linoleoyl ethanolamide, 9,10-dihydroxy-12Z-octadecenoic acid, Curcumol, Deguelin, Naringenin-7-O-glucoside (Fig. 6 and 7).

Discussion

The aqueous and ethyl acetate extracts of *L. nepetifolia* showed an in-vitro inhibitory action in this investigation against the *Colletotrichum* species that cause bean anthracnose. This finding is consistent with other research, such as that by Kowalczyk et al., (2021), Pingale et al., (2013), Qwarse et al., (2017), and Shrivastava & Dwivedi, (2021), which suggested that *L. nepetifolia* may be a source of antimicrobial compounds that are effective against bacterial and fungal infections. Field tests that revealed the extracts dramatically reduced disease incidence and the number of infected pods per plant corroborated the in-vitro results. These findings further highlight *L. nepetifolia* extracts'

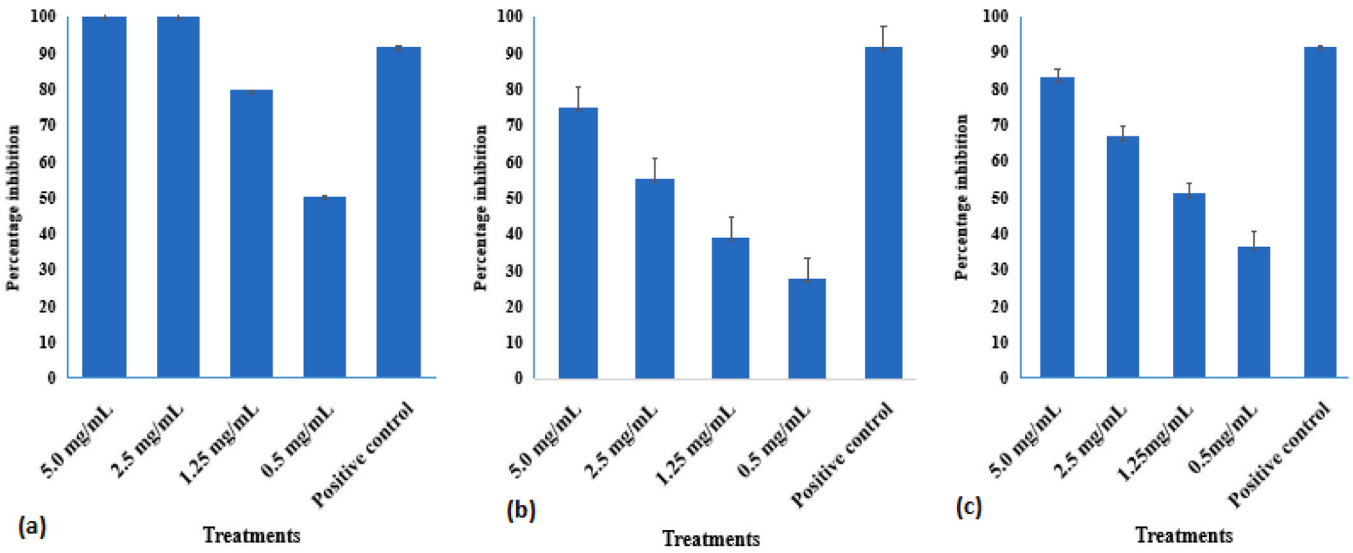

Fig. 1. The effect of L. nepetifolia on mycelial growth of Colletotrichum species (a) ethyl acetate extract (b) aqueous extract (c) methanolic extract.

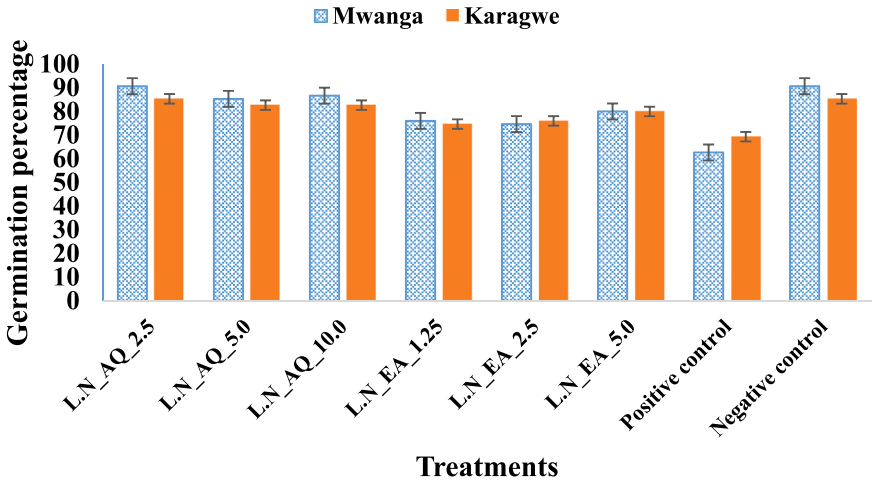

Fig. 2. Mean germination percentage of seeds treated with L. nepetifolia extracts. Extract treatments are in mg/mL. (LN_AQ=L. nepetifolia_aqueous extract, LN_EA = L. nepetifolia_ethyl acetate extract).

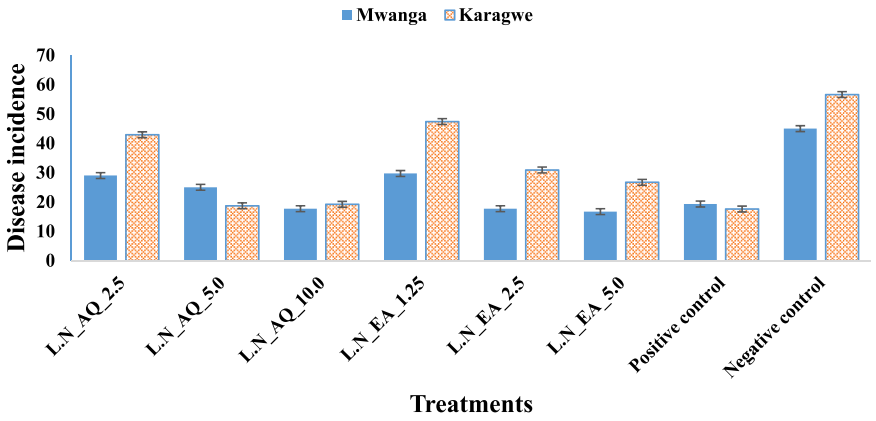

Fig. 3. Effect of aqueous and ethyl acetate extracts of L. nepetifolia on incidence of bean anthracnose. Extract treatments are in mg/mL. (LN_AQ=L. nepetifolia_aqueous extract, LN_EA = L. nepetifolia_ethyl acetate extract).

potential for managing bean anthracnose. All the plots treated with extracts had higher weights of 100 seeds, and the extracts had no adverse effects on seed germination. Again, these findings imply that these extracts can increase yield by increasing the weight of seeds without affecting germination in addition to reducing yield losses caused by bean anthracnose.

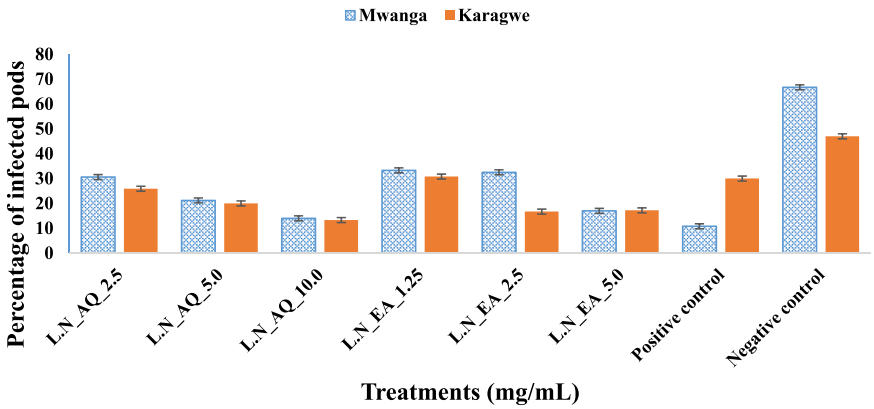

Fig. 4. Effect of aqueous and ethyl acetate extracts on the percentage of infected pods per plant. Extract treatments are in mg/mL. (LN_AQ=L. nepetifolia_aqueous extract, LN_EA = L. nepetifolia_ethyl acetate extract).

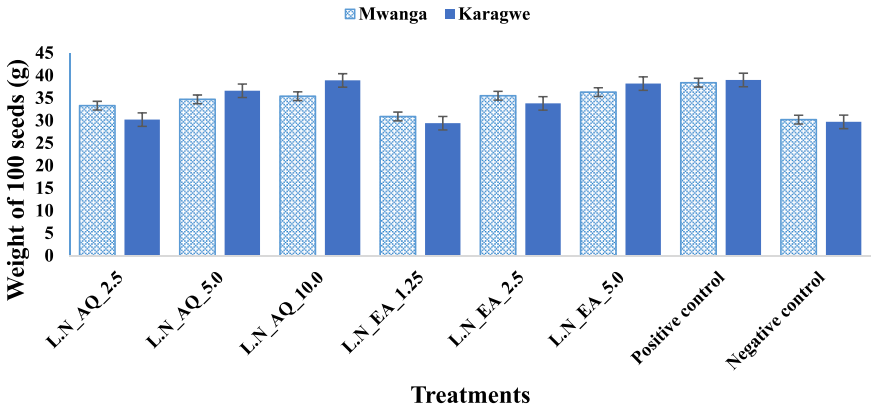

Fig. 5. Effect of aqueous and ethyl acetate extracts on weight of 100 seeds. Extract treatments are in mg/mL. (LN_AQ=L. nepetifolia_aqueous extract, LN_EA = L. nepetifolia_ethyl acetate extract).

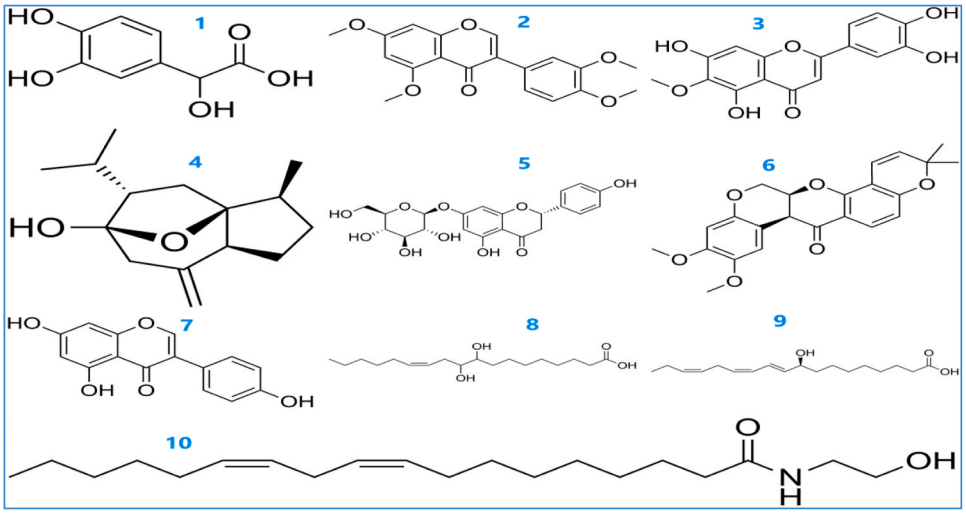

Fig. 6. Structures of compounds identified in the aqueous and ethyl acetate extracts of L. nepetifolia using LC-MS (1 =3,4-Dihydroxymandelic acid, 2 = 5,7,3,4 tetramethoxyisoflavone, 3 = 6-Methoxyluteolin, 4 = Curcumol, 5 = Naringenin-7-O-glucoside, 6 = Deguelin, 7 = Genistein, 8 = 9,10-dihydroxy-12Z-octadecenoic acid, 9 = 9S-hydroxy-10e 12z 15z-octadecatrienoic acid, 10 = Linoleoyl ethanolamide).

In comparison to its aqueous and methanolic counterparts, the study found that *L. nepetifolia's* ethyl acetate extract was the most effective at suppressing the mycelial development of *Colletotrichum* species that cause bean anthracnose. This finding suggests that ethyl acetate is more effective than water and methanol for the extraction of bioactive components from this plant species. Our results are consistent with those of Pintac et al., (2018), who suggested ethyl acetate as a solvent of choice for the extraction of bioactive compounds. Although aqueous and methanolic extracts did not show a higher inhibitory effect compared with ethyl acetate, they had a remarkable effect. According to Yusoff

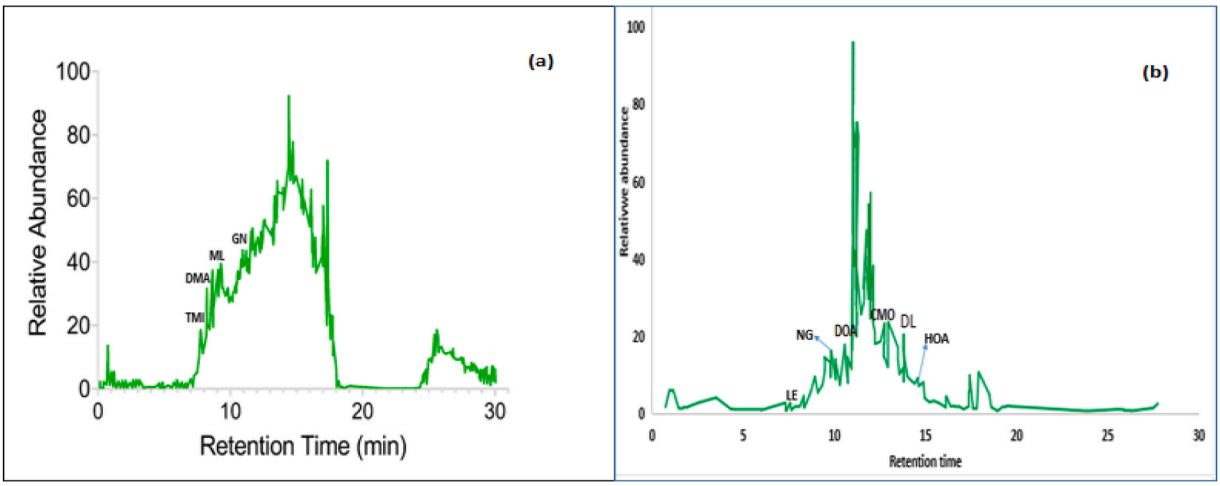

Fig. 7. Chromatogram showing compounds identified in the aqueous (a) and ethyl acetate (b) extracts of L. nepetifolia: mL= 6-Methoxyluteolin, GN=Genistein, TMI= 5, 7, 3, 4-Tetramethoxyisoflavone, DMA= 3, 4-Dihydroxymandelic acid, LE= Linoleoyl ethanolamide, NG = Naringenin-7-O-glucoside, DOA= 9,10-dihydroxy-12Z-octadecenoic acid, CMO = Curcumol, DL = Deguelin, HOA= 9S-hydroxy-10e 12z 15z-octadecatrienoic acid.

et al., (2020), variations in antifungal activity depend on the kind of plant and solvent used, and there is no obvious pattern with solvent polarity. This finding also explains the antifungal activity of extracts from various extraction solvents with varied polarity as observed in this study.

In-vitro results showed that degree of mycelial inhibition increased with the increasing extract concentration for each of the tested extracts. The lowest percentage of inhibition was seen at lower concentrations, whereas the highest percentage of inhibition was shown at higher concentrations. The aqueous extract exhibited comparatively low percentage inhibition, but given the trend and cost, it can still produce the best outcomes at higher concentrations. This prediction was confirmed by a field experiment, which showed that the least disease incidence and number of diseased pods per plant were observed in plots treated with the same extract at 10 mg/mL.

The ethyl acetate and aqueous extracts of *L. nepetifolia* included a number of compounds that were identified by our study. In the ethyl acetate extract, Linoleoyl ethanolamide, Curcumol, Deguelin, 9,10-dihydroxy-12Z-octadecenoic acid, Naringenin-7-O-glucoside, and 9S-hydroxy-10e 12z 15z-octadecatrienoic acid were identified whereas 6-Methoxyluteolin, Genistein, 5,7,3,4-tetramethoxyisoflavone, and 3,4-Dihydroxymandelic acid were found in the aqueous extract. The two extraction solvents, which have different polarities led to the identification of different compounds from the same plant species under the specified conditions. This finding shows that a variety of extraction solvents should be utilized to recover the greatest number of compounds present in a plant species. Ngo et al., (2017) observed similar results after extracting bioactive substances from *Salacia chinensis*.

The majority of the common compounds that *L. nepetifolia* has previously been found to contain and that have been linked to its therapeutic usefulness were not found in this study. For example, Kowalczyk et al., (2021) linked the antifungal activity of *L. nepetifolia* to the presence of rosmarinic acid and coumaric acids, whereas de de Oliveira et al., (2021) reported apigenin, luteolin, cirsiliol apigenin-7-O-glucoside and other compounds as being responsible for the antifungal activity of *L. nepetifolia*. This work found naringenin-7-O-glucoside, a naringenin derivative that has been found in *L. nepetifolia*, and 6-methoxyluteolin-4′-methyl ether, a derivative of 6-Methoxyluteolin found in *Leonotis leonurus* (El-Ansari et al., 2009). The compounds identified in this study, such as Genistein (Harikumar, 2016), 5, 7, 3′, 4′-tetramethoxyisoflavone (Samir et al., 2018), 3, 4-Dihydroxymandelic acid (Shunmugapriya et al., 2017), linoleoyl ethanolamide (Sayre-Chavez et al., 2022), Curcumol (Wei et al., 2019), deguelin (Zhang et al., 2020), 9,10-dihydroxy-12Z-octadecenoic acid, and 9S-hydroxy-10e 12z 15z-octadecatrienoic acid (Cui et al., 2022) have been documented in other plant species and are associated with various bioactivities. Therefore, the observed antifungal activities of its extracts, both in-vitro and under field conditions, as well as their earlier claimed medicinal properties, can be accounted for by the presence of these bioactive substances in *L. nepetifolia* leaf extracts.

Conclusion

Our study demonstrates antifungal activities of ethyl acetate, methanolic and aqueous extracts of *L. nepetifolia* against *Colletotrichum* species associated with bean anthracnose in-vitro. The study further shows that treating bean seeds with ethyl acetate and aqueous extracts of *L. nepetifolia* could reduce the incidence of bean anthracnose, number of infected pods, and hence improving yield. The antifungal activities of ethyl acetate and aqueous extracts could be associated with the presence of Linoleoyl ethanolamide, Curcumol, Deguelin, 9,10-dihydroxy-12Z-octadecenoic acid, Naringenin-7-O-glucoside, 9S-hydroxy-10e 12z 15z-octadecatrienoic acid, 6-Methoxyluteolin, Genistein, 5,7,3,4-tetramethoxyisoflavone, or 3,4-Dihydroxymandelic. These results confirm the antifungal properties of this important plant species and demonstrate the potential of *L. nepetifolia* as the source of antifungal agents for management of bean anthracnose.

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No funding was provided from any source.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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