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ANTIFUNGAL ACTIVITIES OF SOME MEDICINAL PLANTS AGAINST *Colletotrichum lindemuthianum,* THE CAUSAL PATHOGEN OF BEAN ANTHRACNOSE, AND THEIR EFFECT ON SEED GERMINATION AND SEEDLING PERFORMANCE

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ABSTRACT

This study evaluated the *in vitro* potency of *Plectranthus barbatus, Vernonia amygdalina, Conyza bonariensis, Leonotis nepetifolia,* and *Lantana camara* extracts against *Colletotrichum lindemuthianum,* the causal pathogen of anthracnose in common bean (*Phaseolus vulgaris*), as well as assessed their effect on seed germination and seedling performance under greenhouse conditions. *In vitro* assessment of antifungal activities of extracts was carried out using the poisoned food technique. Ethyl acetate extract of *C. bonariensis* was found most effective against the pathogen and completely inhibited its growth at 5.0, 2.5, and 1.25 mg mL^{-1} while it showed 91.2% inhibition at 0.5 mg mL^{-1} level. This was followed by *P. barbatus* and *L. nepetifolia* which completely inhibited the growth at 5.0 and 2.5 mg mL^{-1} level. Methanolic extracts were also effective with highest inhibition observed for *L. camara* (85.1%) at 5.0 mg mL^{-1}, followed by *P. barbatus* (84.7%) and *L. nepetifolia* (83.1%) at the same concentration. Similarly, the aqueous extracts showed remarkable inhibition at the highest concentrations tested. Aqueous extracts of *L. nepetifolia, V. amygdalina,* and *C. bonariensis* inflicted maximum inhibition at 5.0 mg mL^{-1} (75.0, 74.7, and 73.3%, respectively). Extracts had no adverse effect on seed germination and seedling performance, but the test fungicide reduced seed germination significantly ($p < 0.001$). Easy accessibility of the studied medicinal plants and their potential in managing bean anthracnose provides an opportunity to use such plant extracts as seed dressers to manage bean anthracnose in smallholder farmers in Tanzania.

Keywords: Antifungal activity, bean anthracnose, bioactivity, herbal fungicides, growth inhibition, medicinal plants

INTRODUCTION

Colletotrichum lindemuthianum (Sacc. & Magn.) Scrib. causes bean anthracnose, which is one of the most damaging diseases of common bean (*Phaseolus vulgaris* L.) globally. Bean anthracnose is highly prevalent in cool and humid climates (Mwesigwa, 2009) and is principally a seed-borne disease (Yesuf and Sangchote, 2007), though the disease spread by conidia from infected plants through rainwater and wind has also been reported (LeClair *et al*., 2015). Besides common bean, *C. lindemuthianum* has been reported in other crops including lima bean, soybean, mung bean, pea, cowpea, mungo bean, runner bean, faba bean (Yesuf and Sangchote, 2005) and hyacinth bean

(Rajesha *et al.*, 2010). The studies in some countries in the Sub-Sahara African region show that bean anthracnose causes considerable losses that warrant immediate and joint management efforts. Mohammed (2013a) reported the yield loss upto 80% in common bean due to anthracnose with estimated loss of USD $ 304 million in Tanzania alone. The comparable yield losses and disease incidence are also reported from Kenya (Mogita *et al.*, 2013), and Ethiopia (Amin, 2014; Hirpa and Selvaraj, 2016; Mohammed, 2013b).

The main management options for bean anthracnose management include the use of synthetic fungicides and resistant varieties (Mohammed, 2013b). The use of synthetic fungicides is, however, challenged by several factors, including adverse effects to non-target organisms (Cristina *et al.*, 2017), pollution of water sources and are associated with health problems to farmers and applicators (Mwabulambo *et al.*, 2018). Host resistance is also an emerging problem due to the pathogen variability as the pathogen exists in several races. In this case, a cultivar resistant to a particular race may suffer susceptibility to another race (Shenkalwa *et al.*, 2013).

Various plants possess antimicrobial properties against several pathogenic fungi. Masangwa *et al*. (2013) and Mogita *et al*. (2013) reported the release several compounds by various plants that inhibit the growth of *Colletotrichum* species. However, some plants release compounds that show allelopathic effect on other plant species (Laizer *et al.*, 2021). It is, therefore, prerequisite to assess these plant products for such effects before their recommendation to the farmers. *Plectranthus barbatus* (Cordeiro *et al.*, 2022), *Vernonia amygdalina* (Yusoff *et al*., 2020), *Conyza bonariensis* (Lundgren *et al*., 2021), *Leonotis nepetifolia* (Kowalczyk *et al.*, 2021; Shrivastava and Dwivedi, 2021), and *Lantana camara* (Ndala *et al.*, 2019; Mansoori *et al.*, 2020) are known to possess antifungal properties. This study was aimed to evaluate five medicinal plants extracts for their antifungal activity against *Colletotrichum lindemuthianum*, causal pathogen of bean anthracnose. The best performing extracts were evaluated for their effects on seed germination and seedling performance in common bean.

MATERIALS AND METHODS

Plant materials

The medicinal plants were chosen on the basis of their previously reported medicinal values. These included *Plectranthus barbatus*, *Vernonia amygdalina*, *Conyza bonariensis*, *Leonotis nepetifolia*, and *Lantana camara*. All the plants were collected from their natural habitats from the fields at Tengeru Livestock Training Agency, Arusha, Tanzania. Plants were identified by a botanist at the National Herbarium located at the Tropical Pesticides Research Institute, Arusha, Tanzania.

Crude extracts preparation

The collected plant leaves were washed using running tap water, dried under shade for 14 days and finely powdered in a grinder. Known amounts of powdered plant leaves were separately soaked in known volumes of solvents (methanol or ethyl acetate, or sterile distilled water) and shaken on a shaker for 48 h at 150 rpm. The mixtures were then filtered through a two-fold layer cheese cloth, and the filtrates were passed through sterile Whatman filter paper No. 1. The methanolic and ethyl acetate plant filtrates were concentrated in a round bottom flask using rotavapor at 50 and 45^{o}C, respectively. The extracts were left for 48 h in the fume hood at room temperature to remove the excessive solvents. The aqueous filtrate, on the other hand, was concentrated using a freeze-drier. The stocks of each extract were prepared by dissolving the known weight of methanolic and ethyl acetate extracts in dimethyl sulfoxide (DMSO) and sterile distilled water for the aqueous extracts.

Collection of diseased tissues and islation of pathogen

Anthracnose infected common bean pods were collected from diverse bean-growing locations in Tanzania. Small pieces of infected tissues were cut around the sunken lesions (between healthy and

necrotic portions). Surface-sterilization was achieved by immersing the tissues in 1% sodium hypochlorite (NaOCl) solution for 3 min and then rinsing them 3 times in sterile distilled water. After disinfection, tissues were blotted to dry, then placed on potato dextrose agar (PDA) and incubated at room temperature for a maximum of 7 days. The pathogen's typical growths were sub-cultured on freshly prepared PDA and incubated for 4 days at room temperature. The identification of pathogen was done bypathogenicity tests and a method established by (Mathur and Kongsdal, 2003). The pure cultures were stored in a refrigerator at 4^{o}C for further use.

In vitro antifungal testing of plant extracts

The effectiveness of plant extracts against *Colletotrichum lindemuthianum*, causal pathogen of anthracnose in common bean, was evaluated using the poisoned food technique (Kritzinger *et al*., 2005) with slight modifications, wherein the PDA was amended with an appropriate amount of plant extract before pouring in Petri plates when the medium was about 45^{o}C. Each extract of variable concentration (5.0, 2.5, 1.25, and 0.5 mg mL^{-1}) was appropriately mixed with PDA. The poisoned medium was well mixed before being placed into Petri plates and left in the laminar flow cabinet to solidify. After the plates were satisfactorily dry, about 4 mm portion of the prepared 7-days actively growing fungal culture was cut from the edge and placed at the center of Petri dishes with treated medium and incubated at 20-24^{o}C. The standard fungicide, Snow power 45% WP (cymoxanil 4% + mancozeb 12% + copper oxychloride 29%) at the manufacturer's recommended rate of 400 g in 20 L, was used as a positive control, whereas untreated plates (plates without extracts) were used as a negative control. The study was conducted in triplicate, with the treatments organized in a completely randomized design. Observations were made for fungal colony's diameter (mm) at 2, 4, 6, and 8 days intervals after inoculation (DAI). The percent inhibition was calculated using the formula proposed by Tegegne *et al*. (2008) by comparing the recorded size of colonies for treatments with the negative control.

$$\text{Percentage inhibition} = \frac{\text{Mean diameter of negative control} - \text{Mean diameter of treatment}}{\text{Mean diameter of negative control}} \text{ x } 100$$

Effects of plant extracts and fungicide on seed germination

The common bean (*Phaseolus vulgaris*) seeds of a susceptible variety, "Uyole njano" were acquired from a local market in Tengeru, Arusha. The seeds viability was tested by plating on a moist paper towel in triplicates after being washed using tap water, sterilized in 5% NaOCl and rinsed in sterile distilled water. The seeds were incubated for 7 days at room temperature with the towel moistened daily (Laizer *et al*., 2021) and germination was recorded. From the stock solutions, 5 mg mL^{-1} of the best performing extracts were prepared by dilution. The solution of standard fungicide was prepared as per the manufacturer's recommendations (400 g in 20 L water). The pot experiment was performed to test the effect of selected plant extracts and a fungicide (Snow power 45% WP) on seed germination. The seeds were washed using tap water, sterilized by using 5% NaOCl before soaking in distilled water for 30 min. The soaked seeds were transferred to the respective prepared extracts and standard fungicide for 6 h at room temperature. The extracts/ fungicide-treated seeds (20 seeds pot^{-1}) were sown 3 cm deep in pots having a surface area of 2550.97 cm^{2}, filled with a mixture of peat soil and sand. The experiment was conducted in a randomized complete block design with each treatment replicated 4 times. The pots were watered at the two-day interval, and germination recorded daily for 14 days after sowing (DAS). After the 14th day, the germination percentage was determined and calculated as follow:

$$\text{Percentage germination} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds planted}} \text{ x } 100$$

To assess the effect of extracts and fungicide on seedling performance, 14 days old seedlings of common beans were used. Common bean seeds were sterilized using 5% NaOCl, rinsed in distilled water and then treated by soaking in the respective extracts (5 mg mL^{-1}) and the standard fungicide (400 g in 20 L). The treated seeds were then sown in pots (2550.97 cm^{2}) filled with a

mixture of sand and peat soil at the rate of 20 seeds per pot. The pots were arranged in a randomized complete block design with 4 replications. Watering was done after every two days till the 14th day where seedlings were harvested and the data collected. Seedling fresh weight, dry weight, root and shoot lengths were used to evaluate the seedling performance. Ten seedlings were randomly selected from each pot, uprooted, and cleaned of the contaminants. Fresh and dry weight (oven drying at 105°C for 24 h) of seedlings were weighed. Root length was determined by measuring the height of root from the point of soil attachment to the tip of longest root; whereas shoot length was determined by measuring the plant from the longest tip of leaf to the soil level.

Data analysis

A two-way analysis of variance was used to assess the effects of plant extracts and their concentrations on the suppression of pathogenic mycelial growth. The means of seed germination, fresh weight, dry weight, root and shoot lengths, on the other hand, were compared using One-Way analysis of variance (ANOVA). Tukey's HSD test was used to separate between significantly different means. These tests were performed using jamovi statistical software version 1.8.4.

RESULTS AND DISCUSSION

In vitro evaluation of the effect of crude extracts on growth of mycelia

The effects of plant extracts, prepared in various solvents, and their concentrations on the radial mycelial growth of *C. lindemuthianum* were studied using a two-way ANOVA test. The effects of ethyl acetate-based extracts and concentration showed significant interaction ($p < 0.001$). The concentration of plant extracts showed significant effect on the growth inhibition of pathogen. At higher doses (5.0, 2.5 and 1.25 mg mL^{-1}), ethyl acetate-based *C. bonariensis* extract completely inhibited mycelial growth, followed by *L. nepetifolia* and *P. barbatus* extratcs, while *V. amygdalina* extract exhibited least inhibitory effect at all the doses tested (Fig. 1).

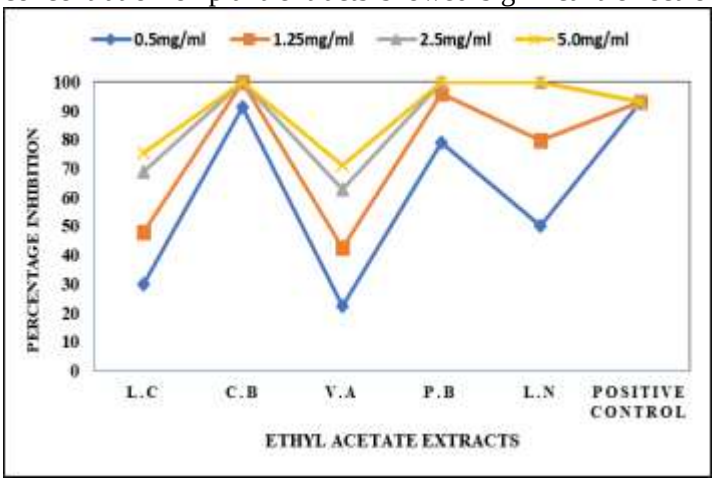

Fig. 1: Effects of ethyl acetate extracts and its concentration on the inhibition of mycelial growth *Colletotrichum lindemuthianum*. L.C = *Lantana camara*, C.B = *Conyza bonariensis*, V.A = *Vernonia amygdalina*, P.B = *Plectranthus barbatus*, and L.N = *Leonotis nepetifolia*)

For methanolic extracts, significant interaction was observed between plant extracts and their concentrations ($p<0.001$). The simple main effect analysis demons trated that both the extract and concentration had statistically significant effect on the mycelial growth inhibition of pathogen ($p<0.001$). *P. barbatus* and *L. camara* showed relatively higher inhibition at lowest and highest concentrations (47.8 and 39.8% and 85.1 and 85.7%, respectively), whereas *C. bonariensis* and *V. amygdalina* had lowest inhibition at 5 mg mL^{-1} i.e. 80 and 79.1%, respectively (Fig. 2). Similarly, the aqueous plant extracts and their concentrations showed significant interaction, and both extracts and concentrations were significant. All the extracts depicted less inhibition at 0.5 mg mL^{-1} level, but *L. nepetifolia, V. amygdalina,* and *C. bonariensis* showed relatively higher inhibition at 5.0 mg mL^{-1} level (Fig. 3).

Generally, ethyl acetate-based extracts showed highest inhibition of *C. lindemuthianum* for all

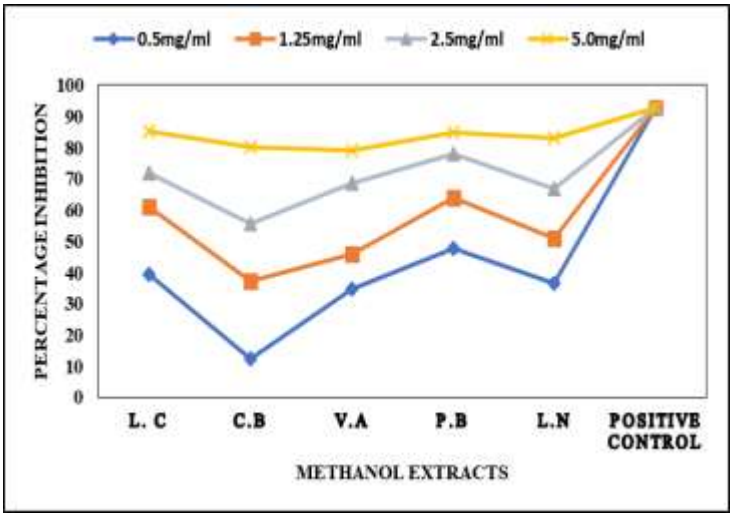

Fig. 3: Effects of methanolic extracts of medicinal plants and their concentration on mycelial growth inhibition of *C. lindemuthianum.* L.C = *Lantana camara*, C.B = *Conyza bonariensis*, V.A = *Vernonia amygdalina*, P.B = *Plectranthus barbatus*, and L.N = *Leonotis nepetifolia*)

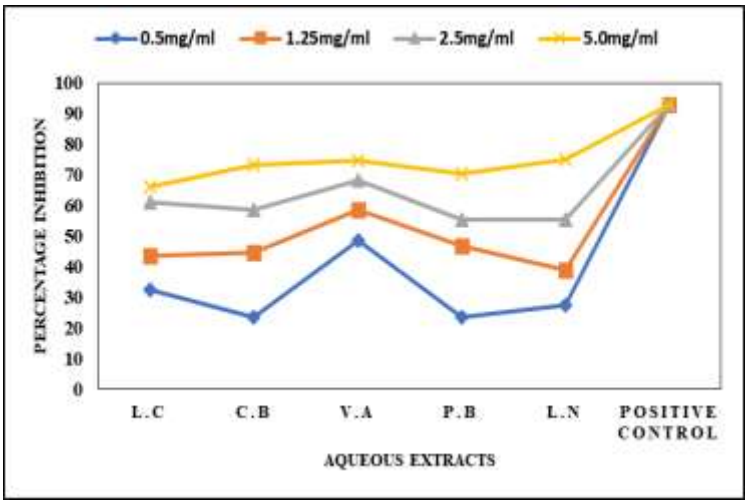

Fig. 2: Effects of aqueous extracts and concentration on mycelial growth inhibition of *C. lindemuthianum.* L.C = *Lantana camara*, C.B = *Conyza bonariensis*, V.A = *Vernonia amygdalina*, P.B = *Plectranthus barbatus*, L.N = *Leonotis nepetifolia*)

the medicinal plants studied as compared to the methanolic- and aquous-based extracts. Ethyl acetate based extracts of *C. bonariensis*, *P. barbatus* and *L. nepetifolia*, for instance, showed highest inhibition of mycelial growth even higher than the standard fungicide at the same concentrations. The results showed that ethyl acetate is an ideal extraction solvent for bio-active compounds from the studied plant species. Similar observations have been reported by Javaid and Rehman (2011) and Ishnava *et al.* (2012) who found that ethyl acetate-based extracts have maximum activity against *M. phaseolina*, *Alternaria* sp., *A. parasi*, *A. nidulans*, *T. harzianum* and *A. flavus* as compared to the methanol, water, or chloroform. Although aqueous extracts did not show strong mycelial growth inhibition at tested concentrations as compared to the ethyl acetate and methanolic counterparts, *V. amygdalina*, *L. nepetifolia*, and *P. barbatus* depicted the potential to manage bean anthracnose if used at higher concentrations. In present study, increase in the concentration of extracts resulted in the increased mycelial growth inhibition. Since water is readily available to the farmers, its use in the extraction of bioactive compounds from medicinal plants is affordable. The observed variation in mycelial growth inhibition of *C. lindemuthianum* among the studied plant extracts maybe attributed to the types and concentration of bioactive compound present in respective plant species. Yusoff *et al*. (2020) havesuggested that variation in antifungal activity depends on the solvent used and the plant species involved; and no specific trend related to solvent polarity.

The antifungal activities of the studied plant extracts indicated that these plant species release bioactive compounds against *C. lindemuthianum*. The antifungal activity of *P. barbatus* observed is in agreement with Runyoro *et al*. (2006), Musila *et al*. (2017) and Cordeiro *et al*. (2022) and the effect has been attributed to the presence of several bioactive compounds including cinnamic derivatives, flavonoids, triterpenes, alkaloids, allagic and rosmarinic acids (Cordeiro *et*

al., 2022). The identified compounds in *V. amygdalina* such as squalene, phytol, triacontane, neo-phytadiene, and heptacosane among others have also been linked to the antifungal activity of extracts from this plant species which act by disruption of the cell membrane, inhibition of cell wall synthesis, cell division and protein synthesis (Yusoff *et al.*, 2020). Several studies have documented the antifungal properties of *C. bonariensis* (Shah *et al*., 2013; Antony and Chacha, 2019). Lundgren *et al*. (2021) reported the antifungal activity of *C. bonariensis* essential oils against *C. musae*, a causative agent of anthracnose of banana, and they linked such activity to sesquicineole, thymol, limonene, and sesquisabinene compounds that reduced the disease development by damaging the cytoplasmic membrane of the pathogen and interfering the enzymatic activity of conidia. Similar observations on the antifungal activity of *L. nepetifolia* have been reported (Pingale *et al*., 2013; Qwarse *et al*., 2017; Kowalczyk *et al*., 2021; Shrivastava and Dwivedi, 2021). The presence of bioactive compounds previously identified, such as rosmarinic acid, coumaric acids (Kowalczyk *et al*., 2021), apigenin, luteolin, cirsiliol apigenin-7-O-glucoside (de Oliveira *et al*., 2021) among others, may account for the observed antifungal activity of *L. nepetifolia* in this study. The *L. camara* extracts did not show strong antifungal activity against *C. lindemuthianum* in *in vitro* assays, its antifungal activity cannot be underestimated (Sreeramulu *et al*., 2017; Kulkarni *et al*., 2019; Ndala *et al*., 2019; Mansoori *et al*., 2020).

Effect of selected crude extracts on seed germination

The tested extracts did not negatively affect seed germination as compared to the untreated seeds (negative control). The seed germination was higher in plant extract treatments than in water treatment (negative control). The seeds treated with aqueous extracts had highest germination while the fungicide-treated seeds had least germination (Fig. 4). Tukey's HSD test revealed a statistically significant difference between the mean percentage germination of the standard fungicide and plant extract treatments ($p<0.001$). Similar findings regarding the potential of plant extracts as seed dressers to enhance seed germination have been documented (Findura *et al*., 2020; Hande Mutlu-Durak, 2021; Talukder *et al*., 2015). Surprisingly, the standard fungicide (Snow Power) led to the reduced seed germination. This indicates that the fungicide is not recommended as a seed dressing agent. This observation may be due to the fungicide toxicity to the seed microbiomes which reportedly play a great role in seed germination and seedling development (Lengai, 2021). The reduction in seed germination upon treatment with some fungicides is associated with the inability of seeds to mobilize the stored starch in absence of microbiomes rather than direct phytotoxicity (Ayesha *et al*., 2021). The antifungal activities, having no negative effect on seed germination as observed in this study; the low toxicity of *C. bonariensis* (Antony, 2019), *L. nepetifolia* (Kowalczyk *et al*., 2021), *P. barbatus* (de Oliveira *et al*., 2021) and *V. amygdalina* (Zofou *et al*., 2011), makes these extracts potential seed dressing agents for the management of bean anthracnose in small farms with low risks to non-target organisms including applicator.

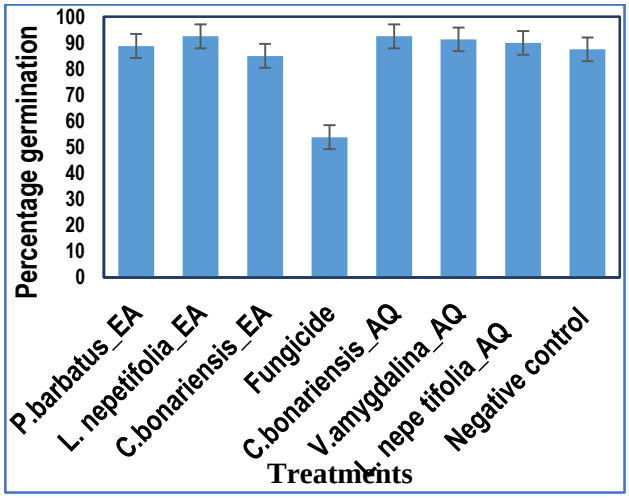

Fig. 4: Effect of selected plant extracts and fungicide on bean seeds germination in pots experiment

Effect of plant extracts on seedling performance

The impact of plant extracts and fungicides on the performance of bean seedling was variable (Table 1). The aqueous extract of *C. bonariensis* recorded highest shoot length whereas *L nepetifolia*

Table 1: Effect of plant extracts on bean seedling germination and seedling growth

Treatments	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
*P. barbatus*_EA	24.30 ± 0.244	8.030 ± 0.165	2.10 ± 0.016	1.00 ± 0.034
*L. nepetifolia*_EA	25.30 ± 0.488	7.750 ± 0.136	2.00 ± 0.115	0.97 ± 0.025
*C. bonariensis*_EA	25.10 ± 0.521	6.420 ± 0.119	2.65 ± 0.191	0.99 ± 0.030
Fungicide	24.30 ± 0.950	5.310 ± 0.044	2.46 ± 0.097	0.98 ± 0.033
*C. bonariensis*_AQ	27.40 ± 0.834	5.270 ± 0.129	2.15 ± 0.100	0.97 ± 0.010
L. nepetifolia AQ	18.80 ± 0.409	5.000 ± 0.200	1.93 ± 0.058	0.99 ± 0.012
*V. amygdalina*_AQ	24.80 ± 0.529	8.260 ± 0.637	2.20 ± 0.231	0.97 ± 0.041
Negative control	23.10 ±1.580	5.340 ± 0.717	1.90 ± 0.231	0.98 ± 0.041

EA - ethyl acetate, AQ - aqueous, S.D - standard deviation; The Values are mean ± S.D.

had the least. The aqueous extract of *V. amygdalina* had highest root length as compared to the other treatments including negative control, followed by ethyl extract of *P. barbatus* and *L. nepetifolia*. Ethyl acetate extract of *C. bonariensis* on the other hand, gave highest fresh weight whereas negative control had the least in this category. Although insignificant, the ethyl acetate extract of *P. barbatus* had relatively highest dry seedling weight as compared to the other treatments.

The present study establishes that the selected plant extracts had no negative effect on the growth of bean seedlings as depicted from higher fresh and dry weight, root, and shoot lengths than untreated seeds (negative control). The root and shoot length and seedling weight contribute to the crop production. Sofi *et al*. (2018) reported that root and shoot length correlates with pod weight and pod set percentage. Longer roots are important in plant growth as they aid in securing the water and nutrients from soil ,whereas healthy shoots enable the plant to withstand environmental stresses and give competitive advantage to plant for air, light, and space (Ngondya *et al*., 2016). Seedling vigour parameters have also been described as key predictors of crop establishment and yield (Adebisi *et al*., 2010). These findings demonstrate that these extracts can be useful in promoting seedling growth and increasing yield of common beans (Mkindi *et al*., 2020).

Conclusion: Our study demonstrates that the ethyl acetate extracts of *C. bonariensis, L. nepetifolia,* and *P. barbatus* and the aqueous extracts of *L. nepetifolia, C. bonariensis, V. amygdalina* and *P. barbatus* have antifungal properties. Thus, they have the potential to manage bean anthracnose caused by *C. lindemuthianum*. The study further demonstrates that these plant extracts have no negative effect on bean seeds germination and seedling development, proving that the extracts have no phytotoxic effect on seed germination and seedling growth. Abundance of the studied on medicinal plants in most of the areas in Tanzania provides a potential affordable management option for bean anthracnose. The relationship between the observed antifungal activity and the growth-promoting effect of the extracts needs to be investigated.

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