

Bio-efficacy of Crude Leaf Extracts of *Eucalyptus globulus* Against *In vitro* and *In vivo* Growth of Chocolate Spot (*Botrytis fabae* Sard.) of Faba Bean (*Vicia faba* L.)

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Abstract: It is widely known that faba bean (*Vicia faba* L.) is seriously attacked by the fungal disease, chocolate spot, caused by *Botrytis fabae* resulting in a yield loss ranging from 50 to 100%. Even though synthetic fungicides are used as one of the effective options for the control of plant diseases, the environmental hazards and economic unfeasibility associated with them necessitate the search for relatively safe natural products. This study was initiated to evaluate the antifungal potential of crude extracts of leaves of *Eucalyptus globulus*, against *in vitro* and *in vivo* growth of *Botrytis fabae*. In the *in vitro* experiment, antifungal assay was set up using different concentrations of the crude extracts. *In vivo* experiment was conducted in the field by planting a faba bean variety, Shallo, (EH011-22-1) and selected plants from each plot were used for extract application and subsequent data collection. Laboratory experiment showed that, compared to the control, extracts obtained from each solvent managed to produce statistically significant ($p < 0.05$) inhibition of mycelial growth. Based on the minimum concentration, inhibition from methanol extract (42.2%) significantly varied from that of ethanol (26.3%). Nevertheless, aqueous extract was nearly statistically the same to that of methanol. On the other hand, at maximum concentration (40%), a maximum inhibition percent of 83.7, which was nearly double of that produced by aqueous extract, was recorded from ethanol extract treated plates. Field experiment has shown that the use of ethanol extract of *Eucalyptus globulus* produced an efficacy of about 58.4%. Number of flowers aborted, number of tillers per m², number of pods per plant, number of seeds per pod and hundred seeds weight were affected due to the disease suppression effect of the three extracts. However, there was quantitative variation in yield though no statistically significant difference was observed. The result of this study showed that extracts of the tested plant species have natural fungitoxic potential and showed a promising future for the development of safe natural alternative fungicides used for the control of *Botrytis fabae* after further pertinent tests and screening of the active principles.

Keywords: Botanicals, Crude Extracts, *Botrytis fabae*, *In vitro*, *In vivo*, *Vicia faba*

1. Introduction

Ethiopia is considered to be the secondary center of diversification of faba bean [4, 39, 42], which occupies an approximate area of 459, 183.5 ha with a total annual production of about 697,798.4 t [8]. The country is also among the major faba bean producing Nations in the World;

taking the second place next to China [11, 12, 39]. The crop is a valuable source of cheap protein for the poorest part of the population who cannot afford animal protein [30]. Moreover, the crop naturally improves soil fertility through biological nitrogen fixation [31].

In spite of the fact that these advantages are obtained from the crop, its yield in Ethiopia is substantially lower than the World average. Diseases of different origin are the major

attributes for this. Among faba bean fungal diseases, Chocolate spot, which is caused by *Botrytis fabae* (Sard.) is the most widespread and highly destructive disease causing a yield loss ranging roughly from 34 - 61% on susceptible cultivars in Ethiopia [15]. However, aggressive infections could yield 100% crop loss [20, 43]. The disease is found to be severely affecting yield wherever it occurs provided that susceptible host and conducive environmental factors prevail [15, 31].

So far, several disease management options have been devised for the protection and control of the disease. Among these are; moderately resistant varieties, cultural control [10], biological control options [32] and synthetic fungicides [16]. Even though the use of synthetic chemicals is one of those frequently employed options, its disadvantages obviously outweigh the benefits gained. Worse situations with regard to resistance development by the pathogens against synthetic chemicals (including *B. fabae* and *B. cinerea*) [22] was reported. Additionally, synthetic chemicals are hazardous to human health and non target beneficial organisms and result in environmental pollution because of their less biodegradability [17]. They are also economically unfeasible, especially by the farmers in the developing countries.

As a new and promising option, the development and use of botanical sources can solve most of the problems listed above. Chemicals from plant based fungicides/pesticides are rich sources of antimicrobial agents [36] and are less persistent and hence easily biodegradable, thus, are considered safe to the environment and human health compared to the synthetic ones [17, 21, 27].

Leaf extracts from the currently investigated plant, *Eucalyptus globulus* Labill. contains 1,8-Cineole which has been proved to be active against filamentous fungi [24]. The current study deals with the natural effectiveness of crude extracts from the leaves of *E. globulus* against *in vitro* and *in vivo* growth of *B. fabae*.

2. Materials and Methods

The experiment was conducted in Sinana Agricultural Research Center (SARC), research station and phytopathology laboratory, Bale Zone, South Eastern Ethiopia during the year 2013.

2.1. Aqueous, Methanol and Ethanol Extraction

Fresh leaf samples of *E. globulus* were thoroughly washed and cleaned and allowed to dry under shade. Thereafter, the dried leaves were pulverized using sterilized blender. Then leaf powder (50 g) of the test-plant was soaked separately into 200ml of distilled water, methanol and ethanol in a 500 ml conical flask and left for 24hrs with intermittent stirring using rotary shaker for uniform mixing [26]. The mixture was then filtered in two steps; using 2-fold cheesecloth and then by sterilized Whatman No. 1 filter paper. The filtrate was centrifuged for 15 min at 6000 rpm. The crude extract was then preserved in an air tight bottle and stored in refrigerator at 4°C until used in antifungal assay [29].

2.2. In Vitro Efficacy Test

2.2.1. Preparation of the Growth Medium

Faba bean dextrose agar (FDA) was used as a medium for isolation of *B. fabae* and subsequent antifungal assay [14]. Four hundred gram of faba bean leaves have been collected, coarsely chopped fresh, mixed with 1l of tap water and sterilized in an autoclave at a temperature of 121°C and a pressure of 15 p. s. i. for 15 minutes. The mixture was then filtered and the filtrate was mixed with 18g agar and 20g dextrose and the volume made up to 1l. The composition was then autoclaved at a temperature of 121°C and pressure of 15 p. s. i. for 20 min, cooled down to about 45°C and poured into sterilized 9 cm diameter petri dishes [14].

2.2.2. Isolation of the Pathogen

The pathogen was isolated from infected faba bean leaves by cutting small portion from the advancing margin. The cut pieces were dipped into 0.5% sodium hypochlorite for 2-3 min [14] and then serially washed by sterilized distilled water three times. The specimens were then plated on the solid medium and incubated at a temperature of 20±2°C for 7 days and then sub-cultured until pure isolate was obtained. The isolate was compared with previous isolates in the center and cross checked with the reference from Barnett and Hunter [6].

2.2.3. Growth Inhibition Test

Different concentrations (5, 10, 20 and 40%) [35] of the crude extract were prepared by diluting with sterilized distilled water and 2ml of the extract from each concentration was transferred to different plates and gently mixed with the molten medium (45°C). After the medium had been solidified, 5mm mycelial disk from the pure culture of the pathogen was cut out using sterile cork borer and aseptically transferred to the medium and put at the center of the plate. The culture was incubated at 20±2°C for about 7 days. Plates with sterilized distilled water and synthetic fungicide (Mancozeb 80WP) made up negative and positive control, respectively. Radial growth measurement was made as soon as the growth in the control (distilled water) reached maximum. The medium was modified by adding 0.2% (v/v) lactic acid to prevent bacterial contamination. Fungi-toxicity of test extracts was calculated in terms of percentage growth inhibition using the following formula according to [38, 13]. The experiment was arranged in CRD in 3 replications.

$$\text{Growth inhibition (\%)} = \frac{DC - DT}{DT}$$

where: DC = average growth diameter from control (distilled water) plates

DT = average growth diameter of fungal mycelia from extract treated plates

2.3. In Vivo Efficacy Test

A faba bean variety Shallo was planted in the field in plots comprising three rows. Five plants were selected and marked from each of the middle rows of each plot for extract

application and subsequent data collection. Those plants were sprayed once with crude extracts of each species from individual solvents in the same concentration (due to vulnerability of active principles to harsh field conditions [34]) to sufficient wetting of the leaves. Randomized complete block design (RCBD) in three replications was used. Plant extract application was performed following the appearance of disease infection [28] using atomizer starting

from 61 DAP on 14th of October 2012. Application of synthetic fungicide, Mancozeb (Pencozeb 80WP) at recommended rate (2.5kg/ha) and distilled water formed positive and negative control plots, respectively. Disease severity was recorded using 1-9 scale according to Bernier *et al.* [5] and then converted for analysis to Percent Severity Index (PSI) using the following formula developed by Wheeler [41]:

$$\text{PSI} = \frac{\text{Sum of Numerical Ratings}}{\text{Number of Plants Scored} \times \text{Maximum Score on Scale}}$$

Where: PSI = Percent Severity Index

Efficacy of each of the plant extracts from each solvents was calculated using the same formula used in *in vitro* experiment given by Sunder *et al.* [38] and Farid *et al.* [13] with some modification.

$$\text{Efficacy (\%)} = \frac{\text{Severity in control plots} - \text{Severity in Treated plots}}{\text{Severity in control plots}} \times 100$$

3. Results

3.1. In Vitro Experiment

Compared to the control (distilled water) methanolic extract of the test plant has significantly ($p < 0.05$) inhibited the growth of mycelia at all concentration levels (Figure 1A). Almost in all extract cases, mycelial growth inhibition was found to significantly ($p < 0.05$) increase with increasing extract concentration. Inhibition effect of the plant extract at 40% concentration (78.7%) was significantly higher than all the lower concentration levels. Similarly, a 20% methanolic extract significantly inhibited mycelial growth (64.8%), but there was no statistical difference between the lower two concentration levels (Figure 1A). Regression analysis showed that increase in extract concentration had highly significantly affected the radial mycelial growth of *B. fabae*. It was revealed that growth inhibition had increased by 1.7% for each unit increase in extract concentration with variance

being explained 77% by the model (Figure 2A). The use of different concentrations of ethanol extract made it possible to get statistically varying degree of inhibition except at 5 (26.3%) and 10% (30.0%) concentrations (Figure 1B). Mycelial radial growth was highly significantly increased as concentration increased. An inhibition of 2.0 mm was obtained for each 10% increase in concentration with the variance being explained 90% by the single point model (Figure 2B). In the case of aqueous extract, no statistical difference was observed in inhibition across the different concentrations. The maximum and minimum concentrations produced comparable growth suppression (Figure 1C). Maximum inhibition percentage obtained by the 40% concentration was almost equal to that from the same concentration in the case of methanolic extract. It was shown by the regression analysis that there was a 0.8% increase in mycelia growth inhibition for each unit increment in the amount of concentration of the extract (Figure 2C).

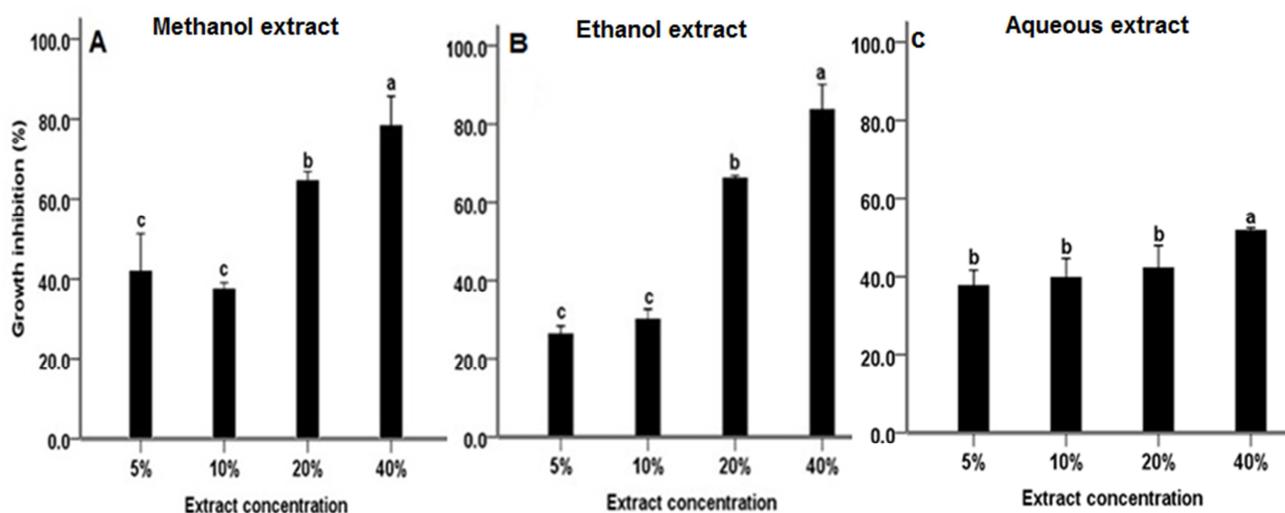


Figure 1. Percentage *in vitro* growth inhibition of *B. fabae* due to methanol (A), Ethanol (B) and Aqueous (C) crude extracts treatment of leaves of *E. globulus*. Bars with the same letter (within the extract type) are not significantly different at $p < 0.05$ level of probability.

3.2. In vivo Experiment

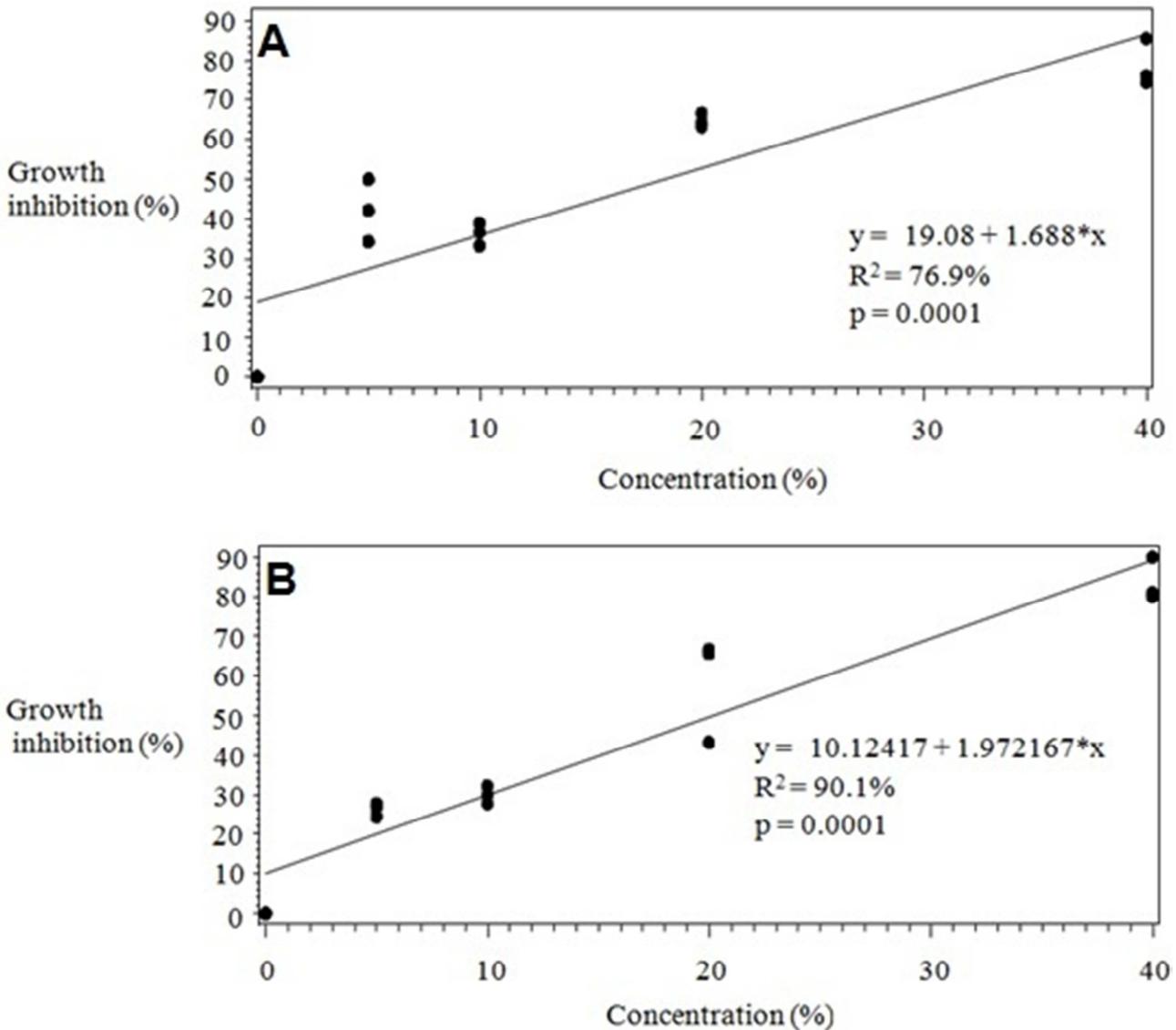
3.2.1. Effect of *E. globulus* Crude Extracts on the Incidence and Severity of *Botrytis Fabae*

In control plots, mean severity of *B. fabae* was 58.8% (Figure 5). Compared to this control, disease severity was significantly ($p < 0.05$) reduced by all solvents extracts of *E. globulus* with severity scores of 24.4%, 26.7%, and 28.9% for ethanol, methanol and aqueous extracts, respectively (Figure 5). There was no significant difference between the three solvents' extracts of *E. globulus* in terms of disease severity reduction. Synthetic fungicide (Mancozeb 80W) lowered disease severity to as low as 18.0%, and though this value is not significantly different from methanol and ethanol extracts, it sufficiently varied from the severity level measured from aqueous extract treated plots (Figure 5). Percentage efficacy was calculated for all the extracts and revealed that *E. globulus* ethanol extract yielded 58.4% efficacy over the control plots. Methanol and aqueous

extracts were 54.6 and 50.9% effective, respectively, in relation to the control plots (Figure 3).

3.2.2. Effect of *E. globulus* Crude Extracts on the Temporal Progress of *B. Fabae*

Figure 2 shows that chocolate spot severity progress over time was differentially affected due to the use of crude botanical extracts of *E. globulus*. At 68 DAP, the lowest severity (15.6%) was recorded on plots treated with methanol extract of *E. globulus* and at 75 and 82 DAP, severity showed no increment (20.0%) after which it rose up to 27.4% at 89 DAP (Figure 2). Methanol and ethanol extracts were more effective in retarding the potential development of the disease and making it unable to temporally increase significantly. Synthetic fungicide (Mancozeb 80WP) was found to reduce the development of the disease through the growth period of the plant, thus producing a more or less stabilized curve (Figure 4).



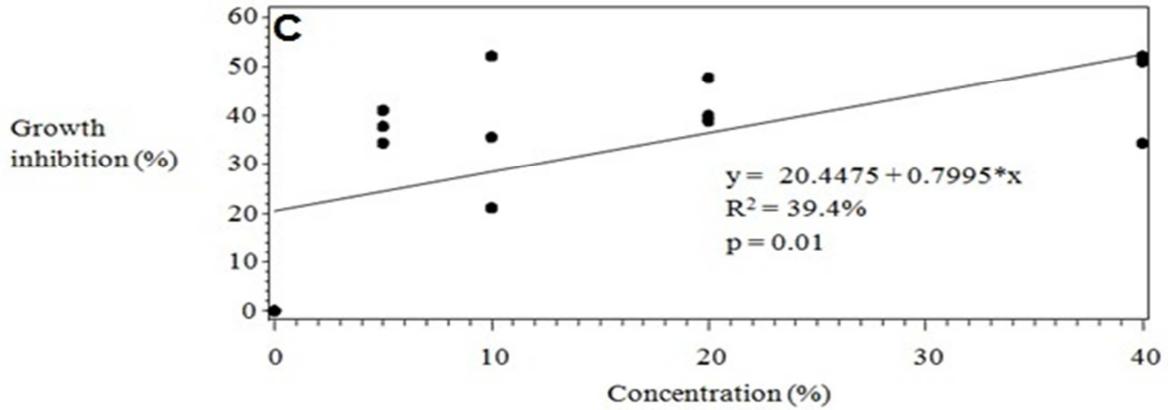


Figure 2. Regression model predicting the relationship between in vitro mycelial Growth Inhibition of *Botrytis fabae* (y) and Crude botanical extract concentration (x) of Methanol (A), Ethanol (B) and Aqueous (C) extracts of leaves of *E. globulus*.

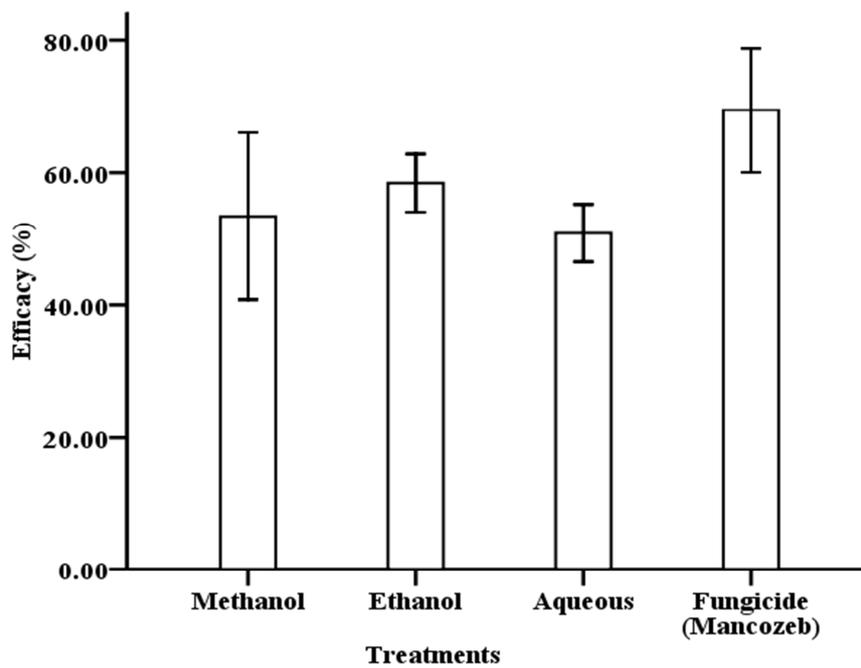


Figure 3. In vivo percent efficacy of *E. globulus* extracts against *Botrytis fabae* at 100% concentration of each extract.

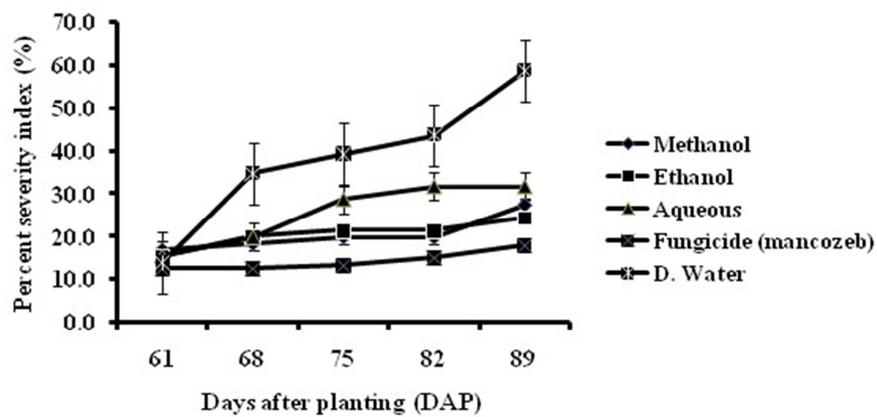


Figure 4. Chocolate spot severity progress curve for methanol, ethanol and aqueous extracts of *E. globulus*. Standard errors are shown in vertical bars drawn across line markers.

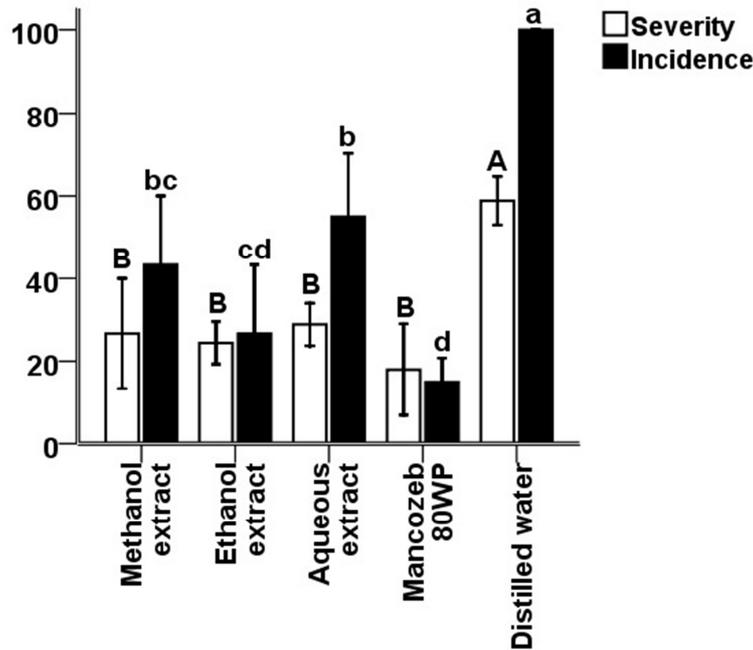


Figure 5. Comparison of percent severity and percent incidence of *B. fabae* field infection on *V. faba* treated with methanol, ethanol and aqueous extracts of leaves of *E. globulus*. Bars with the same letter are non significantly different at $p \leq 0.05$ level of probability. Values represent Mean \pm SE.

3.2.3. Effect of Crude Extracts of *E. globulus* on the Yield and Yield Components of Faba Bean

The potential inhibitory impact of the crude extracts on the severity of the disease in turn produced a remarkable effect on the yield and yield components of the crop. Thus, number of aborted flowers per plant highly significantly varied due to different extracts (Table 1). Methanol extract (5.7), compared to the control (28.0), was the most effective in reducing flower abortion, thus producing the highest number of pods per plant (23.0) which became significantly different from

that produced by untreated plots (11.0). However, non-significantly higher number of flowers were aborted in the case of fungicide application. Likewise, the highest number of tillers (18.5) was obtained from plots treated with methanol extract which was statistically significantly different from that obtained from untreated plots (8.3). An average of 3.3 seeds were counted in each pod, varying non significantly among treatments (Table 1). Similarly, grain yield was found to be statistically the same across treatments.

Table 1. Effect of crude extracts of leaves of *E. globulus* on the severity, yield and yield components of faba bean.

Treatments	PSI (%)	Number of aborted flowers per plant	Number of tillers/m ²	Number of pods/plant	Number of seeds/pod	HSW (gm)	Grain yield (kg/ha)
Methanol	26.7 ^{bc} \pm 3.5	5.7 ^c \pm 3.5	18.5 ^a \pm 2.1	23.0 ^a \pm 2.4	3.2 ^{ab} \pm 0.1	56.1 ^{ab} \pm 2.3	3591.7 ^a \pm 13.8
Ethanol	24.4 ^{bc} \pm 4.9	18.8 ^{ab} \pm 4.9	13.2 ^{ab} \pm 3.0	20.9 ^b \pm 3.4	3.1 ^b \pm 0.1	56.0 ^{ab} \pm 3.6	3197.6 ^a \pm 2.4
Aqueous	29.9 ^b \pm 4.1	24.7 ^a \pm 4.1	16.6 ^a \pm 2.5	16.7 ^{ab} \pm 2.8	3.5 ^a \pm 0.1	52.5 ^b \pm 3.0	3454.8 ^a \pm 19.6
Fungicide (Mancozeb 80 WP)	18.0 ^c \pm 5.3	11.8 ^{bc} \pm 5.3	17.3 ^a \pm 3.2	18.1 ^{ab} \pm 3.6	3.3 ^{ab} \pm 0.1	60.8 ^a \pm 3.9	3404.0 ^a \pm 4.0
Distilled water	55.4 ^a \pm 5.0	28.0 ^a \pm 5.0	8.3 ^b \pm 3.0	11.0 ^b \pm 3.5	3.2 ^{ab} \pm 0.1	60.6 ^a \pm 3.7	2995.7 ^a \pm 2.9
LSD _{p\leq0.05}	10.5	10.6	6.4	7.3	0.3	7.7	1020.1
CV(%)	17.8	30.7	22.3	21.0	4.4	7.0	15.9

PSI=percent severity index; HSW=hundred seed weight; LSD=least significant difference; CV=coefficient of variability; means with the same letter are non significantly different; mean \pm SE of means

4. Discussion

The highest growth inhibition percentage (83.7%) was recorded from 40% concentration of ethanol extract of the plant. Alabi *et al.* [3] studied antimicrobial potential of *E. globulus* leaf extract against fungal disease of cowpea and found that it reduced the disease infection to about 2.4% compared to control (29%). Additionally, Satish *et al.*, [33] studied the efficacy of aqueous and organic solvents (methanol, ethanol, chloroform) extracts of *E. globulus*

against seed borne fungal pathogens caused by different species of *Aspergillus*. Their result indicated that *E. globulus*, among other plant species was highly effective against a number of species of the test pathogen. *E. globulus* leaf extract was recommended by Joseph *et al.*, [18] for the control of the fungal plant pathogen, *Fusarium solani*. A related species, *E. citrodora* was able to control the radial growth of *B. cinerea* 100% in an *in vitro* experiment [40]. In the current study, the highest inhibition was obtained from ethanol, which is followed by methanol and aqueous extracts

in decreasing order, taking the highest concentration into consideration. Obviously, aqueous extract took the lowest degree of inhibition, indicating less water solubility of the bioactive compounds in the plant leaves. Inhibition level equivalent to that obtained from 5% concentration of methanol extract (42.2%) was produced by increasing the concentration by 4 fold in its aqueous extract. Abera *et al.*, [1] in their evaluation of botanical extracts against coffee berry disease causing fungus, *C. kahawae*, *E. globulus* ethanol extract controlled the mycelial growth better than it did with its aqueous extract. The former extract produced an inhibition percentage of 64%, while the later gave 57% compared to the control.

According to Satish *et al.* [33], methanol and ethanol extracts of *E. globulus* were superior to the aqueous in controlling the fungus *Aspergillus flavus*. In another study however, aqueous extract of *E. globulus* leaves was proved to completely inhibit zoosporangium formation of the fungus *Sclerospora graminicola* which is the causative agent of downy mildew of Pearl millet [9]. A maximum of 58.4% efficacy was obtained using ethanol extract. The experiment showed that application of the plant extracts improved some agronomic traits, such as, number of aborted flowers (reduced to 5.7 using methanol extract), number of tillers per m² (18.5 using methanol extract), number of seeds per pod (3.5 using aqueous extract) and yield (3591.7 kg ha⁻¹ using methanol extract) compared to the control. Similarly, Alabi *et al.*'s work revealed that the use of *E. globulus* had positive effect on the agronomic traits of cowpea. Number of pods per plant, hundred seeds weight and yield were improved [3]. The major component in the leaves of *E. globulus* was reported to be 1,8-cineole (eucalyptol) and is shown to have antimicrobial property [19, 7, 25]. Leaves of the plant were also reported to have high amount of oxalic acid [3] which is among chemicals known to act as defense inducers in plants [2, 23].

5. Conclusions

E. globulus is revealed in this study that it is a promising plant species *in vitro* and *in vivo* whose natural potential should be exploited after further detailed physicochemical studies. Results from the *in vitro* and *in vivo* experiments clearly shown that the bioactive compounds in the leaves of *E. globulus* are active against mycelial growth and field severity of *B. fabae* of faba bean, respectively. The success that higher degree of fungal growth inhibition was obtained with minimum concentrations is a clear indication of the immense natural antimicrobial potential that the compounds in the leaves of *E. globulus* bear. Moreover, magnitude of efficacy has been seen to vary with the type of solvent used. Depending on the minimum concentration, *E. globulus* bioactive compounds were best dissolved by methanol.

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