Effect of Crude Ethanolic Extracts from Bottle Brush (*Callistemon viminalis*) against Leaf Spot Fungi and Their Phytotoxicity on Lettuce (*Lactuca sativa* L.)

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Abstract

In response to the public concern about environmental problems as well as fungicide residues on agricultural produces especially leaf vegetables, we investigated the potential use of crude extracts from bottle brush (*C. viminalis*) leaves against leaf spot fungi and their phytotoxicity on lettuce grown in hydroponics. Yield, total phenolic and flavonoid contents of the extract were also evaluated. Regarding the *in vitro* antifungal assay using poisoned food technique, 3×3 factorial in Completely Randomized Design (CRD) was employed with three replicates. Factor A was 3 crude extracts (50% ethanol, 70% ethanol and 95% ethanol crude extract) while factor B was the 3 concentrations (0, 5,000 and 50,000 ppm) of crude extracts. Results showed that all three crude extracts were significantly effective in inhibiting the mycelial growth and sporulation of leaf spot fungi (*Alternaria* sp. and *Curvalaria* sp.). There was a significant interaction between all extracts and their concentration regarding inhibition percent. Increase in crude extract concentrations resulted in a marked increase in their inhibition. The concentration of 50,000 ppm gave the highest effectiveness. For phytotoxicity test, all tested crude extracts did not give any negative effects either on seed germination or growth of lettuce grown in hydroponics. Interestingly, growth promotion about 30 percent was achieved on this regard. Based on quantitative assay, the highest yield of crude extract was obtained from 95% ethanol solvent. Highest total phenolic and flavonoid contents were detected at the highest concentration of all crude extracts.

Keywords: bottle brush extract, phytotoxicity, antifungal activity, leaf spot fungi, *Alternaria*, *Curvularia*
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1. Introduction

Lettuce (*Lactuca sativa* L.), an annual plant, is commercially grown worldwide as leafy vegetable. In Thailand, lettuce is widely consumed as fresh vegetable meanwhile its production in soil and hydroponic cultivation has been increasing due to great market demand and health concern. Leaf spot diseases of vegetable crops caused by fungi such as *Alternaria* sp., *Cercospora* sp., *Curvularia* sp., and *Septoria* sp. are commonly found throughout the world and have significant impact on quantity, quality and commercial value of crops [1]. In southern Thailand, *Curvularia aeria* and *C. lunata* were reported to be causal agents of leaf spot diseases on lettuce and cabbage grown in field, respectively [2-4]. In general, most *Alternaria* species are mainly saprophytic fungi. However, some species have acquired pathogenic capacities collectively causing a range of diseases with

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economic impact on a large variety of important agronomic host plants including vegetables [5]. For example, *A. dauci*, the carrot pathogen, can cause a disease on lettuce and celery grown in the field [6].

Chemical fungicides have often been used to control these diseases, but this conduct is associated with negative impacts on ecosystems, harmful effects on human health and deposition of residue on agricultural produces. Hence, there is a great demand for developing safer alternative and effective measures, such as treatment with natural agents like plant extracts, which are capable of preventing or controlling such diseases to reduce dependency on synthetic fungicides.

At present, much attention has been paid towards plant extract having an inhibitory effect on plant pathogens with none or less negative impacts on growing plants, human and the environment. *Callistemon viminalis*, commonly known as bottle brush tree, belongs to the family Myrtaceae which is a common ornamental small tree with pendulous foliage [7-9]. This plant is on the plant lists of the "Plant Genetic Conservation Project" under the Royal Initiative of Her Royal Highness Maha Chakri Sirindhorn, Thailand. Various parts of this plant used in traditional and folk medicine have been reported to have antibacterial activities [7-9]. However, a few studies have been focused on its antifungal properties for plant disease control especially on leafy vegetables.

Therefore, this research was conducted to evaluate the possible antifungal activities of crude extracts from *C. viminalis* leaves (using solvents with different ethanolic percentages and different extract concentrations) as well as its phytotoxicity on lettuce in order to obtain the safer effective method for disease control.

2. Materials and Methods

2.1 Pathogenicity test

The pathogenicity test was carried out using detached leaf test. The fungi tested in this experiment, i.e., *Alternaria* sp. and *Curvularia* sp.were isolated from lettuce leaves showing leaf spot symptom. The lettuce leaves were washed in tap water and left air-dried. Then, the leaf was inoculated by 5 mm dia mycelial agar plug (7 days old). The PDA agar plug without pathogen was used as a control. The inoculated leaves were kept into the moist plastic box at room temperature. Disease index was rated up to 4 levels of lesion sizes as: 0 = no infection; 1 = 1-5 mm; 2 = 5.1-10 mm; 3 = 10.1-15 mm; and 4 = >15 mm at 3 and 5 days after inoculation (DAI). Disease incidence (DI) and disease severity (DS) of tested lettuce were calculated by the equations:

where $$\%\,\mathrm{DI} = (\text{Number of infected leaves} / \text{Number of total leaves}) \times 100$$

$$\%\,\mathrm{DS} = \frac{\sum(\text{Number of infected leaves} \times \text{Disease index})}{\text{Number of total leaves} \times \text{The highest disease index}} \times 100$$

2.2 Preparation of *Callistemon viminalis* extract and evaluation of its total phenolic and flavonoid contents

The disease free and fresh leaves of *C. viminalis* were collected from Ladkrabang district area, Bangkok, Thailand. The samples were washed thoroughly with water for 3 times and left air-dried. Then, approx. 1.5 kg of air-dried leaves were dried in hot air oven at 45℃ for 3 days. Dried plant leaves were ground and soaked into 95, 70 and 50% ethanol solvent with the ratio of 1:10 (w/v) for 3 days. The extracts were filtered through double-layered cheesecloth and one layer of Whatman No.1 filter paper. The filtrates were collected and concentrated at 40˚C under reduced pressure using

rotary evaporator. All the extracts were then transferred into airtight vials and stored at 4˚C for further use.

The total phenolics and flavonoids of the crude extract were analyzed by Folin-Ciocalteu method [10] and the aluminum chloride method [11], respectively. For total phenolic contents, the 300 µl of extract was mixed with 2.25 ml of Folin-Ciocalteu reagent (10% dilution) and kept for 5 min at room temperature. Then, 2.25 ml of sodium carbonate solution was added to the mixture for 90 min at room temperature. The mixture and absorbance were detected at 750 nm using a spectrophotometer. Gallic acid was used as standard and total phenolic contents were expressed as gallic acid equivalent per milliliter (mg GAE/ml). For flavonoid contents, the 0.5 ml of extract was mixed with 2.25 ml of distilled water. Afterwards, 0.15 ml of 5% $NaNO_2$ solution was added and kept at room temperature for 6 min. Then, 0.3 ml of 10% $AlCl_3$ solution and 1 ml of 1 M NaOH were added and shaken. The mixture and absorbance were measured at 510 nm using a spectrophotometer. Quercetin was used as standard and flavonoid contents were calculated as mg quercetin equivalent per milliliter (mg QE/ml).

2.3 Antifungal activity of *C. viminalis* crude ethanolic extracts

The antifungal activity of crude ethanolic extracts from *C. viminalis* was *in vitro* tested against two foliar fungal pathogens of lettuce, namely *Alternaria* sp. and *Curvularia* sp., using poisoned food assay method. The experiment was carried out in a CRD with a 3×3 factorial arrangement plus one positive control (using 50 ppm carbendazim). Factor A was 50, 70 and 95% ethanolic extracts where factor B was 0, 5,000 and 50,000 ppm. The extract was incorporated into the molten PDA at a desired final concentration and mixed well. Then, the medium was poured into Petri dish. After overnight pre-incubation, the inoculation was done by a mycelial disc of 5 mm dia, which was deposited in the center of the agar plate. After further incubation at 25 ºC, the diameters of fungal growth in control and treatment plates were measured and the antifungal effect was estimated by the following formula:

$$\text{Antifungal activity (\%)} = (Dc-Dt)/Dc \times 100$$

where Dc was the diameter of growth in control plate.
Dt was the diameter of growth in the plate containing tested extract.

Sporulation was determined at the end of incubation according to Islam *et al*. [12] with slight modification. Six discs (5 mm dia) were randomly taken from each Petri dish of fungal culture and macerated in 10 ml of sterile water. Spore suspensions obtained were filtered and homogenized. Spores were then counted under microscope using a haemacytometer. Assay was carried out in three replicates. The percent reduction or stimulation by each extract was determined.

2.4 Phytotoxicity of *C. viminalis* crude ethanolic extracts on lettuce

Phytotoxic effects of the extracts were assessed on seed germination and growth of lettuce in hydroponics as follows:

Seed germination: Phytotoxicity of *C. viminalis* crude ethanolic extract was determined on lettuce seed germination. The dry crude extracts were dissolved into the required concentrations in 10% dimethyl sulfoxide (DMSO). Then, lettuce seeds were soaked into the prepared extract for 15 min. Afterwards, 25 treated seeds of lettuce were placed on sterilized germination paper in 9 cm Petri dishes containing 5 ml sterilized water. The dish was maintained at 25 ºC, 12 h photoperiod in the growth chamber. The experiment was carried out by 3×4 factorials in CRD with 4 replications.

Factor A was 3 crude extracts (50%, 70%, and 95% ethanol crude extract) while factor B was 4 concentrations of crude extracts (0, 5,000, 25,000 and 50,000 ppm). Ten percent of DMSO without the extract was used as control. Seed germination, hypocotyl and radicle length were recorded.

Growth of lettuce in hydroponics: The effect of foliar sprayings of *C. viminalis* crude ethanolic extract was evaluated on lettuce grown in Dynamic root floating technique (DRFT) with nutrient solution (EC = 1.6-1.8 mS/cm, pH= 5.8-6.2). The experiment was laid out as 3×4 factorials in CRD with 3 replications (3 plants per replication). Factor A and B were set as same as in the seed germination test whereas foliar sprayings of 2 ml of the extract were made on leaf of 15 days old lettuce. Phytotoxicity was determined as the occurrences of necrosis and discoloration. Plant growth parameters such as leaf greenness (by SPAD value using a portable chlorophyll meter, SPAD-502, Konica Minolta Sensing, Inc., Japan), leaf size and numbers were weekly monitored after treatment whereas the fresh and dry weight of shoot and root were recorded at the end of the cultivation.

3. Results and Discussion

3.1 Isolation and pathogenicity test

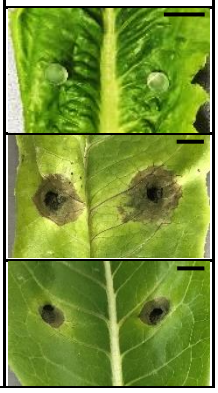
The pathogenicity test on detached leaf test revealed that both tested fungi (*Alternaria* sp. and *Curvularia* sp.) were the causal pathogen of the leaf spot disease of lettuce, showing 100% DI with 85% and 40% DS, respectively at 5 DAI (Table 1). Regarding the symptom, brown circular leaf spots and yellow halo were found on both fungal inoculations. *Alternaria* sp. caused large necrotic and chlorosis symptoms (about 23 mm dia) while *Curvularia* sp. caused small lesion (about 6.6 mm dia) (Table 1). Our findings are in agreement with many researchers who reported that both fungi were leaf and seed-borne pathogen of leafy vegetable crops and especially *Alternaria* sp. leaf spot is a common disease and particularly problematic for plant in the cabbage family [2-3, 13].

3.2 Extract yield, total phenolic and flavonoid contents

Extract yields of *C. viminalis* were shown in Table 2. It was shown that 50% ethanolic extract of *C. viminalis* (50% EECV), 70% ethanolic extract of *C. viminalis* (70% EECV) and 95% ethanolic extract of *C. viminalis* (95% EECV) yielded 4, 6 and 13%, respectively, based on oven dry weight of the leaves (300 g). It can be seen that percentage crude yield of extract increased along with an increase of alcohol percentage in the solvent, i.e., 95% ethanol shown to be the best extraction solvent in terms of crude yield (13%). However, in some cases reported in Salem *et al*. [14], extract yield of *C. viminalis* using methanolic solvent was much higher as 23%. In 2012, Krishna *et al*. [15] reported that the extract yield of *C. citrinus* about 1.75% based on oven dry weight of the leaves (400 g) was obtained by using ethanol solvent.

The total phenolic and flavonoid contents were presented in Table 3. The result revealed a significant interaction between all extracts and their concentrations regarding total phenolic and flavonoid contents. The highest concentration (50,000 ppm) of all tested extracts gave the highest phenolic contents about 1.16-1.23 mg GAE/ml. With regard to flavonoid contents, the 50,000 ppm of 70% EECV significantly exhibited higher content about 3.31 mg % QUE/ml followed by 95%

Table 1. Pathogenicity test of *Alternaria* sp. and *Curvularia* sp. on lettuce leaf.

Treatment	Incidence (%)		Severity (%)		Symptom
	3 DAI[2/]	5 DAI	3 DAI	5 DAI	5 DAI
Control	0c[1/]	0c	0c	0c	
Alternaria sp.	100a	100a	75a	85a	
Curvularia sp.	60b	100b	12b	40b	

[1/]Values are means of three replicates .Values in each column followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P>0.05). Scale bar = 10 mm.
[2/]DAI= Day after inoculation

Table 2. Extract details of *Callistemon viminalis* leaves.

Plant part	Solvent	Solvent volume (ml)	Extract weight (g)	Yield (%)
Leaf (300g)	50% Ethanol	3000	12	4
Leaf (300g)	70% Ethanol	3000	18	6
Leaf (300g)	95% Ethanol	3000	39	13

Table 3. Total phenolic and flavonoid contents of *Callistemon viminalis* leaves.

Extract name	Conc^{n} (ppm)	Total phenolic (mg GAE/ml)	Total flavonoid (mg QUE/ml)
50%EECV	0	0.00f	0.00h
	5,000	0.48c	1.19fg
	25,000	0.92b	1.96e
	50,000	1.16a	3.01b
70%EECV	0	0.00f	0.00h
	5,000	0.20d	1.20f
	25,000	1.00b	2.17d
	50,000	1.23a	3.31a
95%EECV	0	0.00f	0.00h
	5,000	<1e	1.11g
	25,000	1.08b	1.93e
	50,000	1.22a	2.92c
C.V. (%)		2.44	3.67
Value of factor A[2/]		**	**
Value of factor B[3/]		**	**
A×B		**	**

[1/]Values are means of three replicates .Values in each column followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P>0.05).
[2/]Factor A is the ethanol concentration for extraction
[3/]Factor B is the concentration of crude extracts

EECV and 50% EECV about 3.01 and 2.92 mg % QUE/ml, respectively (Table 3). Our results are in agreement with the previous findings [14, 16] who reported that the qualitative phytochemical analysis revealed the presence of different phytochemicals including flavonoids and phenolics in methanol and ethanol crude extract of *C. viminalis*. *Callistemon viminalis* was a rich source of phenolic compounds whereas higher amount of phenolics (about 88.83 mg GAE/g DW), flavonoids (about 65.48 mg GAE/g DW) was found in its ethanol extract [16]. The 44.3 mg GAE/g extract of total phenolics and 45.36 mg GAE/g extract of total flavonoids were detected in methanol crude extract [14]. In addition, its aqueous leaf extract was also reported to contain about 48.35 µg GAE mg^{-1} tissue phenolics [17]. Flavonoid and phenolic contents were also reported to be present in other species of *Callistemon* [18]. Regarding our result on high flavonoids content in 70% EECV, it was in line with Bimakr *et al*. [19] who mentioned that the higher concentrations of more bioactive flavonoid compounds were detected with 70% ethanol due to its higher polarity than pure ethanol. It is worth noted that numerous biological activities (such as antimicrobial activity, antioxidant capacity and so on) of phenolic compounds and flavonoids have been demonstrated by Hasan *et al*. [16], therefore the quantity of the two phytochemical compounds of our crude extracts could play an important role on such biological activities.

3.3 Antifungal activity of *C. viminalis* crude ethanolic extracts

In vitro antifungal activity of the extracts obtained from *C. viminalis* leaves, using different solvents and concentrations, against the tested fungi was shown in Table 4. The growth inhibition of each fungus was presented in Figure 1. All three crude extracts at 5,000 and 50,000 ppm were found to be significantly effective against mycelial growth of the tested fungi at 3 and 5 DAI (Table 4) and at 7 DAI (Figure 1). In addition, there was an interaction between solvent type and extract concentration level on their effects on inhibition percentage of the growth of the two tested fungi. As the concentration level of the crude extracts increased, the percent growth inhibition also increased.

Table 4. Effect of different concentrations of ethanolic extracts from *C. viminalis* leaves on mycelial growth of leaf spot fungi at 3 and 5 DAI.

Factor A	Factor B	Growth inhibition (%)			
		Alternaria sp.		*Curvularia* sp.	
		3 DAI	5 DAI	3 DAI	5 DAI
50%EECV	0 ppm	0.0e[1/]	0.0d	0.0f	0.0g
	5000 ppm	38.4d	31.4c	71.6c	67.0d
	50000 ppm	89.9a	89.6a	86.1b	83.7b
70%EECV	0 ppm	0.0e	0.0d	0.0f	0.0g
	5000 ppm	50.8c	43.4b	67.3d	61.9e
	50000 ppm	84.8b	84.4a	85.5b	80.1c
95%EECV	0 ppm	0.0e	0.0d	0.0f	0.0g
	5000 ppm	53.5c	47.9b	58.5e	49.2f
	50000 ppm	89.1a	88.7a	94.4a	91.8a
C.V. (%)		5.32	7.73	2.40	3.46
Value of factor A		**	*	*	**
Value of factor B		**	**	**	**
A×B		**	**	**	**

[1/]Values are means of three replicates .Values in each column with in each pathogen followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P>0.05).

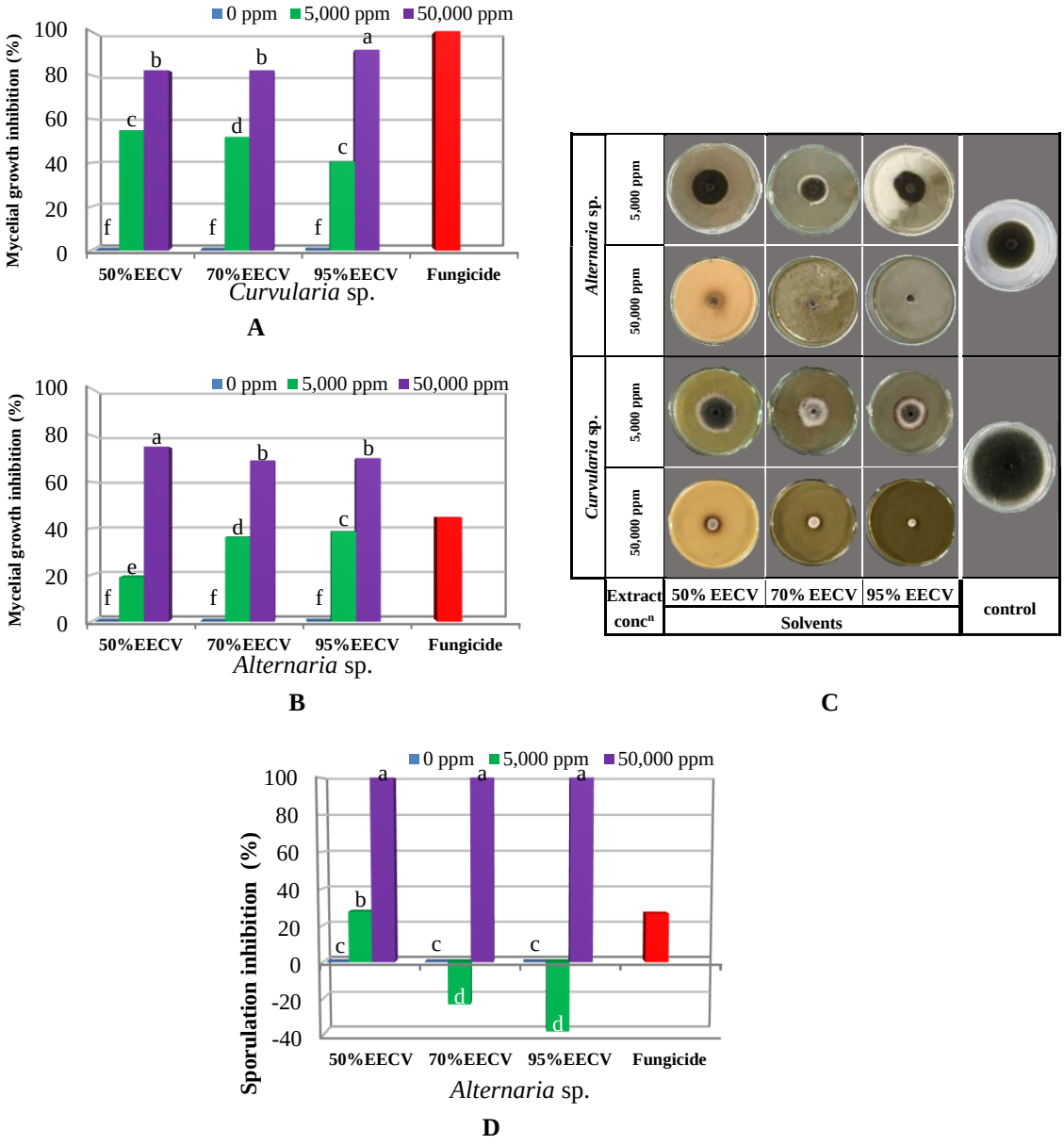


Figure 1. Growth inhibition of *Alternaria* sp. and *Curvularia* sp. by crude ethanolic extract from *C. viminalis* leaves at 7 DAI. Values are means of three replicates. Values followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P>0.05)
A) Mycelial growth inhibition of *Curvularia* sp. (%), B) Mycelial growth inhibition of *Alternaria* sp. (%), C) Colony morphology of *Curvularia* sp. and *Alternaria* sp. grown on PDA with crude extracts, and D) Sporulation inhibition or stimulation of *Alternaria* sp. (%)

At 7 DAI, the highest concentration (50,000 ppm) of the 50% EECV showed the greatest inhibitory effect (about 75%) on growth of *Alternaria* sp., followed by 95% and 70% EECV (about70%) whereas only 44.8 % growth inhibition was recorded from positive control (50 ppm carbendazim) (Figures 1A, 1C). Unlike *Alternaria* sp., 95% EECV was shown to be the most significantly effective against *Curvularia* sp. (about 91%) at the same concentration of 50,000 ppm followed by 50% and 70% EECV (about 82%), while 50 ppm carbendazim completely inhibited fungal growth (Figures 1B, 1C). In regard to sporulation assessment, only *Alternaria* sp. has been conducted since the growth of *Curvularia* sp. was almost completely inhibited by the extract. The result showed that its sporulation was totally inhibited at the highest concentration (50,000 ppm) of the tested extracts whereas it was stimulated at 5,000 ppm of 70% EECV and 95% EECV (Figure 1D).

From the result on *Alternaria* sp., this fungus seemed to be less sensitive to carbendazim fungicide (only 44.8% inhibition) but more sensitive to the extracts (about 75% inhibition) (Figure 1A). This finding was in agreement with the reports in USA and France [20-21] mentioning that *Alternaria* spp. were insensitive to many classes of fungicides recently recovered from fields. From our findings, it would be noted that a marked antifungal activity of the all tested crude extracts of *C. viminalis* leaves was observed which may be attributed to the presence of phenolics and flavonoids contents. *Callistemon viminalis*, especially its flowers are rich in polyphenols, flavonoids, saponins, steroids contents which possess good antibacterial and antifungal activities [8, 22]. A number of studies [8, 12, 23-24] reported that phytochemical extracts of *C. viminalis* leaves exhibited strong to moderate antibacterial effects and methanol extracts in some cases showed a stronger effect than ethanolic extract, which could be explained by the differences in the compounds between these two extracts. However, we have still focused on using ethanolic solvents for the safety reason [25]. In terms of antifungal activity of *Callistemon* plant, Nguefack *et al*. [26] reported that 70% ethanol extract from *C. citrinus* leaf proved to be effective against *Alternaria padwickii* and *Bipolaris oryzae* under both laboratory and field conditions.

Stimulation of *Alternaria* sporulation observed at 5,000 ppm of 70% EECV and 95% EECV was in line with the finding of Tiaiba *et al*. [27] who reported that the fungus *Ascochyta pisi* could sporulate on the medium containing 1.5% of plant extracts at levels sometimes exceeding the control.

3.4 Effect of *C. viminalis* crude ethanolic extracts on lettuce

Phytotoxicity of *C. viminalis* crude ethanolic extracts was assessed on seed germination (by seed treatment) and growth of lettuce (by foliar spray). With regard to the assessment of phytotoxic effects on lettuce seed germination, it revealed that the crude extracts at all tested concentrations did not have any significant adverse effect on seed germination at 2, 3 and 5 DAT compared to control (Table 5). On the contrary, especially the lowest concentration (5,000 ppm) of all tested ethanolic extracts (50% EECV, 70% EECV and 95% EECV) significantly stimulated the germination to 100% at 5 DAT compared to control showing about 91%. There was no interaction between the three extracts and their concentrations on percent seed germination. In addition, the 50,000 ppm of all the extracts as well as all 3 concentrations of 95% EECV significantly increased the seedling length compared to control (Table 5 and Figure 2).

Table 6 presented the effect of crude ethanolic extract of *C. viminalis* on lettuce growth. The result was in line with that of seed germination trial. Importantly, phytotoxicity (necrosis and discoloration) was rarely detected on lettuces treated with the extracts except for the concentration of 25,000 and 50,000 ppm of 50% EECV which showing only few necrotic lesions on the young leaf. Overall, the extracts did not give any negative effects on lettuce growth throughout the experiment. On the other hand, the extracts seemed to exhibit the positive effect such as stimulation

Table 5. Effect of different concentrations of ethanol extracts on seed germination and seedling growth of lettuce

Factor A	Factor B	Seed germination (%)			Seedling length (mm)	
					Radicle	Hypocotyl
		2 DAT[2/]	3 DAT	5 DAT	5 DAT	5 DAT
50% EECV	0 ppm	91.8ab[1/]	91.8c	91.8b	24.4e	13.0ef
	5000 ppm	92.0ab	96.0abc	100.0a	32.7cd	12.1f
	25000 ppm	97.8a	100.0a	100.0a	33.3bcd	13.3e
	50000 ppm	92.0ab	96.0abc	96.0ab	31.2d	14.8d
70% EECV	0 ppm	91.8ab	91.8c	91.8b	24.4e	13.0ef
	5000 ppm	92.0ab	96.0abc	100.0a	33.8bcd	12.9ef
	25000 ppm	96.0ab	98.0ab	98.0ab	33.7bcd	15.0d
	50000 ppm	96.0ab	100.0a	100.0a	36.0ab	16.5ab
95% EECV	0 ppm	91.8ab	91.8c	91.8b	24.4e	13.0ef
	5000 ppm	90.0b	96.0abc	100.0a	34.7bc	17.2a
	25000 ppm	90.0b	96.0abc	96.0ab	37.5a	16.1bc
	50000 ppm	93.8ab	94.0bc	93.9ab	37.7a	15.1cd
C.V. (%)		3.51	2.26	2.66	14.74	13.10
Value of factor A		ns	ns	ns	**	**
Value of factor B		ns	**	**	**	**
A×B		ns	ns	ns	**	**

[1/]Values are means of three replicates. Values in each column followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P>0.05).
[2/]DAT= Day after treatment

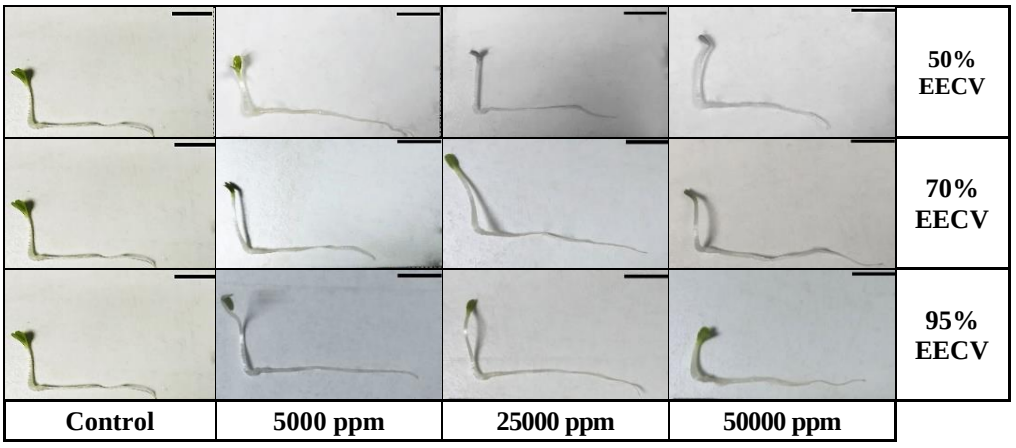


Figure 2. Effect of crude ethanolic extract from *C. viminalis* leaves on lettuce seedling at 5 DAT
Scale bar = 10 mm

Table 6. Effect of crude ethanolic extracts from *C. viminalis* leaves on lettuce growth in hydroponics

Factor A	Factor B	Phytotoxicity		Plant parameters					
		3 DAT[2/]		Leaf number		Leaf size (cm)		SPAD value	
		Necrosis	Discoloration	7 DAT	21 DAT	7 DAT	21 DAT	7 DAT	21 DAT
50% EECV	0 ppm	-	-	8.4ab[1/]	16.6b	9.2d	17.3a	36.3d	35.1ab
	5000 ppm	-	-	8.4ab	19.3ab	11.8a	18.3a	37.3cd	33.6b
	25000 ppm	+++	-	8.0b	17.6ab	11.2ab	17.7a	39.2b	35.6ab
	50000 ppm	+++	-	9.0a	19.6a	9.6cd	16.4a	41.9a	34.8ab
70% EECV	0 ppm	-	-	8.4ab	16.6b	9.24d	17.3a	36.3d	35.1ab
	5000 ppm	-	-	8.2ab	20.0ab	11.1ab	17.7a	38.5bc	35.2ab
	25000 ppm	-	-	8.5ab	20.3a	11.9a	18.3a	38.0bc	36.4ab
	50000 ppm	-	-	8.4ab	19.6a	10.1bcd	18.4a	38.5b	36.7ab
95% EECV	0 ppm	-	-	8.4ab	16.6b	9.2d	17.3a	36.3d	35.1ab
	5000 ppm	-	-	9.0a	19.0ab	10.8abc	18.3a	38.7bc	34.7b
	25000 ppm	-	-	8.8a	19.3ab	10.2bcd	18.5a	39.4b	36.4ab
	50000 ppm	-	-	8.8a	19.0ab	9.6cd	18.7a	38.4bc	38.5a
C.V. (%)				8.81	7.41	12.83	19.98	3.62	12.62
Value of factor A				ns	ns	ns	ns	ns	ns
Value of factor B				ns	**	ns	ns	**	ns
A×B				ns	ns	ns	ns	**	ns

[1/]Values are means of three replicates .Values in each column followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P>0.05).

[2/]DAT= Day after treatment

on some growth parameters and yield. At 21 DAT, the extracts were shown to increase the leaf numbers, while a significant increase was recorded at 50% EECV (5,000 ppm) having 19.6 leaves and 70% EECV (25,000 and 50,000 ppm) having 20.3 and 19.6 leaves compared to control (16.6 leaves). Leaf size and leaf greenness expressed by SPAD values were not significantly affected by the extracts (Table 6).

With regard to lettuce yield, fresh and dry weights of stem and root of the treated lettuces were significantly greater than control (Figures 3 and 4). The extract at 5,000 ppm of 70% EECV significantly stimulated lettuce growth in terms of stem fresh weight over control about 30 percent. The enhancing effect on some growth parameters and yield was probably due to phenolic compound present in the extract. Our findings were in accordance with Bali *et al.* [17] who reported that no phytotoxic effect of 1% and 2% aqueous leaf extract of *C. viminalis* was observed on rice whereas its growth and yield attributes increased, probably due to the release of allelochemicals (mainly phenolics). On the contrary, the aqueous leaf extract of *C. viminalis* still exhibited phytotoxicity as well as inhibited germination and growth of rice weed [17]. In addition, quite a number of reports mentioning that seed germination, growth and yield attributes of lettuce crop were not affected by negative effects of the plant extracts such as clove, colander and cinnamon extracts [28]; orange, mango, jabuticaba and guava leaf extracts [29]; *Ocimum tenuiflorum* leaf extract [30]. In regard to *C. viminalis*, our results seemed to contradict with earlier finding of Oliveira *et al*. [31] which stating on allelopathic activity of *C. viminalis* and negative effect of its essential oil on lettuce seedlings and causing a reduction in the length of shoots and root system. This contradiction result was probably due to the differences between phytochemical compounds in essential oil and ethanolic crude extract which were responsible for growth-enhancing or growth-retarding effect.

Overall, our results seem to be very promising as the crude ethanolic extracts from *C. viminalis* showed significant inhibition against the two tested fungal pathogens. The extracts showed no adverse effect on lettuce seed germination, less or no phytotoxicity on lettuce leaves with significant growth-enhancing effect on the lettuce grown in hydroponics about 30% above control.

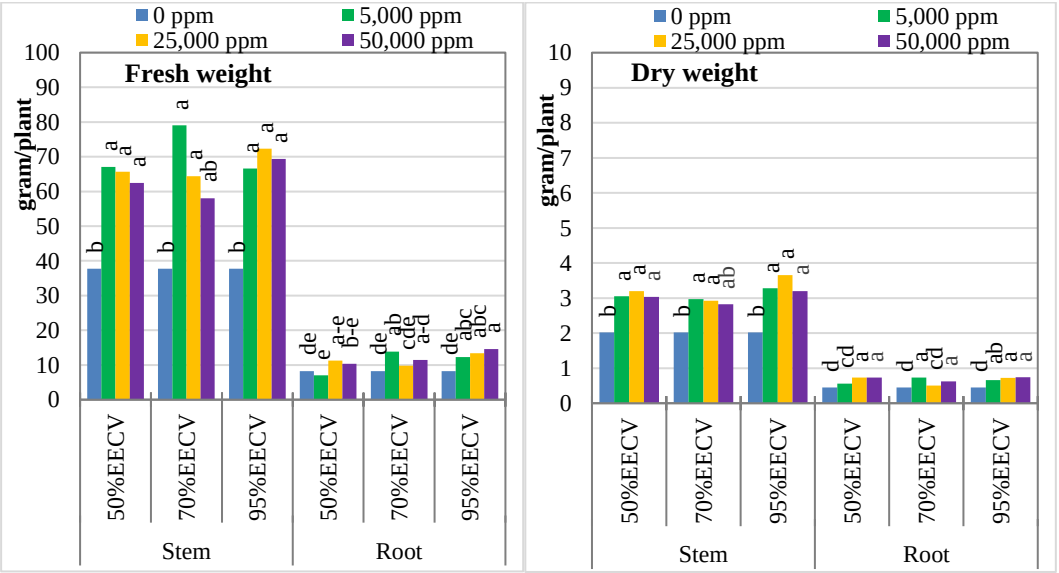


Figure 3. Yield of lettuce grown in hydroponics treated with ethanolic extract from *C. viminalis* leaves. The same letter on column bar is not significantly different according to Duncan's Multiple Range Test (P>0.05)

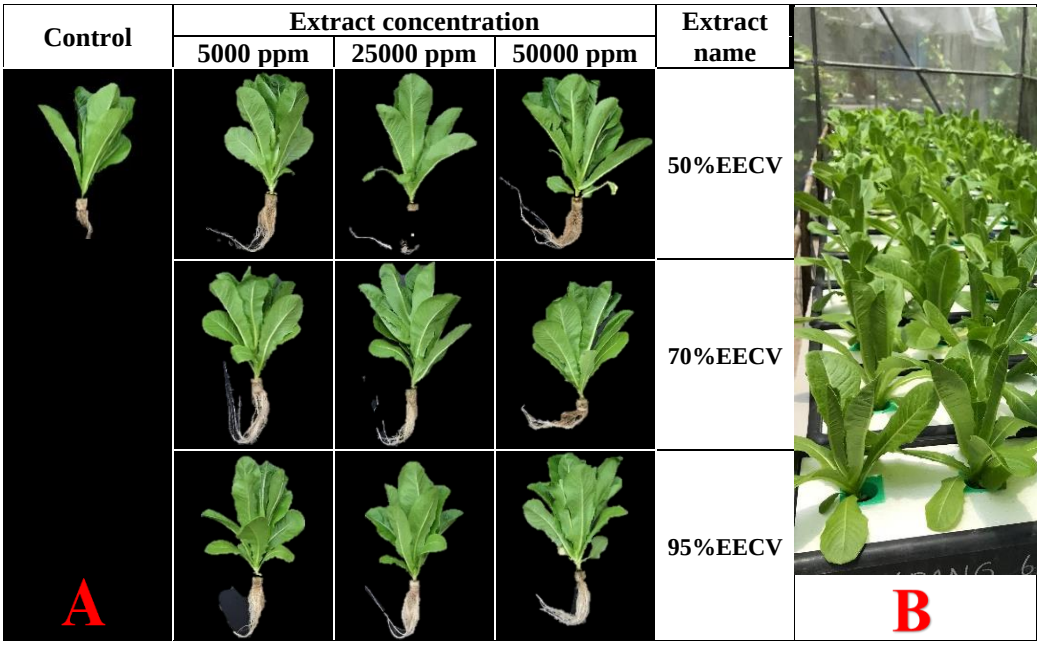


Figure 4. Effect of crude ethanolic extracts from *C. viminalis* leaves on yield of lettuce grown in hydroponics. A = Representative of lettuce treated with crude ethanolic extract from *C. viminalis* leaves, B= All treated lettuce grown in hydroponics

4. Conclusions

In conclusion, crude extracts from *C. viminalis* leaf (using 50%, 70% and 95% ethanol as solvent) were found to have high antifungal activities against *in vitro* mycelial growth and sporulation of leaf spot fungi (*Alternaria* sp. and *Curvularia* sp.). The highest concentration (50,000 ppm) of 50% and 95% ethanol crude extracts gave the strongest inhibitory effect. No phytotoxicity of crude extract was noted either during seed germination or growth of tested lettuce in hydroponics. Interestingly, foliar spraying of the extracts induced positive effects on the growth and yield of lettuce in hydroponics more than control treatment. For quantitative assay of crude extract, its highest yield was obtained from 95% ethanol solvent. Highest total phenolic and flavonoid contents were detected at the highest concentration of the tested crude extracts.

Our findings provided evidence that *C. viminalis* crude extract could be a new potential source for the development of an alternative natural fungicide to manage some plant pathogenic fungi. Further research would be necessary to precisely identify the chemical substances in the extract responsible for antifungal activity as well as plant growth-enhancing effect.

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