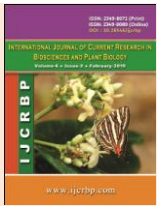
EXCELLENT PUBLISHERS

International Journal of Current Research in
Biosciences and Plant Biology

Volume **6** • Number **2** (February-2019) • ISSN: 2349-8080 (Online)

Journal homepage: www.ijcrbp.com



Original Research Article **doi:** https://doi.org/10.20546/ijcrbp.2019.602.005

In vitro allelopathic activity of aqueous leaf extract of *Hyptis suaveolens* (L.) Poit. against growth parameters of plant seeds and *in situ* analysis of rhizosphere microflora

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Article Info

Date of Acceptance:
22 January 2019

Date of Publication:
06 February 2019

Keywords

Allelopathy
Germination
Hyptis suaveolens
Inhibition
Rhizosphere

ABSTRACT

The allelopathic effect of aqueous leaf extract of *Hyptis suaveolens* were tested against *Oryza sativa* and *Brassica juncea* seeds and plant growth parameters such as seed germination, speed of germination, mean germination time, phytotoxicity, radicle and plumule length, seed vigour index, percentage of toxicity and relative germination ratio were studied. Different concentrations (20, 40, 60, 80 and 100 %) of *H. suaveolens* aqueous extracts were irrigated in test plates and the control plate was irrigated with sterile distilled water. For each treatment, three different replicates were tested with 10 seeds each. 5 ml of each of aqueous extracts were added to Petri dishes laid with filter paper every day to avoid drying out of filter paper during the course of experiment. The Petri plates were kept in a germinator (25±3˚C, 70% humidity and 12 h photoperiod) for 7 days. Germinated seeds (considered when radical emerges ≥ 1mm) were daily counted for 7 days and measurements of both radicle and plumule were noted. To evaluate the allelopathic activity against microorganism, rhizosphere soil samples were collected from four different sites from *H. suaveolens* and from a control non-phenolic plant, *Crotalaria pallida*. Soil samples were serially diluted to different concentrations and were plated on correspondingly labeled nutrient agar and potato dextrose agar plates. Analysis of rhizosphere soil of *H. suaveolens* and *Crotalaria pallida* (control plant) were also studied from four different locations and found that the bacterial and fungal count of rhizosphere soil of *H. suaveolens* was low when compared to *C. pallida* rhizosphere soil.

Introduction

Hyptis suaveolens (L.) Poit. belongs to Lamiaceae family is a soft suffrutescent and ruderal weed that normally grows along the roadsides and the wet margins of ponds. The plant is native to tropical America but now distributed throughout the world from tropical to subtropical regions and, therefore, the plant is sometimes regarded as pantropical weed (Afolayan, 1993; Sarmiento, 1984).

Plants release various compounds into their surroundings that have either deleterious or beneficial effects on other plants in their vicinity. This naturally occurring chemical interaction between plants is known as allelopathy (Rice, 1984). Allelopathic potential is an important attribute to the success of an invasive species in natural ecosystems, particularly when the species produces novel biochemical weapons (Callaway and Ridenour, 2004).

Allelochemicals (residues, exudates, and leachates by many plants from leaves, stem, roots, fruit and seeds) are a type of secondary metabolites which impose environmental stress on other plants growing in their vicinity. Allelopathy is a characteristic feature of certain plants, algae, bacteria, corals and fungi. The non-nutritional secondary metabolites produced by an organism of one species that affect the growth and population biology of individuals of other species are known as allelochemicals (Minorsky, 2002; Callaway and Ridenour, 2004). Interactions associated with allelochemicals produced by invasive or native plant species have the potential to impact seed germination, seedling growth, development and establishment of neighboring plant species, as well as of the same species, in both natural and agricultural systems (Dorning and Cipollini, 2006; Lara-Nunez et al., 2006) and also inhibit the growth of recipient soil microorganisms.

The plant materials such as dried roots, leaves, stems, branches, fruits and seeds left behind after completion of life cycle is generally termed as residue. Litter is one of the important forms of decomposing residue. The decomposition of plant residues adds a large quantity of allelochemicals to the rhizosphere (Goel, 1987), which is influenced by nature of the residue, soil type and the conditions of decomposition (Mason-Sedum *et al*., 1986). The decomposing plant materials may not get evenly distributed throughout the soil and hence as the roots will grow through the soil and wherever and whenever they come in contact with decomposing residue; they may get affected by allelochemicals. The compounds released into the soil are subjected to transformation by soil microflora and produce biologically more active products than original compound (Blum and Shafer, 1988). These residues influence not only the crop emergence, growth and productivity but also influence similar aspects of weed growth. Moreover the microbial population of rhizosphere is significantly affected by the interactions between plant roots, soil, and microbes that significantly alter soil physical and chemical properties (Nihorimbere et al., 2011). Plant root exudates mediate the interactions between plant roots and the microbial communities in the rhizosphere (Badri et al., 2013; Chaparro et al., 2013).

The present investigation was carried out to evaluate the allelopathic activity of aqueous leaf extract of *H. suaveolens* on the growth and development of *O. sativa*, *B. juncea* seeds and to enumerate the rhizosphere bacterial and fungal population from *H. suaveolens* and a control plant *C. pallida*.

Materials and methods

Plant materials

The plant, *Hyptis suaveolens* was collected from S.D.V. College of Arts and Applied Science campus, Alappuzha, Kerala, India. The plant material was identified by Dr. Shaji P. K., Scientist, Environmental Resources Research Centre (ERRC), P.B. No. 1230, P.O. Peroorkada, Thiruvananthapuram, Kerala State, India. The plant leaves were washed several times with water, shade dried and then pulverized to coarse powder in an electric grinder. The powder was then stored in airtight bottles for further studies. The test plant seeds of *Oryza sativa* and *Brassica juncea* were purchased from local market.

Preparation of plant extract

One hundred gram of dried leaf powder of *H. suaveolens* were weighted and extracted in 100 ml sterile distilled water. The powder was kept in sterile distilled water for 24 hours in a reagent bottle at room temperature and was filtered using Whatman filter paper No 1. The pH of the extract was adjusted to 7 and this extract was further diluted to 20, 40, 60, 80 and 100 % and used for further studies.

Surface sterilization of seeds

The seeds were surface sterilized separately in 0.1% mercuric chloride and washed thoroughly with distilled water twice.

Seed germination

Ten surface sterilized seeds of *O. sativa* and *B. juncea* were placed separately in 9 cm Petri plates. Different concentrations of *H. suaveolens* aqueous extracts were irrigated solely with concentrations of 20, 40, 60, 80 and 100 % along with control plate with sterile distilled water. For each treatment, three replicates were tested with 10 seeds each. 5 ml of each of aqueous extracts were added to Petri dish laid with filter paper every day to avoid drying out of filter paper during the course of experiment. The Petri plates were kept in a germinator (25±3˚C, 70% humidity and 12 h photoperiod) for 7 days and the percentage germination was also calculated. Germinated seeds (considered when radical emerges ≥ 1mm) were daily counted for 7 day. The speed of germination, final germination percentage (G %) and mean germination time (MGT) (i.e., time from imbibition to radicle emergence) were calculated as follows.

Speed of germination

The speed of germination was calculated using the formula

$$S = [N_1/1 + N_2/2...] \times 100$$

Where N_i is the number of seeds germinated on i^{th} day.

Mean germination time

The Mean Germination Time was calculated using the method of Ganaie et al. (1992):

$$MGT = \sum (n \times d)/N$$

Where, n is the number of seeds which germinated after each period in days (d) and N is the total number of seeds germinated at the end of experiment.

Phytotoxicity

The phytotoxicity (PT) of the target species was expressed as a percentage of the germination at different concentrations with respect to control, higher values indicating lower toxicity (Cayuela et al., 2007).

$$PT = [1-(allelopathic/control)]/100$$

Radicle and plumule length

Mean radicle and plumule length was measured after germination per replication with a centimeter (cm) ruler from the first day to the seventh day.

Seed vigor index

Seed vigour is an important quality parameter which needs to be assessed to supplement germination and viability test to gain insight into the performance of a seed in the field or in storage. Seed vigour is defined as the sum total of those properties of the seed which determine the level of activity and performance of the seed during germination and seedling emergence. Seed vigor index (SVI) was calculated by using formula

$$SVI = \text{Germination percentage} \times \text{seedling length}$$

Percentage of toxicity

The percentage of toxicity of the seedling was calculated by the formula proposed by Chiou and Muller (1972).

$$\text{Phytotoxicity} = \frac{\text{Radical length of control} - \text{Radical length of treated sample}}{\text{Radical length of control}} \times 100$$

The relative germination ratio

The Relative Germination Ratio (RGR) was calculated using the method described by Rho and Kil (1986).

$$RGR = GRt / GRc \times 100$$

Where, RGR is the relative germination ratio, GRt is the germination ratio of plants under treatment and GRc is the germination ratio of plants under control.

Analysis of rhizosphere microorganisms

One gram of soil sample from the rhizosphere region of *H. suaveolens* and soil sample from a control plant (*Crotalaria pallida*) were collected from four different locations to ascertain the total number of microorganisms (bacteria and fungi) present. The soil sample was serially diluted to different dilutions such as 10^{-2}, 10^{-3},

and 10^{-4}. Using pour plate technique each dilution was plated in correspondingly labeled Petri plates in triplicates. Nutrient agar plates were incubated at 37°C and potato dextrose agar plates were incubated at 25°C. In the case of nutrient agar plates, the microbial colonies were counted for each dilution after 24 h of incubation and in the case of potato dextrose agar, the microbial colonies were counted for each dilution after 48 h of incubation.

Results and discussion

Aqueous extract of *H. suaveolens* inhibited only 10 % seed germination (Table 1). Similarly, radicle length of the seedling was decreased from 6.45cm to 5.80 cm with an increase in concentration of leaf extract from 0.0% to 100%. The decrease was negligible and similar observation was noted for the length of plumule as well i.e. 5.04 cm–4.03 cm (Tables 2 and 3).

Table 1. Effect of aqueous extract of *H. suaveolens* on seed germination of *O. sativa* on 3rd day.

Concentration of extract (%)	Number of seeds germinated	Number of seeds sown	Percentage of germination	Percentage of inhibition
Control	10	10	100	0
20	9	10	90	10
40	10	10	100	0
60	9	10	90	10
80	10	10	100	0
100	10	10	100	0

Table 2. Average radicle length (cm) of *O. sativa*.

Plant	Days	Control (water)	*H. suaveolens* leaf extract concentrations (%)				
			20	40	60	80	100
O. sativa	1	0	0	0	0	0	0
	2	0.82	0.88	0.78	0.42	0.33	0.30
	3	1.38	1.94	0.86	0.68	0.50	0.55
	4	4.05	2.90	2.05	1.50	1.35	1.23
	5	4.88	4.46	4.20	3.90	3.85	3.80
	6	5.50	5.22	5.30	4.98	4.90	4.80
	7	6.45	6.30	6.28	6.10	5.85	5.80

Table 3. Average plumule length (cm) of *O. sativa*.

Plant	Days	Control (water)	*H. suaveolens* leaf extract concentrations (%)				
			20	40	60	80	100
O. sativa	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	1.25	1.06	1.02	0.86	0.72	0.60
	4	1.84	1.60	1.35	1.30	0.98	0.80
	5	3.94	3.66	3.58	3.22	2.86	2.70
	6	4.83	4.61	4.72	4.32	3.99	3.84
	7	6.68	6.47	6.34	5.90	5.82	5.75

The *in vitro* analysis of aqueous leaf extract of *H. suaveolens* on *B. juncea* revealed that the percentage of germination was significantly reduced from 80–40% with increase in extract concentration. This observation was in agreement with the length of radicle and plumule observed. The length of radicle reduced significantly from 5.04 cm to 4.03 cm with an increase in leaf extract from 0.0% - 100%. Similarly the plumule length was also reduced significantly from 6.22 cm to 3.90 cm. Values obtained are given from Table 4-6. In a similar report, the dry leaf-residue of *H. suaveolens* significantly reduced the germination and growth parameters (number of leaves, branches, length of the leaves, plant height, capitula/ plant and seeds/five capitula) of *Parthenium hysterophorus* plant in pot culture (Kapoor, 2014). Mominul Islam, 2014, isolated the phytotoxic substance, suaveolic acid (14α-hydroxy-13β-abiet-8-en-18-oic acid), from *H. suaveolens* and reported that at concentrations greater than 30 µM, suaveolic acid found to exhibit phytotoxicity against the shoot and root growth of garden cress, Italian ryegrass, and barnyard grass and lettuce shoots.

Table 4. Effect of aqueous extract of *H. suaveolens* on seed germination of *B. juncea* on 7th day.

Concentration of extract (%)	Number of seeds germinated	Number of seeds sown	Percentage of germination	Percentage of inhibition
Control	8	10	80	20
20	7	10	70	30
40	6	10	60	40
60	5	10	50	50
80	4	10	40	60
100	4	10	40	60

Table 5. Average radicle length (cm) of *B. juncea*.

Plant	Days	Control (water)	*H. suaveolens* leaf extracts concentrations (%)				
			20	40	60	80	100
B. juncea	1	0.77	0.62	0.68	0.59	0	0
	2	1.02	0.92	0.89	0.81	0.80	0.74
	3	1.20	0.85	0.54	0.40	0.40	0.45
	4	1.89	1.48	1.25	1.18	1.08	1.02
	5	2.96	2.79	2.52	2.37	2.22	2.10
	6	4.27	4.02	4.01	3.97	3.77	3.68
	7	5.04	4.88	4.79	4.54	4.22	4.03

Table 6. Average plumule length (cm) of *B. juncea*.

Plant	Days	Control (water)	*H. suaveolens* leaf extracts concentrations (%)				
			20	40	60	80	100
B. juncea	1	0	0	0	0	0	0
	2	1.56	1.48	1.33	1.21	1.10	0.92
	3	2.20	2.14	1.98	1.68	1.21	1.01
	4	2.70	2.55	2.15	1.90	1.30	1.10
	5	3.91	3.86	3.89	3.77	3.24	2.62
	6	4.89	4.78	4.65	4.45	4.39	3.47
	7	6.22	5.98	5.67	5.22	5.01	3.90

Table 7. Speed of germination, mean germination time and phytotoxicity of *O. sativa* and *B. juncea*.

Plant	Parameters	Control	Concentration of leaf extract (%)				
			20	40	60	80	100
O. sativa	Speed of germination	783.3	433.3	733.3	700	583.3	533.3
	Mean Germination Time (days)	3	2.7	3	2.7	3	3
	Phytotoxicity (%)	Nil	10	0	10	0	0
B. juncea	Speed of germination	833.3	600	766.6	566.6	250	283.3
	Mean Germination Time (days)	2	2	2	2	2	1.8
	Phytotoxicity (%)	Nil	12.5	25	37.5	50	50

From the Table 7, it is evident that the aqueous leaf extracts of *H. suaveolens* have significant impact on speed of germination of both the test plants. At 100% concentration the speed of germination of *O. sativa* showed a difference of 250 with respect to control. However, *B. juncea* showed a difference of almost 550. Thus it is clearly evident that *B. juncea* showed the highest degree of inhibition, when compared to *O. sativa*. Similar pattern of inhibition was observed for mean germination time. *B. juncea* showed a difference of 1.2 at 100% extract concentration, while *O. sativa* showed no difference at all.

Phytotoxicity values obtained was very high for *B. juncea* when compared to *O. sativa*. At 100% concentration, *B. juncea* showed a phytotoxicity of 50, while *O. sativa* showed a value of 0. It is very clear from the experimental data that when the concentration of the extract was increased, phytotoxicity values also increased (Table 7). Similar results were reported on the growth of *Phaseolus vulgaris* L. subjected to aqueous leaf, stem and flower extracts of *Parthenium hysterophorus* L. Although aqueous flower extracts showed maximum phytotoxic effect than leaf and stem extracts (Tahseen et al., 2015). The allele-chemicals often regulate the distribution and vigour of plants. Allelopathy plays an important role in agricultural ecosystems and in a large scale, in the plant covers among the crop-crop, crop-weed and tree-crop covers. These interactions are detrimental and occasionally, are useful and gave attention to allelopathy in natural and agricultural ecosystems. Today, allelopathy is recognized as appropriate potential technology to control weeds using chemicals released from decomposed plant parts of various species (Naseem et al., 2009).

Table 8. Seed vigour index, percentage toxicity and relative germination ratio.

Plant	Parameters	Control	Concentration of leaf extract (%)				
			20	40	60	80	100
O. sativa	Seed vigour index	651.68	636.47	631.34	615.9	590.82	585.75
	Percentage toxicity	Nil	2.33	3.88	5.43	9.30	10.08
	Relative germination ratio	Nil	100	100	100	100	100
B. juncea	Seed vigour index	384.22	347.58	393.07	209.52	173.81	175.9
	Percentage toxicity	Nil	3.17	4.96	9.92	16.27	20.04
	Relative germination ratio	Nil	93.33	80	60	53.33	53.33

Control showed the highest values for seed vigour index each day. Highest value was showed at 7th day in control plate, 651.75. Lowest set of values were observed in 100% concentration. The lowest value at 7th day was 585.75 showed in 100 %. Seed vigour index was found to be decreasing with the increase in the concentration of aqueous extract. The seed vigour index values of *O. sativa* showed a gradual decrease as the concentration increased. The results of seed vigour index on 7th day are given in table 8. Similar observation was recorded for *B. juncea* as well. Highest value was at 7th day in control plate, 384.22. Lowest set of values were showed in 100%. The lowest value at 7th day was 175.9 showed in 100%. As the concentration of plant extract was increased, the values of seed vigour index also decreased. The values of seed vigour index of 7th day are given in Table 8.

Similar reports on the growth of tomato, radish, cucumber and barnyard grass being inhibited via water extract of *Lantana camara* (Liu and Jia, 2002), and inhibition in root initiation, number of roots and root length of hypocotyls of mung bean and pea was observed when treated with leaf extract of *Eucalyptus urophylla* (Huang et al., 1997). Literature also revealed that the inhibitory effect of the aqueous extract of five weed species namely *Alternanthera sessilis*, *Echinochloa colona* L., *Tridax procumbens* L., *Parthenium hysterophorus* L. and *Cyprus tuborus* upon germination of crop plants like black gram, cluster bean, cotton, ladies finger and rice (Rao, 1987). The findings of the present study was also confirmed the weedicidal effect of the aqueous extract of *Hyptis suaveolens* thereby showing its allelopathic potential.

Percentage toxicity for both plants increased as the concentration of leaf extract increased. This is due to the increased allelopathic activity as the extract concentration is increased. *B. juncea* showed the highest value for percentage toxicity. For 100% extract, *B. juncea* showed the toxicity percentage of

20.04 while, *O. sativa* exhibited a value of 10.08. Percentage toxicity of two plants of 7th day is given in Table 8.

It is to be noted that the value for relative germination percentage for *O. sativa* was 100 % across all concentrations on 7th day of experiment. On the other hand, *B. juncea* showed reducing values as the concentration increased. For 20 % and 100% extract, the values obtained were 93.33 and 53.33 respectively. This clearly indicated the degree of allelopathic effect of extract on the seed germination of *B. juncea*.

Rhizosphere microbial analysis

The results showed that the microflora of rhizosphere soil samples from *H. suaveolens* were low when compared to the *C. pallida*. In Site I, 10^{-3} dilution gave a bacterial count of 99 × 10^3 and 266 × 10^3 colony forming units (CFU) /ml for *H. suaveolens* and *C. pallida* respectively (table 9) and gave a fungal count of 16 × 10^3 and 28 × 10^3 CFU/ml respectively (Table 10). In site II, bacterial count observed for 10^{-3} dilution was 266 × 10^3 CFU/ml and TNTC (too numerous to count) for *H. suaveolens* and *C. pallida* respectively.

Table 9. Bacterial count under *H. suaveolens* and *C. pallida*.

Dilution	Rhizosphere bacterial colonies under test and control (CFU/ml)							
	H. suaveolens				***C. pallida***			
	Site I	**Site II**	**Site III**	**Site IV**	**Site I**	**Site II**	**Site III**	**Site IV**
10^{-1}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-2}	TNTC	TNTC	231	210	TNTC	TNTC	218	322
10^{-3}	99	266	68	93	266	TNTC	121	164
10^{-4}	42	72	6	8	72	264	16	18

Table 10. Fungal count under *H. suaveolens* and *C. pallida*.

Dilution	Rhizosphere fungal colonies under test and control (CFU/ml)							
	H. suaveolens				***C. pallida***			
	Site I	**Site II**	**Site III**	**Site IV**	**Site I**	**Site II**	**Site III**	**Site IV**
10^{-1}	110	120	132	65	TNTC	TNTC	TNTC	TNTC
10^{-2}	43	50	39	41	79	118	228	174
10^{-3}	16	18	13	14	28	42	10	19

In the case of fungal population, the colony count observed was 18 × 10^3 CFU/ml and 42 × 10^3 CFU/ml. Microbial count from other two sites site III and IV) are given in table 9 and 10. From the above set of readings, it can be concluded that the leaf leachates from the fallen leaves and root exudates of *H. suaveolens* may contain phenols and terpenoids which may have inhibited the soil microflora of the rhizosphere of *H. suaveolens*. The release of allelochemicals of different chemical classes from allelopathic plant species includes tannins, cyanogenic glycosides, several flavonoids and phenolic acids such as ferulic, p-coumaric, syringic, vanillic, and p-hydroxybenzoic acids (Einhellig 1995). Suaveolic acid, a major compound found in *H. suaveolens,* may get released into the surrounding environment through the decomposition of their aerial plant parts (leaf or stem) and accelerate the invasion or dominance of *H. suaveolens* (Mominul Islam et al., 2014). The rhizosphere is the region that is directly influenced by the roots, and this is where most of the interactions between microorganisms and plants occur (Hirsch et al., 2003). It is characterized by a greater proliferation of microbial populations due to the transfer of C from the root to the soil as exudates, secretions, lysate, and mucilage (Baudoin et al., 2003). Under certain conditions phytotoxic substances may get released from the phytotoxic plants and suppress the germination, growth, and establishment of neighbouring plants by affecting their physiological properties (Yu et al., 2003; Weir et al., 2004) or indirectly by modifying the rhizosphere soil properties through influencing the microbial biomass carbon and microbial community (Yu et al., 2005; Meier et al., 2008; Zhou et al., 2013).

Conclusion

From the present study, it is evident that the aqueous leaf extract of *H. suaveolens* had negative effect on seed germination and further growth of

O.sativa and *B. juncea*. Among them *B. juncea* showed the highest degree of negative allelopathy. Thus it is clear that the allelochemicals from the leaf extract of *H. suaveolens* had more impact on the germination and subsequent development of seedlings of *B. juncea* when compared to that of *O. sativa*. Analysis of the rhizosphere soil samples of *H. suaveolens* revealed that the root exudates along with leaf leachates reduced the rhizosphere bacterial and fungal population when compared to control plant. The reduction in bacterial and fungal population along the rhizosphere of *H. suaveolens* can be attributed to the presence of certain allelochemicals.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

The authors are grateful to The Manager and Principal, S.D.V. College of Arts and Applied Science, Alappuzha and The Director, K.V.M. College of Science and Technology, Cherthala, Kerala, India, for providing necessary facilities and support.

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How to cite this article:
Pratap Chandran, R., Ig Barry, A., Reshma, S., Vineetha, J., Neeraja, U., Aswathy, S., 2019. *In vitro* allelopathic activity of aqueous leaf extract of *Hyptis suaveolens* (L.) Poit. against growth parameters of plant seeds and *in situ* analysis of rhizosphere microflora. Int. J. Curr. Res. Biosci. Plant Biol. 6(2), 61-69.
doi: https://doi.org/10.20546/ijcrbp.2019.602.005