

Full Length Research Paper

Allelopathy of *Teucrium Royleanum* Wall. Ex Benth. from Pakistan

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Teucrium royleanum was tested by four variant assays using lettuce seeds, showing excellent inhibition. The plants leaf leachates were assayed by Sandwich and Homogenated Sandwich Methods while Dish Pack Method was used for the evaluation of essential oils. The above stated methods confirm the presence of allelochemicals in *T. royleanum* and were further evaluated in bioassay guided way by total activity approach. The methanol subsequent fractions; hexane, chloroform, ethylacetate, butanol and water fractions were assayed by the Total Activity Method. The essential oils, hexane and chloroform fractions played a decisive role in our findings. The results from this study suggested that the secondary metabolites from *T. royleanum* can be potential candidates for the phytotoxicity (Allelopathy) and can be utilized on commercial scale for the development of new herbicides or weedicides.

Key words: Allelopathy, *Teucrium royleanum*, lettuce seeds, leaf leachates, extracts essential oils.

INTRODUCTION

Teucrium is a genus comprising some 300 species four of which are wild growing in Pakistan (Ali and Nasir, 1990). Different volatile secondary metabolites are released from one plant specie in its surrounding resulting in either stimulation or inhibition on the near by another plant specie termed as allelopathy (Rice, 1992; Sajjad et al., 2007; Khan et al., 2009). Generally the allelopathy is accepted as an important ecological factor in determining the chemistry of plant communities (Lorber and Muller, 1976; Mizutani, 1989; Seigler, 1996). The dependence of allelopathic effect is upon release of certain compounds into the environment known as allelochemicals (Whittker and Feeny, 1971). These are biosynthesized in the plants

as secondary metabolites such as tannins, phenolics acids, lignins, alkaloids, flavonoids, coumarins, and terpenoids, and may be present in all tissues including leaves, stems, roots rhizomes, flowers, fruits, and seeds and even trichomes and pollens (Waller, 1987).

Allelopathy is not a matter of controversy any more as different methods are available to evaluate the allelopathic potential of different medicinal plants, highlighting the significance of allelopathy with the corresponding biological control of herb and enhance crop productivity (Fujii et al., 1991, 1992, 2003, 2004; Khan et al., 2009).

In our studies on the allelopathy of *T. royleanum* we have utilized four different methods namely, Sandwich and Homogenated Sandwich, Dish Pack and Total activity methods. The first two stated methods (Sandwich and Homogenated Sandwich) were used for the initial

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screening to know the effect of secondary metabolites in the plant leaves on the lettuce seed.

The genus *Teucrium* is famous for the presence of essential oils, which have been recognized since ancient times to exhibit biological activities (Demetzos et al., 1995; Zygadlo et al., 1995; Donata et al., 2005; Djilas et al., 2006; Erdemgil et al., 2007; Fisgin et al., 2009). However, there is no literature available on the allelopathy essential oil of the *T. royleanum* neither on any members of its genus hence was evaluated by the Dish Pack Method (Sekine et al., 2007).

Plants are natural factories, different parts have different classes of secondary metabolites and hence all the compounds cannot be accumulated in essential oil (extracted from aerial plant). Therefore, in a separate set of experiments the plant was grinded and subjected to extraction and subsequent solvent solvent partition to make *n*-hexane, chloroform, ethylacetate, butanol and water subfractions which were evaluated by the approach of Total Activity (Hiradate et al., 2004) for the presence of possible allelochemicals.

The objective of our research was to know the allelopathic potential of *T. royleanum*, and to screen it for the presence of some environmentally friendly weedicides (allelochemicals). Our final findings suggested that some interesting weedicides can be isolated from the bioactive subfractions.

MATERIALS AND METHODS

Plant material collection

The aerial parts of *T. royleanum* were collected at flowering stage during June 2003 from a locality in Shamoza, Swat, Pakistan. A voucher specimen (No. Shabir 2651979(PUP)) was identified by Professor Dr. Abdul Rashid and deposited in the Herbarium of the Department of Botany, University of Peshawar, Peshawar Pakistan. The plant material was packed in boxes containing silica gel to avoid any kind of moisture intrusion inside during transportation from Pakistan to Japan.

Leaf leachates assay

Two assays namely Sandwich (Fujii et al., 2003) and a newly established method of Watanabe lab as Homogenated Sandwich Method were used for the preliminary screening of the *T. royleanum*. Details of these two methods are as follows:

Sandwich method

Agar Powder (Nacalai Tesque Kyoto, Japan) having gelling temperature of 30~ 31°C was used for this methods and 0.75% (w/v) solution was prepared in distilled water. Two different weights (10 and 50 mg) of plant leaves were taken and placed in each well of the multidish plastic plate (6 – wells micro-plat following Fujii et al., 2003, 2004 methods). Then 5 ml of 0.75% of agar solution was added into each well. It was kept for 30-60 min at room temperature (ca. 25°C) for solidification followed by another 5 ml agar addition to each well. The plant material becomes embedded between two agar layers. The experiment was performed in triplicate. To ensure

the re-use of microplate, these were washed with 10% contaminin for 20 min followed by keeping in sonicator for 20 min.

Homogenated sandwich method

Agar solution of 0.5 and 1% was prepared and autoclaved at 115°C for 15 min. After autoclaving, the agar solution was well shaken and was cooled down to ca. 45°C in a water bath. Like sandwich method two different weights 60 (10 mg/well) and 300 mg (60 mg/well) of the plant material was taken and fine grinded using liquid nitrogen. The grinded material was placed in 50 ml Falcon tubes and 20 ml of distilled water (DW) was added subsequently. This was centrifuged (Kobota 5220) at a speed of 3000 rpm for 20 min, resultant supernatant was transferred into a new Falcon Tube while the residue was discarded. Further the supernatant was filtered using Syringe driven filter unit (Millipore Corporation, Bedford USA) of 0.45 µm (Millex-HV PVDF membrane) with the help of 50 ml disposable syringe and transferred into a new falcon tube. Then 1% of equal volume of 20 ml agar solution was added to the filtrate. Now 5 ml of this was added to each well of the 6-well multidish plastic plate (3.5 cm x 3.5 cm D x W x H) which made the same concentration of leaf leachates as sandwich method that is, 10 and 50mg /well. It was left for 30 min to ensure the solidification of the solution. 0.5% agar solution was prepared in distilled water separately, 5 ml of which was added to each well of multidish plastic plate. The solution was left for solidification.

Five lettuce seeds (*Lactuca sativa* L. Great Lakes 366, Takii Seed Co. Ltd. Japan) were added to each well of the multidish plastic plate and then sealed with plastic tape, labeled and kept in an incubator (BIOTEC 300-L) (Shimadzu Rika Institute Co. Ltd. Kyoto, Japan) in order to grow the seedlings for 72 h at 25°C under dark. The results are based on the inhibition and promotion of hypocotyls and roots of lettuce seedlings. The negative or lesser values represent promotion of the root or hypocotyls in comparison with controls. The roots and hypocotyls were measured and percentage of seed germination was also recorded. The experiment was repeated in triplicate.

Essential oil extraction

The essential oil isolation was carried out by means of hydrodistillation process for 3 h using a Clevenger-type apparatus (1.8% v/w). The essential oils extracted were obtained with diethyl ether, the latter then evaporated to give essential oil which was dried with anhydrous magnesium sulphate.

Dish pack method (Sekine et al., 2007)

Essential oil of *T. royleanum* was taken in one well of 6-well multi dish plate and lettuce seeds on filter paper in rest of the five wells. 50 µl of essential oil was weighted and was added into 0.25 ml sample cup (11.0 x 13.5 x 16.3 mm). The cup was placed in the lower left well of the 6 well multi dish plate (0 mm distance well). In the rest of the five wells filter paper was placed. To each well 0.7 ml distilled water was added followed by placement of 7 seeds for germination. The plates then were sealed with plastic tapes and incubated for 72 h in dark at 24°C. After the incubation (72 h) the lengths of hypocotyl and roots were recorded.

Total activity (Hiradate et al., 2004)

The air dried plant material (1.5 Kg) was extracted with 20% Water: Methanol for 15 days. Then it was suspended in water and was

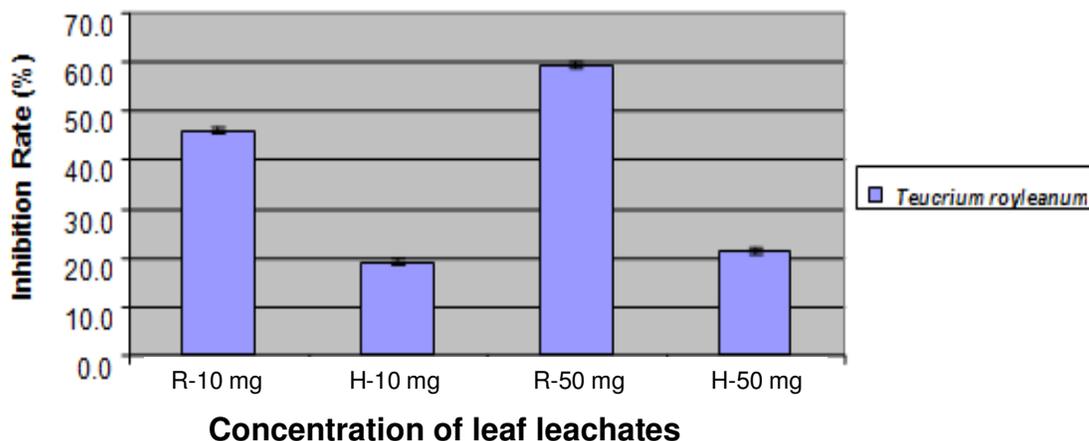


Figure 1. Effects of leaf leachates on the inhibitions of lettuce seeds by Sandwich method.

subjected to solvent-solvent partition to obtain hexane, chloroform, ethylacetate, butanol, and water sub-fractions. Total activity was measured with the help of the following formula:

$$\text{Total activity} = 1/\text{EC}_{50} \times \text{Concentration}$$

EC₅₀ value calculation

1000 ppm (1 mg/ml) of stock solution was prepared in 1% DMSO for each of the above sub-fractions. Working standards: 3, 10, 30, 100, and 300 ppm solutions of each sub-fraction were prepared. A glass Petri dish (27 mm ϕ) was taken and a filter paper (27 mm ϕ , Type Roshi Kaisha, Ltd, Tokyo) was placed inside. The different working standards solutions were added to the filter paper in the Petri dish, followed by the placement of seven lettuce seedlings (*Lactuca sativa* cv. Great Lakes 366) on the filter paper, and was incubated for 72 h at 20°C in the dark. The inhibitory effects at each concentration of all sub-fractions were calculated by measuring the length of both roots and shoots and comparing it with the control.

Statistical analysis

For the resultant data from the aforementioned experiments, means, standard deviation and standard error were calculated to determine the inhibition pattern of different concentrations. The extension rate is the actual rate of root and hypocotyl while inhibition rate shows the suppression of growth against control. The negative values present stimulation while higher inhibition rate shows restraint of lettuce seeds. One way ANOVA was applied to know the significance of concentration on the rate of inhibition of lettuce seeds.

RESULTS AND DISCUSSION

Leaf leachates assay

Dried leaves of *T. royleanum* were subjected for the preliminary screening of phytotoxicity by Sandwich and Homogenated Sandwich method.

Sandwich method

The lengths of both the roots and hypocotyl were measured, the inhibition rates showed higher phytotoxicity. The test plant has inhibited the lettuce roots and hypocotyl elongation of germination to 60 and 22% respectively at 50 mg concentration. While at 10 mg roots and hypocotyl were inhibited to 45 and 20%, respectively (Figure 1). *T. royleanum* leaf leachates exhibited more phytotoxicity at 50 mg compared to 10 mg concentrations to both the roots and hypocotyl elongations (Figure 1).

Homogenated sandwich method

The inhibitions of the roots and hypocotyl of lettuce seeds were 50 and 5% at 300 mg, respectively while at 60 mg, the roots were inhibited to 42% and hypocotyl to 18% (Figure 2).

Comparison of sandwich and homogenated sandwich methods

Two different approaches were used in both the methods. In Sandwich Method, the plant leaves were sandwiched between the agar layers so all the phytotoxic volatile and nonvolatile compounds leaches out from the leaves slowly thus were in direct contact with the lettuce seedlings. While in the Homogenated Sandwich Method the powdered plant material was taken in a falcon tubes containing water thus only the water soluble compounds were taken in consideration which were responsible for showing inhibitory activity. Comparing the results of both the assays (Figure 3) the Sandwich Method exerts more inhibitory effects which may be due to the absence of the more volatile oils (monoterpenes or other volatile

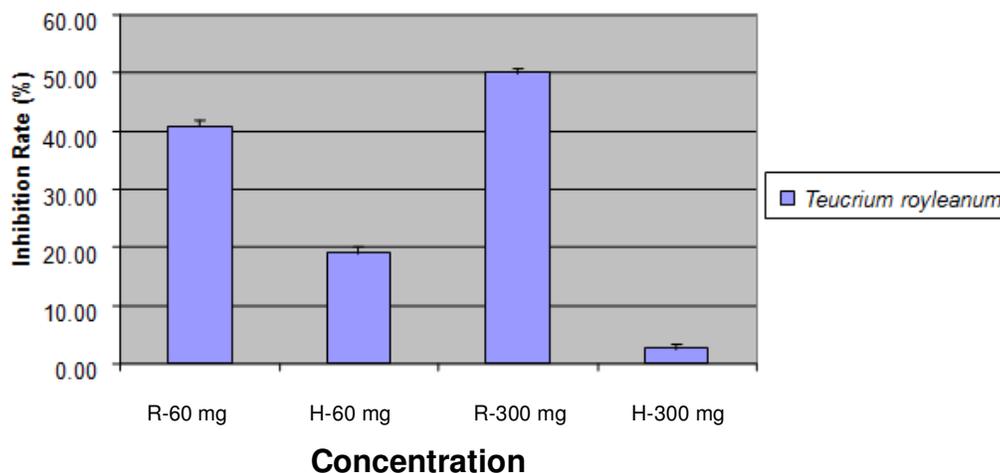


Figure 2. Effects of leaf leachates on the inhibitions of lettuce seeds by Homogenated Sandwich method.

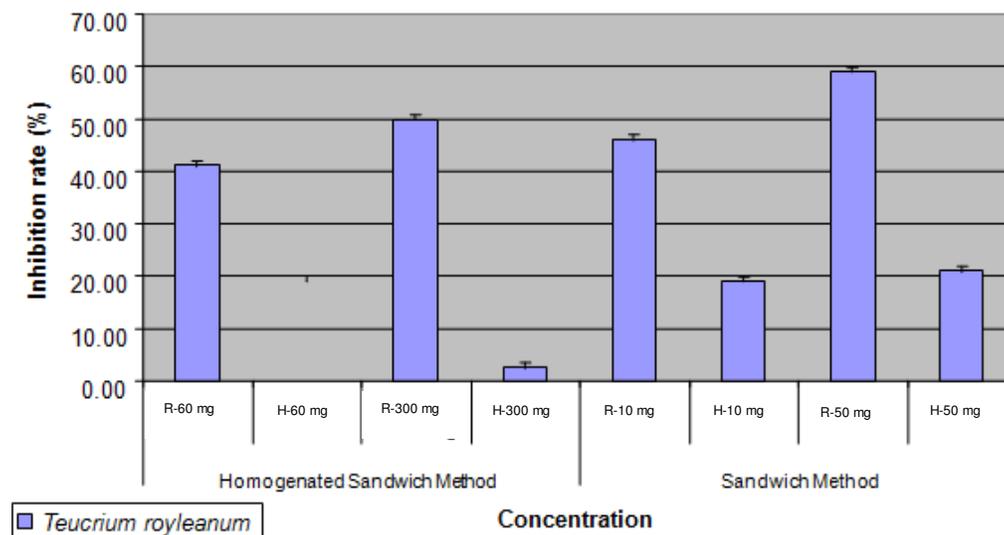


Figure 3. Comparison of the effects of leaf leachates on the lettuce seeds inhibition by both methods.

sesquiterpenes) from the aqueous extract (Homogenated Sandwich Method consideration) due to its insoluble nature in water.

These preliminary results showed the presence of some phytotoxic compounds in the leaves of the test plant. These results prompted us to check the presence of phytotoxic compounds in the volatile oils and in the subfractions of hexane, chloroform, ethylacetate, butanol and water.

Essential oils allelopathy

The essential oils were subjected to phytotoxic studies using Dish Pack Method. The nearest 2 wells to essential

oils have the distance of 41 mm. The upper middle well had distance of 58 mm, furthest lower right 82 mm and furthest upper right had 92 mm distance. The data was recorded according to the distance of the wells from the essential oils that is how far it is from each well. Results showed that essential oils of *T. royleanum* exhibited inhibitory rates of 65 to 100% on shoots and 47 to 65% on roots growth of lettuce seeds. The essential oil showed maximum inhibitory effects at 65 and 100% on both the roots and hypocotyl in 41 mm wells respectively (Figure 4).

Nearest wells were affected to great extents as these compounds are released by processes such as volatilization. The volatiles oil and terpenoids has the tendency to strongly inhibit the germination (Asplund,

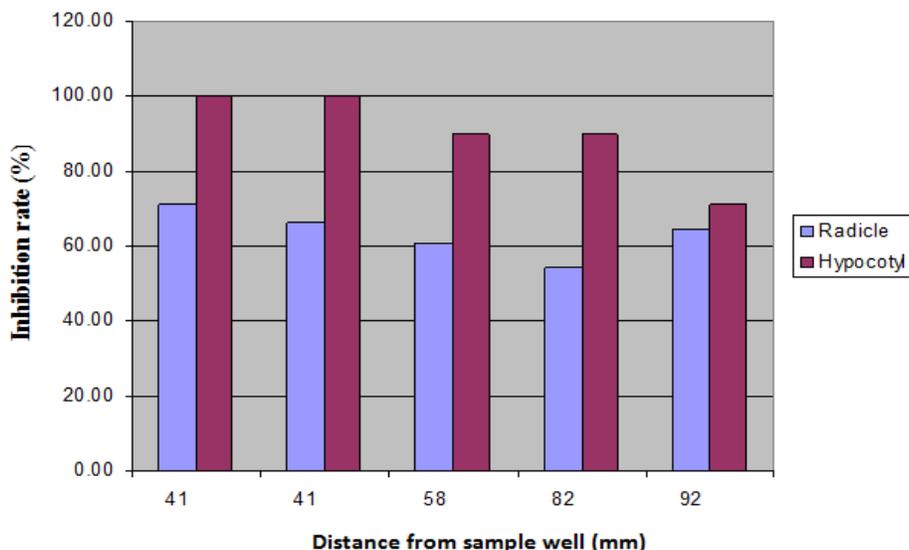


Figure 4. Inhibition rates of lettuce roots and hypocotyl by essential oils of *Teucrium royleanum*.

Table 1. Inhibitory effect of various extracts of *Teucrium royleanum* on the growth of root and hypocotyls.

Concentration	Hexane		Chloroform		Ethylacetate		Butanol		Water	
	R	H	R	H	R	H	R	H	R	H
0 ppm	0	0	0	0	0	0	0	0	0	0
3 ppm	-0.2	3.7	-11.2	9.2	4.2	-3.7	-5.9	-1.8	-15.6	-3.7
10 ppm	-7.3	-6.7	-7.3	1.2	-17.8	-4.3	-10.3	6.7	-29.7	0.6
30 ppm	-7.7	9.2	-4.6	-1.8	-17.4	7.4	15.2	19.6	-12.1	5.5
100 ppm	43.3 (c)	54.0 (b)	-13.0	6.7	-28.4	7.4	1.5	8.0	-13.4	3.7
300 ppm	100.0 (a)	100.0 (a)	35.4 (c)	58.9 (b)	-2.0	8.6	4.6	12.9	-10.3	6.1
ANOVA test	SS	dF	MS	F	P-Value	F crit				
Between groups	8588.083	9	954.2314	1.94149	0.067083	2.073349				
Within groups	24574.73	50	491.494	-	-	-				

R= Root, H = Hypocotyl; ppm = parts per millions; a = highly significant, b = significant and c = moderate level of inhibition rate by the extract's concentration. SS = Sum of squares; dF = denominator of factors; MS = means of square; F = factor; P-value = alfa value; F crit = factorial critical value.

1968; Moral, 1970; Fische, 1991; Kohli and Singh, 1991; Lorber and Muller, 1976; Muller and Muller, 1964; Souto et al., 1995; Vaughn and Spencer, 1993). The GC-MS analysis of *T. royleanum* in our on going project in a separate set of experiment showed that major chemical constituents are monoterpenes hydrocarbons 17.1%, oxygenated monoterpenes 7.6%, sesquiterpene hydrocarbons 42.3%, and oxygenated sesquiterpenes 21.0% (Saroglou et al., 2007). Thus the inhibition caused by the essential oil can be attributed to the presence of these terpenoids.

Methanol and subsequent subfractions

From the preliminary results of above mentioned studies, it was confirmed that *T. royleanum* contains allelochemicals. After confirming its presence in essential

oils, the test plant was further subjected to extraction with 20% methanol and subsequently partitioned into *n*-hexane, chloroform, ethylacetate, butanol, and water sub-fractions. Hexane fraction showed slightly stimulatory effects on roots at 3, 10, and 30 ppm but 100 and 300 ppm concentrations exhibited 43.3 and 100% inhibitory effects, respectively (Table 1) while shoots inhibition was recorded in all the concentration except 10 ppm (Table 1). The sub-fraction of chloroform showed stimulatory effects in all the concentration except higher concentration of 300 ppm for roots and inhibition was caused in shoots lengths except at 30 ppm.

Since the other sub-fractions of ethylacetate, butanol, and water have not shown 50% of the inhibition to the growth of lettuce seed, therefore, they have no EC₅₀ value and the compounds may be absent or of low concentration in these subsequent fractions (Table 1). ANOVA results showed that inhibitory effects were

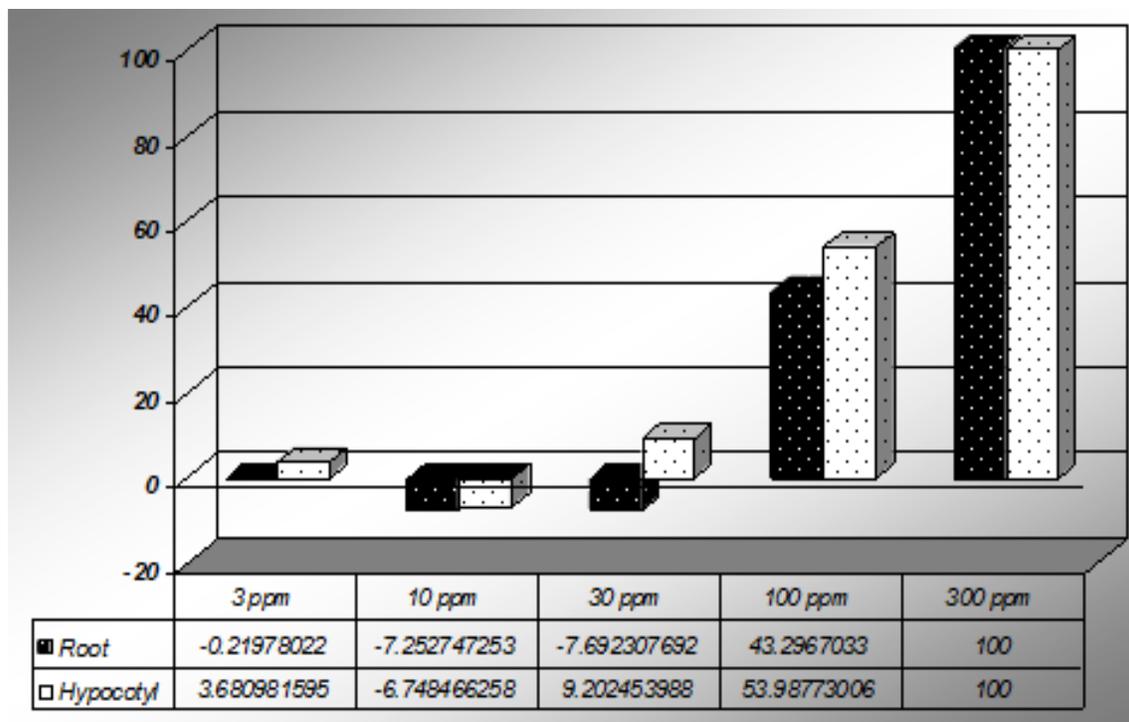


Figure 5. Effect of various concentration of hexane on the lettuce roots and hypocotyls germination.

significantly concentrations dependent. Higher concentrations had higher inhibitory effects and vice versa (Table 1). The probability value (P-value) was 0.1 for the ANOVA. The resultant P-value is 0.067083, which is less than the 0.1 probability level, thus presenting highly significant value (Table 1). The higher concentration of leaf leachates furnish significant inhibitory values which can help in identification of allelopathic potential of the plant species (Hiradate et al., 2004).

EC₅₀ values of all the subfractions of hexane, chloroform, ethylacetate, butanol and water was calculated and the results of the inhibitory activity on the lettuce seeds germination showed that the major activity of the original plant was accumulated into the hexane and chloroform fractions (Figures 5 and 6).

Although the focus of our study was on the inhibition but negative values for some of the concentrations of hexane and chloroform and almost all concentrations of ethylacetate, butanol and water subfractions showed stimulatory effects rather than inhibition. Our results are in confirmation (Rice, 1984; Batish et al., 1997) that allelochemicals are inhibitory to plants at one concentration but are stimulatory to the same plant or different plant at another concentration.

The EC₅₀ value of hexane extract was 53.9 while the concentration which has caused 50% inhibition of the lettuce seed is 100 ppm. The 300 ppm showed 100% inhibition. The total activity of the hexane extract was

1.85, calculated by the formula: Total activity = $1/EC_{50} \times$ Concentration.

The EC₅₀ value of chloroform extract is 300 ppm and the total activity of the extract was 5.09, the activity suggests the possible presence of allelochemicals. The genus *Teucrium* was extensively studied for neoclerodane diterpenoids (Burno et al., 2002; Rodriguez et al., 1995; Fraga et al., 1995; Bruno et al., 1999; Sattar et al., 1995; Coll et al., 2005; Bedir et al., 2003; Malakov and Papano, 1996). Beside terpenes, some flavonoids have also been reported from this genus by De la Torre et al. (1988), Xie et al. (1992) and Oganasyan et al. (1985). Our results strongly favored the presence of terpenes and flavonoids in *T. royleanum* because the same classes of compounds exhibiting some outstanding allelochemicals properties (Inderjit and Dakshini, 1991, 1992, 1994; Rice-Evans and Packet, 1998; Baruah et al., 1994; Batish et al., 2002). Thus it may be concluded that terpenes and flavonoids are responsible for the allelopathy which are found in the chloroform fraction of the tested plant.

Our results of essential oils, hexane and chloroform showed the possible presence of phytotoxic terpenes and flavonoids in the *T. royleanum*. The global approach is to identify and isolate environmental friendly weedicides and herbicides. Our further analysis for the targeted isolation and characterization of potential allelochemicals is in process. However, here we contribute to the global goal of introducing environmental friendly herbicides and

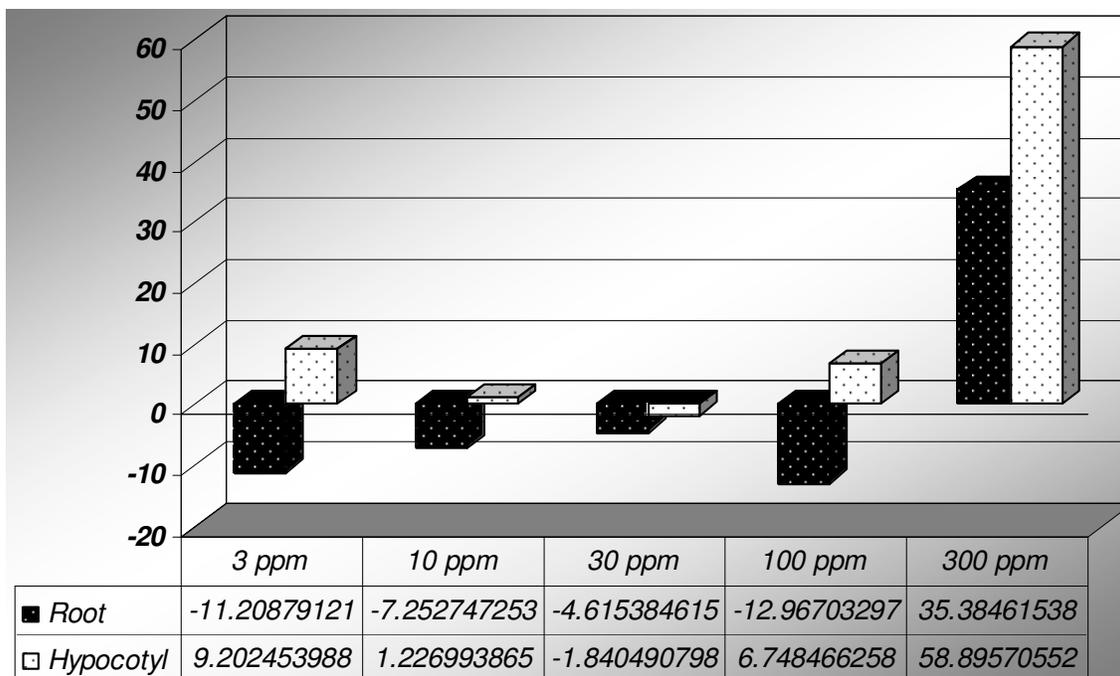


Figure 6. Effect of various concentration of chloroform on the lettuce roots and hypocotyls germination.

weedicides.

Conclusion

These results indicated that *T. royleanum* contains important chemical constituents and is suitable for the isolation of compounds which can be utilized as environmentally friendly herbicides or weedicides on commercial scale.

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