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INORGANIC PROFILE AND ALLELOPATHIC EFFECT OF ENDEMIC *INULA KOELZII* FROM HIMALAYA PAKISTAN

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Abstract

Inula koelzii of Astereaceae, an endemic plant growing at Ladakh and Kashmir regions, has been observed a potential allelopathic plant species. Further observing its allelopathic role, four subfractions (hexane, chloroform, butanol and water) were extracted from 80% MeOH of the aerial plant material. While using EDX technique for inorganic profile of each subfraction, it revealed 18 different elements i.e., C, O, Cl, K, Ca, Cr, Mn, Fe, Zn, Ag, Cd, Co, Ni, Cu, Al, Si, S and P. However, C, O, K, Fe, Zn and Co was present in highest percentages in each subfraction. Overall, chloroform ($CHCl_3$) revealed a composition of 13 different elements and the most abundant were Cl, K, Ca, Zn, Co, Ni, Cu and Al. To know the allelopathic effect of each subfraction, dish pack method was used for bioassay with a concentration gradient of 10, 30, 100 and 300ppm. The significant chloroform subfraction had highest specific activity compared to other four fractions and presented 85% inhibitory effect to germination of lettuce seeds. Hexane subfraction presented dose-response effect, while butanol and water subfraction presented stimulatory effects.

Introduction

Various chemical species are released from one plant species in its surrounding effecting (either positive or negative) the growth and function of other plants is known as allelopathy (Rice, 1992; Sajjad *et al*., 2007; Khan *et al*., 2009). Problems pertaining to sustainable agriculture have been pursued while understanding allelopathy of various crops and plant species (Chou, 1999; Fujii *et al*., 2003; Inderjit & Duke, 2003). However, this multidisciplinary field has still been entangled with many unsolved issues at larger field scale (Inderjit & Duke, 2003).

More attention has been paid to allelopathic behaviors of biologically active secondary metabolites in plant and microorganisms, while fewer studies have been observed on elemental allelopathy (Morris *et al*., 2009). The allelopathic effect cause by inorganic constituents/elements in plant surrounding is known as elemental allelopathy. It has been conceptualized and reported that the inorganic contents may be concentrated within the rhizosphere to a level at which it can effect the neighboring plants while not effecting the host itself (Zhang *et al*., 2005; Morris *et al*., 2009). Efforts have to be strengthened to work on the role of such elements which can be helpful to promote sustainable agriculture practices. Although the interference of Zn, Ni, S, Cl and Na salts has been studied by various scientists like Boyd & Jaffre (2001), Bottoms (2001), Duda *et al*., (2003), and Zhang *et al*., (2005) etc., but still there is need to explore the plant wealth for their elemental profile and respective allelopathic behavior.

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In this regard, various techniques/methods have been developed and plants species has been analyzed (Gavlak *et al*., 2003). To know the elemental composition, SEM-EDX is also one of the method among many others, which has been knowledgeable enough to identify/quantify elemental contents of plants samples (Maunsbach & Harvey, 1992; Khan *et al*., 2006). There is almost less report on plant specimens while we have not found any report to correlate the allelopathy aspects.

In the present study, *Inula koelzii* Dawar. & Qaiser of Astereaceae is endemic to Ladakh and Kashmir regions (i.e. Pakistan and India) (Qaiser & Abid, 2005). It is a perennial herb, with bright golden flower found near river beds (Dawar, 1998). Previously the plant has been reported to carry allelopathy potential as the leaf leachates of the plant suppressed the growth of lettuce seeds (Khan *et al*., 2009). To further specify the role and effect of *I. koelzii*, the present study was carried out. The aim of the study was to elaborate the effect of various subfractions on the growth of root and hypocotyl of lettuce seedlings and to determine the elemental composition of individual subfraction. The present work is, although, based on preliminary findings, however, extensive work will further help to elucidate the role and scope of elemental allelopathy.

Materials and Methods

Plant material: *Inula koelzii* Dawar & Qaiser, was collected from Deosai National Park, Northern Areas of Pakistan from geographical location of 35º03’ 23.26” N- 75º27’39.69” E (Khan *et al*., 2009). The aerial parts of the plant species were packed in polyethylene bags with silica gel, shade dried at room temperature for 10 days and were stored at 10ºC.

Extraction: The dried plants of *I. koelzii* (520 g) was soaked in MeOH and extracted for 30 days at 5 ºC in the dark. The MeOH extract was evaporated to dryness in vacuo and suspended in 500 ml of water in separating funnel. The water suspension was partitioned three times with hexane. The residual water fraction was then partitioned three times with chloroform and butanol. The remaining water layer was evaporated to dryness using in vacuo conditions.

Elemental analysis: The elemental analysis was carried out at Centralized Resource Laboratory (CRL), Department of Physics, University of Peshawar, using Energy Depressive X-Rays spectrometer (EDX), Model (Inca-200), Company Oxford Instruments (U.K) using the established method (Ferreira *et al*., 1995; Bibi *et al*., 2006; Khan *et al*., 2006).

Allelopathic assay: Allelopathic potential of the subfractions was carried out following Hiradate (2006). *I. koelzii* was reported as a potential allelopathic plant species among other four plants while analyzing the role of method and concentration reported previously (Khan *et al*., 2009) following sandwich and homogenated sandwich methods (Fujii *et al*., 2003). Lettuce seeds (*Lectuca sativa* Great Lakes 366, Takii Seed Co. Ltd, Japan) were used to know the effective concentration at 50% inhibition (EC_{50}) using various concentrations. EC_{50} is the effective concentration of an extract/compound that induces 50% of inhibition of the tested organism (Hiradate, 2006). The inhibitory effect of extracts was studied through specific activity method. Specific activity is expressed by EC_{50}. If the extract exhibit small EC_{50} value, it presents high specific activity. Specific activities of extract were recorded following Hiradate (2006) and Golisz *et al*., (2007).

Four different concentrations of 3, 10, 30, 100 and 300ppm of each subfraction were prepared by dissolving it in 2% DMSO. Initial concentration was 1000ppm. A filter paper (27 mm ø, Type Roshi Kaisha, Ltd, Tokyo) was placed in a glass Petri dish. The dilutions were subjected on the filter paper and thus allowed to spread over it. Seven lettuce seeds were placed on it and the dishes were sealed and packed for incubation for 72 hours at room temperature.

For each plant species, mean, SD variance (Fuji *et al.,* 2003) and standard error were calculated to determine the inhibition pattern at various concentration levels. While using EC_{50}, specific activity of each extract was determined. ANOVA analysis were done using MS Excel 2003.

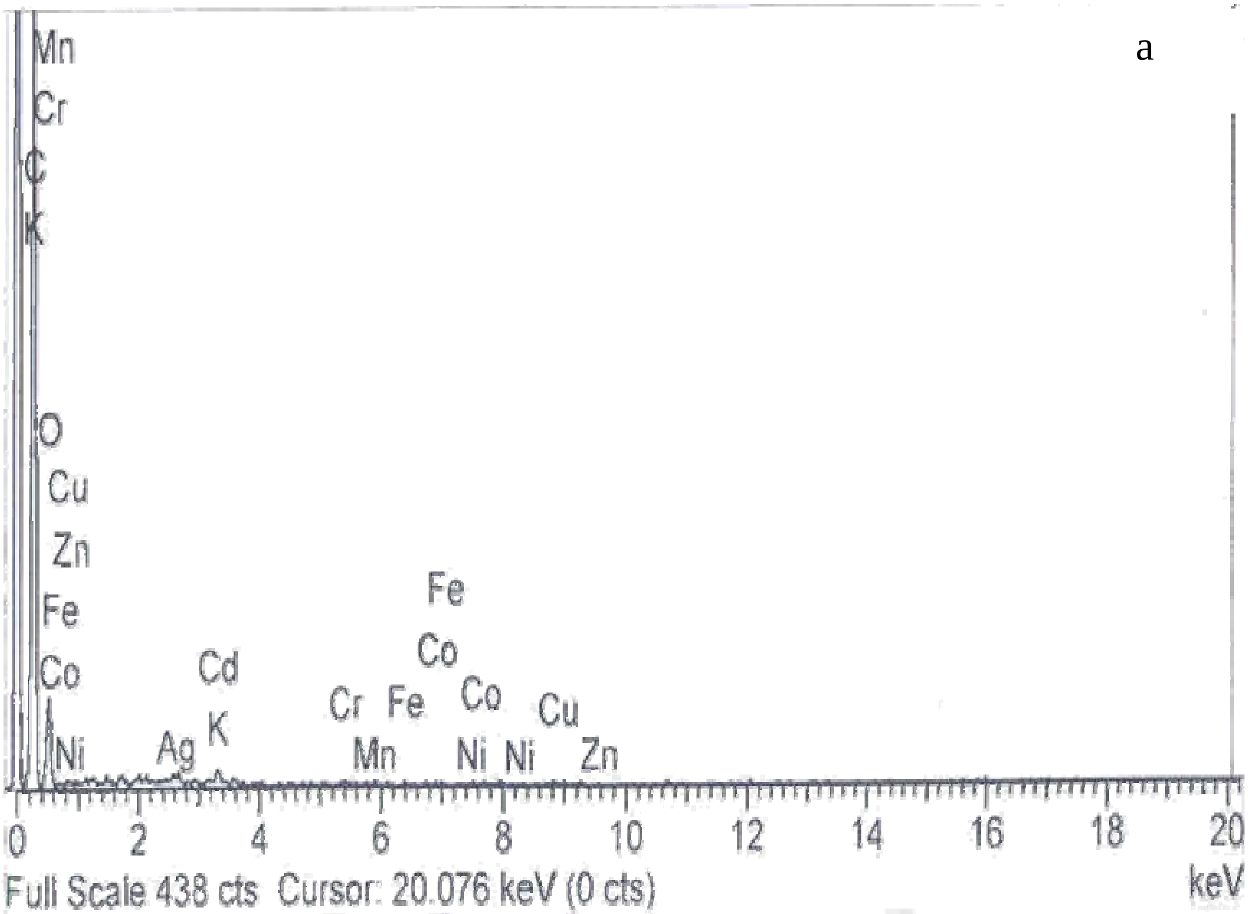
Results and Discussion

Elemental analysis: In case of elemental compositions, overall 18 elements are present in the different subfractions of the plant, while carbon and oxygen were the most predominant in their percentages (Table 1). The highest total number of elements was found in $CHCl_3$ subfraction. In case of $CHCl_3$ subfraction, highest percentages of Cl, K, Ca, Zn, Co, Ni, Cu and Al compared to other subfractions. However, it did not have Cr, Mn, Ag and Cd.

Table 1. Elemental analysis of each of the subfractions of *I. koelzii* (in percent)

Elements	Hexane		$CHCl_3$		*n*-butanol		Water	
	Weight	Atomic	Weight	Atomic	Weight	Atomic	Weight	Atomic
C	67.77	75.31	41.08	51.37	49.17	57.62	39.62	49.67
O	28.6	23.85	47.28	44.38	46.26	40.69	48.57	45.71
Cl	nd	nd	2.18	0.92	0.81	0.32	3.96	1.68
K	0.9	0.31	4.06	1.57	0.95	0.35	6.89	2.65
Ca	nd	nd	0.67	0.25	0.2	0.07	0.3	0.11
Cr	0.31	0.08	nd	nd	0.09	0.02	nd	nd
Mn	0.28	0.07	nd	nd	0.43	0.11	nd	nd
Fe	0.69	0.16	0.3	0.08	0.54	0.13	nd	nd
Zn	0.02	0	0.24	0.05	0.11	0.02	0.1	0.02
Ag	0.41	0.05	nd	nd	nd	nd	nd	nd
Cd	0.6	0.07	nd	nd	nd	nd	nd	nd
Co	0.26	0.06	0.84	0.21	0.24	0.06	nd	nd
Ni	0.03	0.01	0.37	0.09	nd	nd	0.16	0.04
Cu	0.13	0.03	1.72	0.41	nd	nd	0.35	0.08
Al	nd	nd	0.21	0.08	0.94	0.49	nd	nd
Si	nd	nd	0.5	0.27	nd	nd	nd	nd
S	nd	nd	0.35	0.17	nd	nd	nd	nd
P	nd	nd	nd	nd	0.26	0.12	nd	nd

Nd = Not detected. Inorganic constituents are presented in ascending order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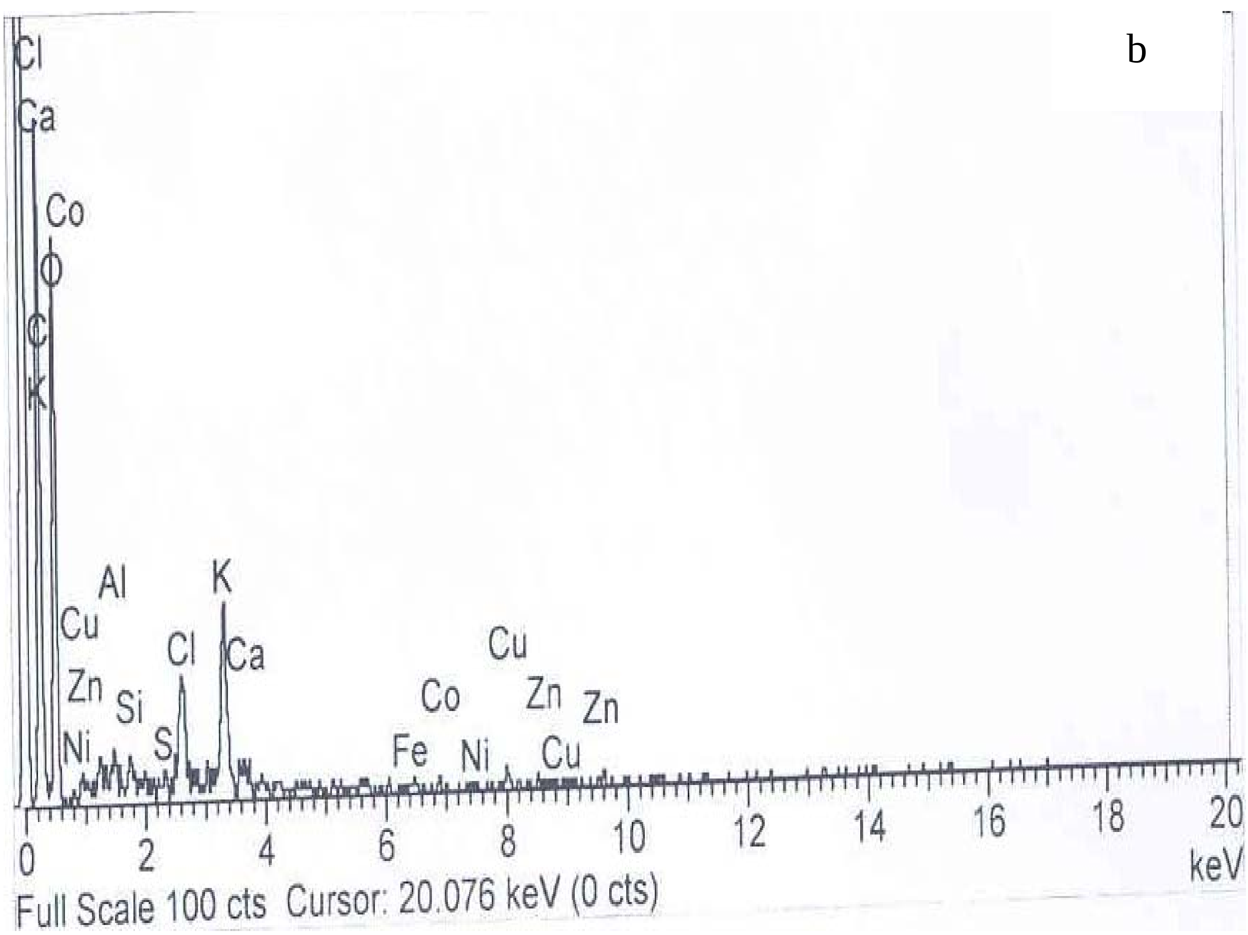
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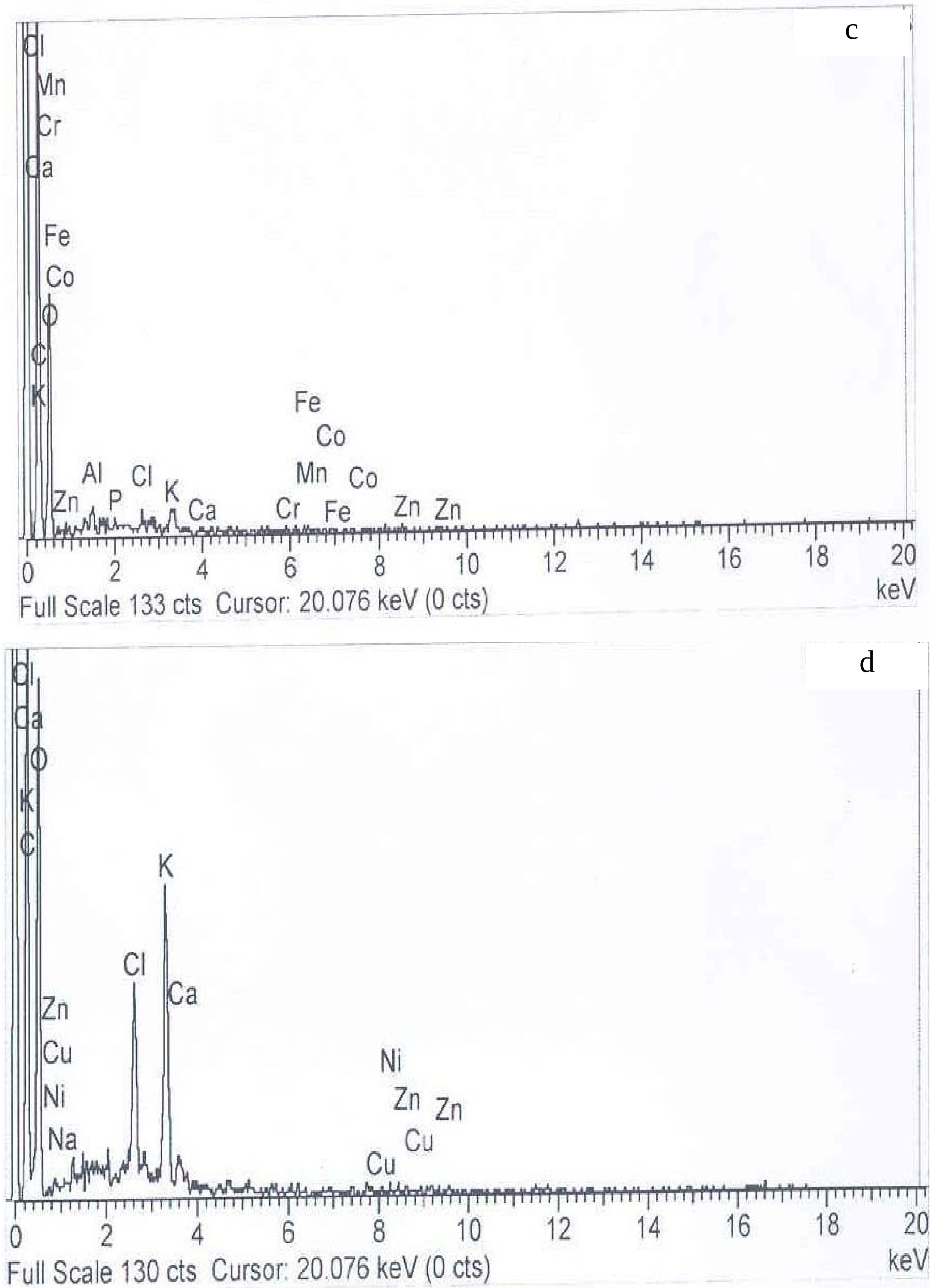


Fig. 1. Inorganic profile of the selected subfractions using SEM-EDX technique. Diverse nature of elements was recorded in the hexane (a), chloroform (b), butanol (c) and water (d) subfractions. Full scan of the subfraction was done to obtain complete informations.

Table 2. Specific activity of all subfractions of *I. koelzii*

Extract	Effective concentration	EC_{50} activity	Specific activity
Chloroform	100 ppm	63.1	1.517
Hexane	300 ppm	54.39	5.517
n – Butanol	-	-	-
Water	-	-	-

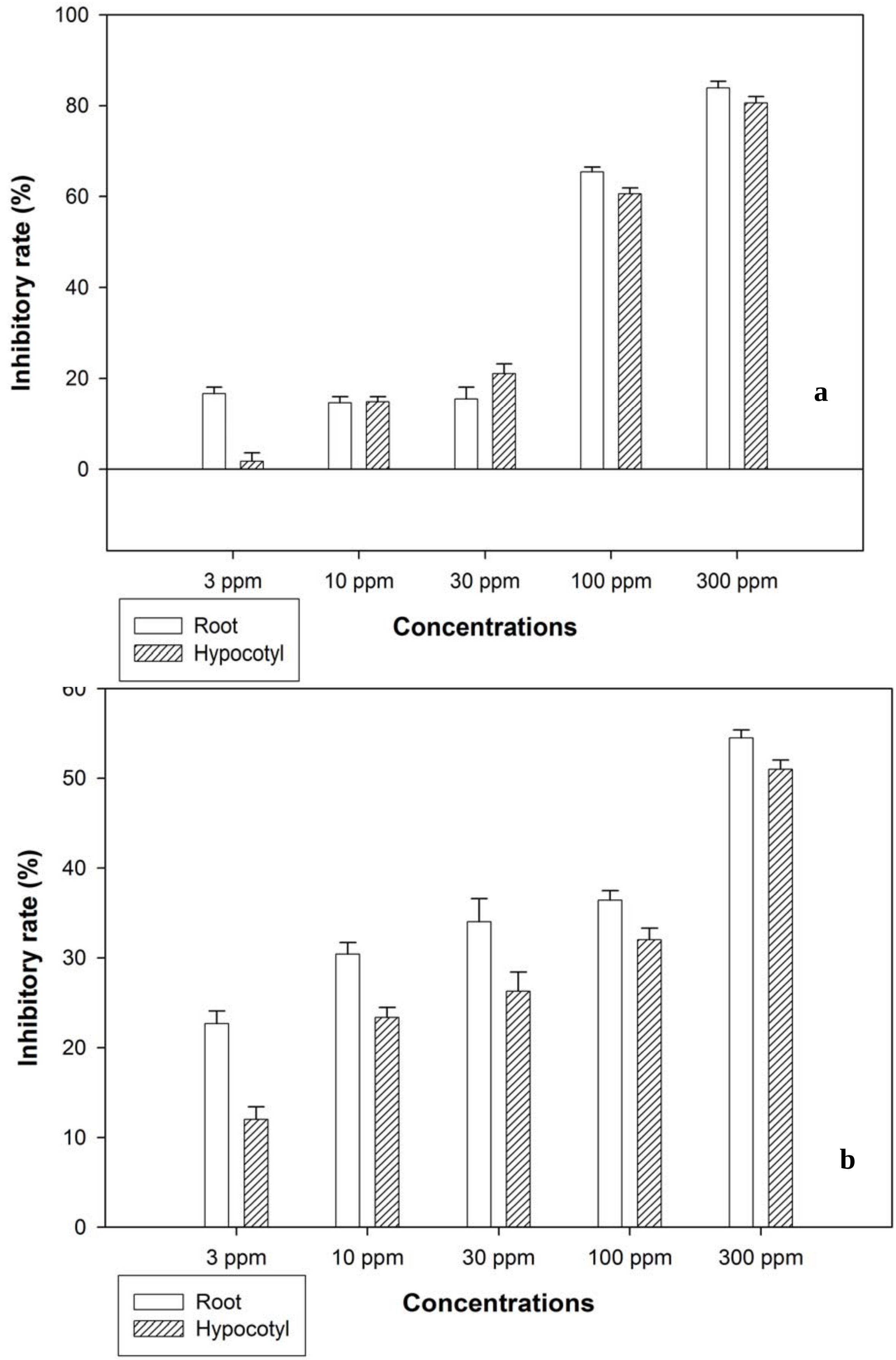
- = no activity; EC_{50} is the effective concentration of an extract/compound that induces 50% of inhibition of the tested organism (Hiradate, 2006). The inhibitory effect of extracts can be studied through specific activity method. Specific activity is expressed by EC_{50}.

Table 3. ANOVA analysis of concentration and subfractions

Source of variation	SS	df	MS	F	P-value	F crit
Concentration	6397.510	5	1279.502	5.312074	0.000982	2.485145
Subfractions	9370.812	7	1338.687	5.557793	0.000232	2.285233
Error	8430.336	35	240.8667			
Total	**24198.66**	**47**				

Allelopathic effect: The allelopathic effect of each sub-fraction (hexane, chloroform, butanol and water) revealed that chloroform was the most active. All the sub-fractions presented fumble response from 85% inhibition to stimulatory effect towards the growth of lettuce seeds (Figure 2). Chloroform ($CHCl_3$) sub-fraction has the highest specific activity (Table 2) compared to others. The specific activity of the $CHCl_3$ fraction is highest which is according to the Hiradate (2006) and Golizs *et al*., (2007). It inhibited the growth of root and hypocotyl for 10, 30 and 100 and 300ppm. The inhibition rate was recorded 85% for 300 ppm (Fig. 2a). It was also observed that growth of hypocotyl of lettuce seeds was lesser inhibited as compared to roots by the various concentrations of hexane sub-fractions. The allelopathic effect was in dose dependent manner for hexane sub-fraction. The inhibition rate was 57% at 300 ppm concentrations. Higher concentration (300 ppm) of the fraction suppressed the seed germinations while at lower concentrations (100, 30, 10 and 3 ppm) the effect was in descending order (Fig. 2b). The butanol subfraction has shown minimal inhibition level to only roots while at against hypocotyl growth its was stimulatory to inhibitory as the concentration increased (Fig. 2c). The different concentrations of water subfraction have shown simulative effect compared to other subfractions (Fig. 2d).

The ANOVA analysis further clarifies our results, elaborating that concentration plays a significant role towards inhibition or promotion. While it further demonstrates that the results revealed after treatment with individual subfractions varies significantly (Table 3). The same findings were observed previously as well (Khan *et al*., 2009).



Inhibitory rate (%)
100
80
60
40
20
0
3 ppm
10 ppm
30 ppm
100 ppm
300 ppm
Concentrations
Root
Hypocotyl
a
Inhibitory rate (%)
60
50
40
30
20
10
0
3 ppm
10 ppm
30 ppm
100 ppm
300 ppm
Concentrations
Root
Hypocotyl
b

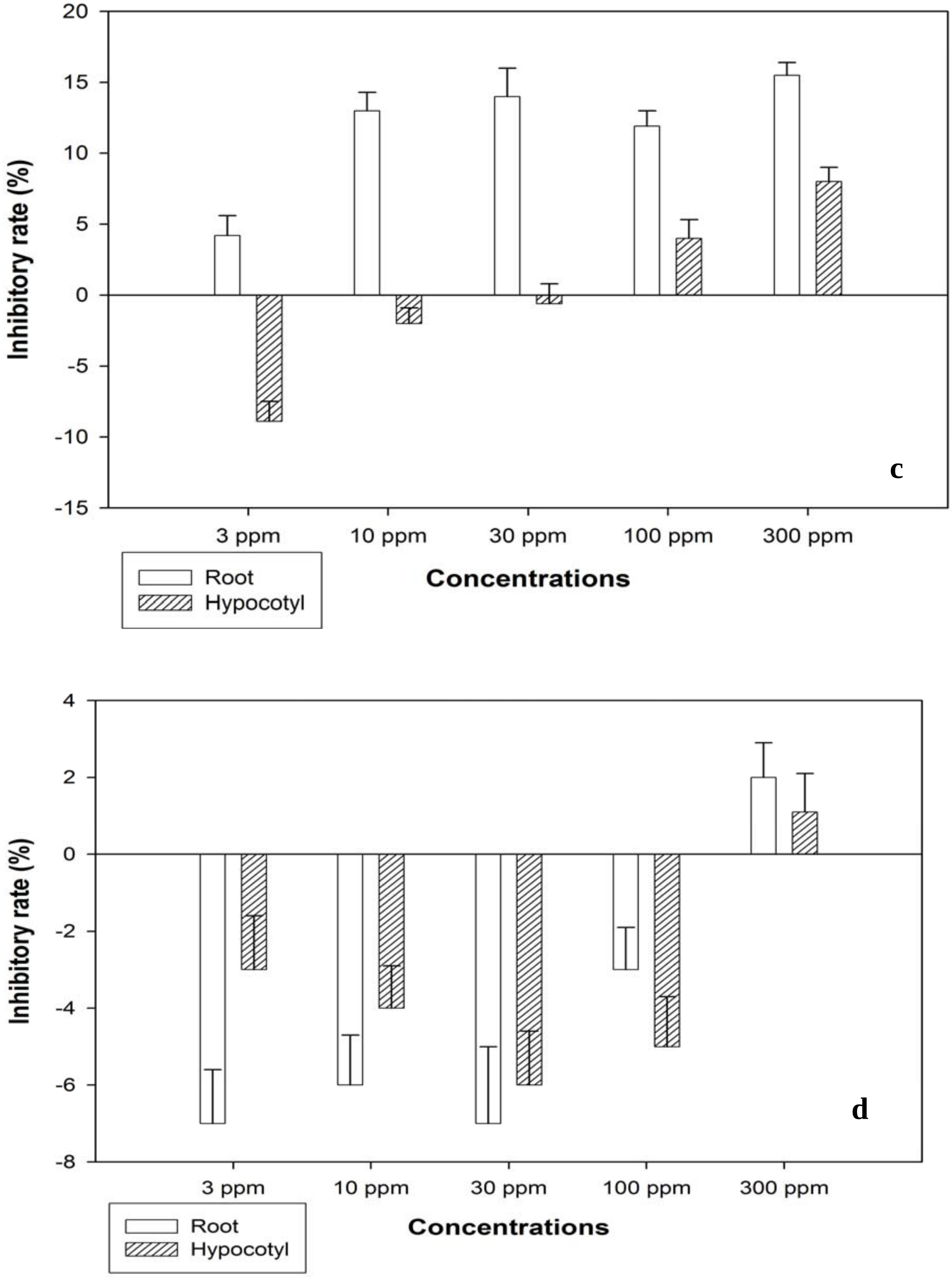


Fig. 2. Allelopathic effect of the subfractions of 80% MeOH; the lettuce seeds were treated against different concentrations (3, 10, 30, 100 and 300 ppm) of four subfractions (hexane, chloroform, *n*-butanol and water). The chloroform subfraction demonstrated highly significant effect towards the growth of lettuce (a). 100 ppm was the effective concentration to cause 50% inhibition towards the growth of lettuce seeds. Hexane subfraction offered inhibitory effect towards the germination of lettuce (b). At 300 ppm, 50% inhibition towards the growth of lettuce seeds the lettuce seeds did not germinated. While butanol and water presented stimulatory effect (c & d respectively). None of the concentration of both the subfractions showed 50% inhibition towards lettuce seed's growth.

Discussion

Chloroform sub-fraction being the most bioactive in allelopathic, showed significant inhibition rate to the grow th of root and hypocotyl of lettuce seeds. Although the focus of the allelopathy study was on the inhibition concentrations of buOH and water subfractions showed stimulatory effects. The results are in confirmation with Rice (1992) and Batish *et al*., (1997) that chemicals are inhibitory to plants at one concentration but are stimulatory to the same plant or different plant at another concentration.

Looking at the elements present in each subfraction and their inhibitory effect, it has been previously reported in many studies that heavy metal accumulation can affect plant growth vigorously if their concentration reach beyond threshold level. Example like Cd, Cr, Co, Ni, and Co etc have been observed to cause plant toxicity (Jamal *et al*., 2006; Osman *et al*., 2008; Liu *et al*., 2009; Farooqi *et al*., 2009). Since these elements also play a crucial role in plant growth as essential nutrients but still their higher can develops stress on the metabolic functions of the plants, as was observed in lettuce seed in this study, affecting photosynthetic activity and growth inhibition (Leyval *et al*., 1995; Claire *et al.*, 1999; Mullar *et al*., 2000). Chloroform had the highest composition of elements (Cl, K, Ca, Zn, Co, Ni, Cu and Al) (Table 1). Among these elements cupper was highest (1.72%), followed by Co (0.84%), which bears phytotoxic behavior (McBride & Martinez, 2000). Concentration of Cu and Co higher than 17 to 21ppm can affect the seed germination of lettuce seed.

Leaf and petiole chlorosis was particularly striking in plants deficient in Fe. Due to immobility of Fe within plan tissues, interveinal chlorosis of the newer leaves and the ramets occurred. Plants under server Fe deficiency appeared almost bleached-white on the youngest organs. In contrast to Fe, Mg deficiency was apparent only on the leaf blades (Newman & Haller, 1998). It has also been suggested that *A. repens* may interfere with neighboring plants by concentrating zinc (Zn) in the top soil layers through the accumulation of high Zn levels in stems and leaves, which are then released into soils with litter deposition and decomposition (Moris *et al*., 2009).

However, it has also been observed that low concentration of some metals like chromium was stimulatory to the growth of some plants while for other inhibitory role is observed (Sharma & Aery, 2000). Therefore, the same can be predict for butanol and water subfractions, having Cr presence but still there is no inhibition for the lettuce seedlings. Al has been reported to carry stimulatory effect and the same was observed in n-butanol subfraction (Jamal *et al*., 2006). Si is not considered an essential element for higher plants but has been reported to cause cell wall incrustation (Neumann & Nieden, 2001) and has been found in the chloroform subfraction. There are numerous studies explaining the toxicity and role of these elements in corresponding ecology, but still many field observations has to be recorded to understand it.

From the results, it has been observed that *I. koelzii* is an important allelopathic plant and the selected subfractions of the plant carry allelopathic behavior against the lettuce seeds (Khan *et al*., 2009). The elemental composition of the plant is diverse and the literature shows both the inhibitory and stimulatory role of elements to the plants, thus the subfractions of *I. koelzii* shows inhibitory to stimulatory allelopathic effects against the growth of lettuce seedlings. However, this quantum effort can lead to the new ways to understand the elemental allelopathy. Hence the SEM-EDX is potential technique and can be extended for wider analysis of plant’s composition.

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