Effect of Heavy Metals on the Growth and Development of *Silybum marianum*, in Various Polluted Areas of Peshawar, Pakistan

[1]I. HUSSAIN*, [1]L. KHAN, [1]T. MEHMOOD, [2]I. KHAN , [2]W.ULLAH AND [2]H. KHAN
[1] *PCSIR Laboratories Complex, Jamrud Road Peshawar-25120, Pakistan*
[2]*Department of Chemistry, University of Peshawar, Peshawar-25120, Pakistan*

(Received 23rd December, 2004, revised 13th May, 2005)

Summary: The effects of heavy metals on the growth, development and accumulation of active constituents of *Silybum marianum* were studied at three different areas, situated at a distance of 3 and 4 km from the main polluted area. The concentration of Cd, Cr, Pb, Cu, Mn, Zn, and Fe was studied in the soil as well as in the plants collected. The plant parts including roots, stems, leaves, seeds, oil and silymarin extracted from the seeds were evaluated. Oil obtained from the seeds was not contaminated with heavy metals, on the other hand silymarin obtained from the three spots were relatively contaminated with heavy metals. It was observed that heavy metals in soil and air pollution reduced the contents of cellular and acellular constituents of *Silybum marianum*. This study showed that *Silybum marianum* is suitable for the control of environmental pollutants, however, for pharmaceutical utilization; it should be collected from areas not contaminated with heavy metals.

Introduction

Heavy metal pollution of agricultural soil and air is one of the severe ecological problems of the world and in Pakistan in particular. The biggest sources of pollution in Pakistan are poor sanitation, food contamination due to pesticides as well as vehicular emissions, which pollute air, water and soil. Vegetables and animals production result in contaminated produce with excessive levels of Chromium, Cadmium, Lead, Copper, Manganese and Zinc. These toxic levels can enter the nutrition chain and subsequently the human diet. Heavy metals are considered harmful for human beings. For example Cadmium causes oesteomalcia and pyelonephristis. Similarly, Lead may cause renal tumors and other carcinomas. However, Copper, Iron and Zinc are less toxic than former ones. Unluckily very little work has been done regarding heavy metals accumulation in medicinal plants or herbal drugs. For example Wong et al. estimated the concentration of nine metals (Cd, Co, Cu, Fe, Mn, Ni, Pb, Zn, and Hg) in 42 medicinal plants [1]. Similarly, Kwapulinski et al., and Chizzola and Franz determined the heavy metals concentration of various medicinal plant species growing in Poland and Austria, respectively [2,3]. However, in Pakistan, no such study has been reported.

There are many investigations in the world concerning the effect of heavy metals on the yield, accumulation and damage to various plants [4]. Although several chemical and physical approaches for cleaning the soil from transmission of the metal ions into insoluble forms are available, however, all of them are quite expensive and they can hardly be applied to thousands of hectors. Most of these studies have been reported over the last two years. Heavy metals concentration in some essential oil and medicinal species has been reported by several authors discussing the problem from other points of view [5].

A plant, *Silybum marianum*, commonly known as milk thistle is distributed throughout southern Europe, Australia, and North America and found wild in N.W.F.P. and the Punjab areas of Pakistan [6]. The plant and its seeds are used for medicinal purposes in the treatment of various liver diseases [7].

The objective of this investigation was to study the effects of increasing heavy metal polluted air and soil on the growth, productivity and medicinal contents of *Silybum marianum* as well as the possibilities for growing this plant in highly polluted areas.

Results and Discussion

Chromium

Chromium is one of the known environmental toxic pollutants in the world. The main sources for

*To whom all correspondence should be addressed.

Chromium contamination are tanneries and steel industries, sewage sludge applications and fly ash [8]. Besides these, Chromium plating and alloys in motor vehicles is considered to be a more probable source of Chromium [9]. At elevated concentration it could be toxic for plants and animals. Concentration between 5 - 30-mg kg^{-1} is considered as critical for plants and could cause yields reduction [10].

Soil samples collected from three different spots (Table 1) showed significantly different amount of Chromium. High Chromium concentration was found in the soil from spot 1. In case of plants collected from spot 1, the concentration was 1.19-mg kg^{-1} (Table 2). Chromium concentration in the plant parts from spot 1 and spot 3 (control) were in the order: roots > leaves > stems, while in case of plant from spot 2, it was in the order: leaves > roots > stems.

Seeds from the three spots (Table 3) accumulated significantly different amount of Chromium. High Chromium concentration was found in the seeds from spot 1 (1.02-mg kg^{1}).

All the oil samples showed almost negligible amount of chromium. Silymarin obtained from the three spots accumulated significantly different amount of Chromium (Table 3). High Chromium concentration was found in the silymarin obtained from spot 1 (Table 3) followed by silymarin from spot 2.

Table –(Plant height and weight)

Area	Height	Weight
Spot-1	98 cm	80.5 g
Spot-2	135 cm	82.0 g
Spot-3 (controlled)	144 cm	90.0 g'

Lead

Lead is also regarded as very hazardous for plants, animals and for the microorganisms in particular. Soil collected from spot 1 contained 0.32 mg kg^{-1}, from spot 2 about 0.20 mg kg^{-1} and the soil from spot 3 (control), 0.02 mg kg^{-1}, with significant differences from spot 1. Lead concentration in plants from spot 1 was very high, beyond the threshold level of this element in plant [11] and as compared to the plants obtained from spot 2 and 3, respectively. The main sources of lead pollution of agricultural soils and plants are lead mines, vehicular emissions and washing, sewage sludge applications etc. Due to, soil and air pollution, Lead concentration in plant parts was in the order of roots > leaves > stems. Obviously, the high Lead concentration in plant roots was due to the uptake from the available Lead in the soil. Contrary to this, the high Lead concentration in the above ground parts is due to airborne Lead [12].

The plants from three different environments accumulated different amounts of Lead, the most sensitive was being spot 1 and the least one was spot 3. The seeds and oils from the three spots contained negligible amount of Lead. However, in case of Silymarin (Table-3) different amount of Lead

Table 1. Heavy metal concentration in soil; mg kg^{-1}, nd: not detected

Soil	Pb	Cr	Cd	Fe	Cu	Mn	Zn	Co	Ni
Spot-1	0.32±0.03	0.22±0.04	0.04±0.01	11.37±1.86	0.34±0.05	0.96±0.12	1.35±0.07	0.69±0.07	0.29±0.02
Spot-2	0.20±0.03	0.08±0.08	0.03±0.01	19.6±2.76	0.24±0.02	0.94±0.04	0.85±0.06	nd	0.18±0.014
Spot-3	0.02±0.01	0.04±0.02	0.01±0.002	14.28±1.06	0.17±0.02	4.68±0.69	0.20±0.024	nd	0.08±0.015

Table –2. Heavy metals concentration in plant parts (root, stem and leaves): mg kg^{-1}, nd: not detected.

1) Root									
Root	Pb	Cr	Cd	Fr	Cu	Mn	Zn	Co	Ni
Spot-1	0.05±0.013	1.19±0.05	nd	34.85±1.43	1.54±0.08	2.28±0.60	1.26±0.07	nd	0.44±0.06
Spot-2	0.03±0.007	0.18±0.06	nd	14.2±2.00	0.68±0.09	1.46±0.09	0.98±0.10	nd	0.32±0.07
Spot-3	0.01±0.004	0.16±0.004	nd	49.13±4.42	0.38±0.04	0.40±0.04	0.15±0.02	nd	0.19±0.031
2) Stem									
Stem	Pb	Cr	Cd	Fe	Cu	Mn	Zn	Co	Ni
Spot-1	0.02±0.004	0.37±0.04	nd	22.80±2.13	1.31±0.06	2.03±0.10	0.99±0.07	nd	0.31±0.018
Spot-2	nd	0.12±0.017	nd	12.46±1.42	0.63±0.04	0.51±1.04	0.89±0.04	nd	0.37±0.02
Spot-3	nd	0.08±0.02	nd	9.98±1.08	0.30±0.08	1.02±0.08	0.2±0.02	nd	0.08±0.02
3) Leaves									
Leaves	Pb	Cr	Cd	Fe	Cu	Mn	Zn	Co	Ni
Spot-1	0.02±0.004	0.79±0.04	nd	62.24±2.82	0.72±0.03	5.68±0.83	1.46±0.06	nd	0.13±0.01
Spot-2	0.02±0.002	0.94±0.04	nd	59.85±3.12	0.49±0.04	5.84±0.87	2.24±0.05	nd	0.43±0.09
Spot-3	0.20±0.004	0.09±0.017	nd	18.23±1.67	0.25±0.05	1.90±0.09	0.97±58.34	nd	0.16±0.02

concentration was found. High Lead concentration (0.05 mg kg^{-1}) was observed in the silymarin from spot 1 while in spot 2, it was 0.02 mg kg^{-1}.

Copper

Copper concentration in soils from spot 1 was higher than spot 2 and spot 3. Plants grown on the three spots contained significantly different amounts of Copper, due to different Copper concentration in the soil of three spots. (Table 1). The Copper concentration in plants to a greater extent is due to the available Copper in the soil. The Copper concentration in plant obtained from spot 1 was higher than spot 2, which is in fact having more Copper concentration than spot 3 (Table 2). Roots accumulated significantly more Copper than other plant parts, which is in accordance with the results recorded by some authors [13], and as it is very clear from our results (Table 2). Thus, the Copper concentration in plant parts was found in the following order roots > stems > leaves.

Copper concentration in the seeds from the three spots was significantly different (Table 3). More Copper was found in the seeds from spot 1 and least in the seeds from other two spots. In the oil, obtained from the three spots Copper was found in lower concentration (Table 3). Surprisingly signifycant amount of Copper was found in the silymarin obtained from the three spots. High Copper concentration was found in the silymarin sample from spot 1 followed by spot 2 and slightly different concentration was found in the silymarin sample from spot 3.

Zinc

Recently there has been great concern about zinc pollution although it is one of the essential elements for plant growth. Most plant species were found to be tolerant to excessive Zinc concentration. Sources for Zinc contamination of the soil and plants are non-ferrous smelters, sewages, burning of fossil fuels and agro-chemicals [14].

Soils obtained from the three spots contained significantly different amount of Zinc (Table 1). Zinc concentration in the soils from the three spots was in the order spot 1 > spot 2 > spot 3. Thus the polluted soil (Spot 1) contained high metal concentrations than spot 2 and spot 3. The plants growing in the three spots accumulated significantly different amounts of Zinc. In most plant parts Zinc, concentration was found higher but no Zinc toxicity symptoms were found. Generally, Zinc concentration in plant parts was in the order leaves > roots > stems. The higher Zinc concentration was considered to be due to air pollution, as roots had a lower concentration [15,16]. Plants from spot 1 accumulated significantly more Zinc than plant from spot 2, which is in turn having more amount of Zinc than spot 3.

Seeds obtained from the three spots (Table 3) accumulated different amount of Zinc. High Zinc concentration was found in the seeds from spot 1 that is followed by seeds from spot 2 and spot 3. Thus, the Zinc concentration was also found to be of the order spot 1 > spot 2 > spot 3.

Oil obtained from the seeds of three spots contained negligible amount of Zinc (Table 3). High Zinc concentration was recorded in silymarin. Thus Zinc concentration is above the critical level in silymarin obtained from spot 1. Silymarin obtained from spot 2 contained significantly lower Zinc concentration than spot 1 but higher than spot 3 (Table 3). Thus, the Zinc concentration of silymarin from the three spots was in the order spot 1 > spot 2 > spot 3.

Cobalt and Nickle

Soil collected from spot 1 (Table 1) has Cobalt concentration of 0.69 mg kg^{-1}, however, signifycantly negligible Cobalt concentration was found in the soils from spot 2 and spot 3. In case of plants from the three spots, the Cobalt concentration was below detection limit.

Slightly Nickel contamination was found in the soil samples from the three spots (Table 1). However, significantly different amount of Nickel was found in the plant parts from the three spots. Thus a similar Nickel order was found as spot 1 > spot 2 > spot 3 as shown in table 2.

In the seeds part (Table 3) from the three spots, higher Nickel amount was found in the seeds from spot 1. Relatively, very low Nickel concentration was found in the seeds from spot 3 and no Nickel contamination was observed in the spot 2 seeds. In case of the oil obtained from the three spots, the Nickel concentration was found to be below detection limit (Table 3). However, in case of silymarin significantly different amount of Nickel concen-

Table 3. Element concentration in seed, oil and silymarin; mg kg^{-1}, nd: not detected.

1) Seeds

Seeds	Pb	Cr	Cd	Fe	Cu	Mn	Zn	Co	Ni
Spot-1	0.02±0.004	1.02±0.30	0.021±0.003	15.52±2.77	1.76±0.80	nd	3.92±1.04	nd	3.44±0.47
Spot-2	0.01±0.002	0.13±0.016	0.011±0.003	2.19±0.67	1.04±0.09	nd	3.19±1.38	0.16±0.04	0.27±0.05
Spot-3	0.01±0.003	0.05±0.02	0.01±0.005	3.69±1.37	0.66±0.19	nd	2.17±0.25	Nd	nd

2) OIL

Oil	Pb	Cr	Cd	Fe	Cu	Mn	Zn	Co	Ni
Spot-1	0.05±0.02	0.02±0.001	0.02±0.005	0.20±0.07	0.63±0.21	nd	0.01±0.002	nd	nd
Spot-2	0.02±0.003	0.01±0.002	0.034±0.007	0.25±0.052	0.32±0.171	nd	0.01±0.004	nd	nd
Spot-3	0.01±0.001	nd	0.02±0.007	0.35±0.07	0.01±0.005	nd	0.05±0.028	nd	nd

3) Silymarin

Silymarin	Pb	Cr	Cd	Fe	Cu	Mn	Zn	Co	Ni
Spot-1	0.05±0.014	0.12±0.015	nd	6.13±1.76	0.73±0.20	nd	12.4±3.61	0.10±0.00	0.24±0.07
Spot-2	0.02±0.006	0.108±0.023	nd	5.53±0.97	0.80±0.13	nd	3.21±1.25	0.16±0.02	0.16±0.02
Spot-3	0.02±0.005	0.04±0.005	nd	4.47±1.68	0.59±0.20	nd	0.55±0.16	nd	0.14±0.042

tration was found i.e., 0.24, 0.16 and 0.14-mg kg^{-1} from spot 1, 2 and 3, respectively.

Cadmium

As can be seen from (Table 1), there were statistically significant differences in Cadmium concentration in the soils from spot 1, spot 2 and spot 3. Cadmium concentration in the soil from spot 1 was higher than spot 2 and spot 3.

The most common sources for Cadmium in the soils and plants are phosphate fertilizers, non-ferrous smelters, Lead and Zinc mines, sewage sludge application and combustion of fossil fuels [17,18].

Critical level of Cadmium in soil is between 3 - 5-mg kg^{-1} [19]. At this level in most cases it cannot cause toxic or excessive accumulation. Concentration in plants, or the lowest level of the element concentration in plants, which can cause yield reduction is between 5 - 30-mg kg^{-1}.

Surprisingly Cadmium was not detected (Table 2) in plant samples collected from the three spots. This may be due to a very low level (below detection limit) of Cadmium present in the available soil for plant growth.

Iron

Soil samples collected from the three spots showed significant differences between heavy metals (Table 1). This was not because of the pollution. Iron is very essential for plants and animals. Its deficiency can cause various types of diseases; however, its high concentration also affects plant growth. The plant samples from the three spots have different Iron concentrations between them. Iron concentration in the plant parts from spot 1 and spot 2 were in the order; leaves > roots > stems, however, it was found different in plant sample from spot 3, which is in the order: roots > leaves > stem having the order spot 1 > spot 2 > spot 3 (Table 2).

High Iron concentration was found in seeds from spot 1, 15.52-mg kg^{-1} followed by spot 3 seeds 3.7 mg kg^{-1}. Oil obtained from the three spots accumulated low concentration of Iron. However, in case of silymarin obtained from the three spots contained high concentration of Iron (Table 3). Thus Iron concentration of silymarin from the three spots were in the order spot 1 > spot 2 > spot 3.

Manganese

Manganese is also an essential element for plants and animals. Its uptake is being controlled metabolically. Soils derive Manganese from the parent material and its contents in the rocks are higher than the concentration of other micronutrients apart from iron [20]. Some of the sources for Manganese in the soils are fertilizers, sewage, sludges and non-ferrous smelters. Critical Manganese concentration in the soils is rather high 1500–3000 mg kg^{1} [10], while as critical concentration in plants is in the range of 300–500 mg kg^{-1}

The concentration of Manganese in the soils from the three spots (Table 1) was significantly different. There were significant differences in Manganese concentration in plants from the three spots and between the plant parts. Manganese concentration in the plants obtained from the three

Table 4. Extraction of oil and silymarin from seeds

Area	Oil (%)	Silymarin (%)
Spot 1	22.0	1.1
Spot 2	23.6	1.44
Spot 3 (Control)	25.7	1.72

spots was of the order leaves > roots > stems. Manganese concentration in the plant parts was also in the sequence spot 1 > spot 2 > spot 3.

The yield of oil obtained from the three spots can be seen from (Table 4). High yield of the oil (25.70 %) was obtained from spot 3, and from spot 2 was 23.60 %. Lower yield was recorded from spot 1 (22.0 %). The lower yield obtained from spot 1 and spot 2 was because of the high soil and air pollution.

Heavy metals of soil and air pollution have also affected the yield of silymarin obtained from the three spots (Table 4). Thus the yield of silymarin obtained from the three spots was found in the order spot 3 > spot 2 > spot 1.

As screened out, the heavy metals pollution of soil and air which has greatly affected the plants to which they were exposed and particularly the most biologically active component, the silymarin, which is used as a drug against hepatitis [6,7], had greatly contaminated with the heavy metals like Lead, Chromium, Zinc and Iron etc.

Experimental

The combined effects of heavy metals on growth, productivity and medicinal contents of Silybum marianum in highly polluted areas were investigated. Phytochemical screening was performed on specimens collected from the three areas i.e. polluted; less polluted and controlled area, relatively unpolluted.

Sampling areas

Spot 1 (polluted area)

In this area the plant is exposed to both polluted soil and air pollution.

Spot 2 (less polluted area)

This area is situated at a distance of 3 km from polluted area; the plant is exposed to less polluted soil and air.

Spot 3 (control area, relatively unpolluted)

This area is located at a distance of 4 km from polluted area; the plant is grown relatively in unpolluted air and soil.

Post harvest treatment of plant material

Plant samples were collected from the three areas. Plant parts like roots, stems, leaves and seeds were rinsed with de-ionized water, dried, crushed and powdered. The dried samples were stored in bottles for further processing.

Soil samples were taken in plastic envelopes, dried and stored. During all these steps of sample processing necessary measures were taken in order to avoid any loss or contamination with heavy metals.

Acid digestion of soil samples (Aqua regia digestion)

Weighed 1 g of air-dried and sieved (< 2mm) soil and was taken in a flask. To this 15 ml of Aqua Regia was added and swirled to wet the sample. It was kept overnight. Next day the flask was heated at 50 °C for 30 minutes. The temperature was raised to 120 °C and the heating was continued for 2 h. The flask was cooled and added 10 ml of 0.25 M HNO_3 [21].

The solution was filtered through a Whatman No. 542 filter paper. The flask and filter paper were washed with small aliquots of 0.25 M HNO_3. The filtrate and washings were transferred to a 50-mL flask and made up to the mark with 0.25 M HNO_3.

Acid digestion for plant samples

Weighed 1 g of crushed powdered part from each part of plant like root, stem, leaf and seed was taken in a china dish and was heated in the furnace for 4 h keeping the temperature at 550 °C. The contents of the china dish were cooled in a dessicator. Then 2.5 ml 6 M HNO_3 solution were added to the dish to dissolve its contents. The solution was transferred to a 20-mL flask and was diluted up to the mark [21].

The analysis for heavy metals was done by flame atomic absorption spectrophotometer (Polarized Zeeman Hitachi-2000).

Extraction of oil from seeds

Dried capitulums of Silybum marianum were thrashed, seeds were separated and powdered material extracted three times with n- hexane through solvent recycling at room temperature for 10 h. After evaporation of n- hexane under reduced pressure yielded oil [22].

Extraction of Silymarin

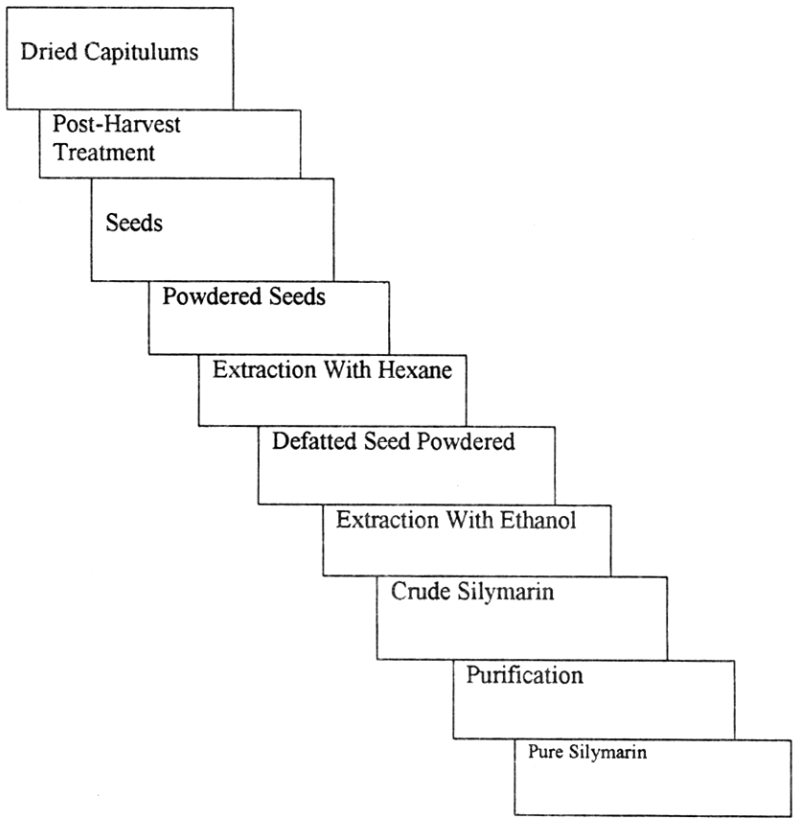
After the removal of oil, the defatted material was then extracted four times with ethanol by percolation and recycling at room temperature for 12 h. The combined alcoholic extract was filtered, concentrated under reduced pressure and gradually added to water under intensive stirring. The product salted out from the aqueous extract with sodium chloride. The precipitated material was filtered, washed with water, dried in an electric vacuum oven and powered. The crude material was yielded.

The crude product was dissolved in absolute ethanol with stirring and shaking. The solution was filtered and then cooled to 10 °C. The resultant turbid solution was filtered and the filtrate dried under reduced pressure, residue obtained was dissolved in acetone, the solution filtered and dried under reduced pressure. The yield of purified silymarin was obtained [22] as shown in table 4.

Acid digestion for oil samples (dry ashing)

Weighed 1 g of the oil sample and transferred in a pre-weighed china dish. The sample was ignited until reduced to dry char. The charred mass was then heated in electrical muffle furnace at 400 °C for 4 h to remove the carbon residue completely. The china dish was cooled in a dessicator. Then 2.5 ml 6 M HNO_3 solution was added to dissolve the contents of the china dish. The solution was transferred to a 20-mL flask and made volume up to the mark with distilled water [23].

Flow Chart for the Extraction and Purification of Silymarin from Silybum marianum



Acid digestion for silymarin

Weighed 1-g silymarin from each of the three spots. Each sample was taken in a china dish and was heated in the furnace for 4-h keeping the temperature at 550 °C. The contents of the china dish were cooled in a dessicator. Then 2.5 ml 6 *M* HNO_3 solution was added to the china dish to dissolve its contents. The solution was transferred to a 20-mL flask and was diluted to the mark of 20 mL with distilled water [21].

Samples were analyzed for HNO_3 extractable Pb, Cr, Cd, Fe, Cu, Mn, Zn, Co, and Ni by using flame atomic absorption spectroscopy (Polarized Zeeman Hitachi-2000).

For the studied elements we established the following sensitivity and detection limits respectively of the used FAA apparatus.

Element	Range mg kg^{-1}
Pb	0.2 - 1.0
Cr	0.5 -3.0
Cd	0.2 - 1.0
Fe	0.5 - 5.0
Cu	0.5 - 3.0
Mn	0.5 - 2.50
Zn	0.05 - 5.0
Co	1.0 - 5.0
Ni	0.5 -4.0

References

1. M.K.Wong, P.Tan and Y.C .Wee, *Biol.Trace Element Res.*, **36**, 135 (1993).
2. J.Kwapulinski, J.Miroslawki, D.Wiechula and R. Rochel, *Bromatologia chemia Toksykolo Giczna*, **29,** 243 (1996).
3. R.Chizzola and C Franz, *J. App. Bot.* **70,** 52 (1996).
4. V.D. Zheljazkov and N.E. Nielson, *Plant and soil,* **178,** 59 (1996).
5. V.D. Zheljazkov and N.E. Nielson, *J. Essent. Oil. Res.*, **8,** 259 (1996).
6. M. Ikram, N.Ahmed and R.Bibi, Bulletin No. 4, PCSIR Labs., Pesh. Pak. 4 (1984).
7. V.H Wood and J.B Herborne, The biology and chemistry of compositae, Academic Press, London, 414 (1977).
8. S.P Mc Grath and S. Smith, Chromium and Nickel in heavy metals in soils, Ed. B.J. Alloway, Blackie, Glasgow, 125 (1990).
9. D.G. Shaheen, Environ. Prot. Agency report, EPA 600/2-75-004. (1975).
10. A.K. Pendias and H.Pendias, Trace elements in soils and plants, 1st. ed. Boca Raton, Fl: CRC Press, (1984).
11. V.D. Zheljazkov, Heavy metal content in some essential oils and plant extracts, International society for horticultural Science, Amherst, USA. 429 (1995).
12. K.Isserman, *Environ. Pollot.*, **12,** 199 (1977).
13. T.C. Broyer, C.N.Johnson and R.E. Paul, *Plant and soil,* **36**, 301 (1972).
14. L. Kiekens, Zinc in heavy metals in soils', Ed.Bj. Alloway, Blackie, Glasgow, 261 (1990).
15. W. Baumeister and W.Ernst, Mineralstoffe und pflanzenwachstum. Fisher Stuttgart, Germany, 416 (1978).
16. A.Faber and J. Niezgoda, *Rocz. Glebozn,* **33,** 93 (1982).
17. B.J. Alloway, Cadmium in heavy metals in soils', Ed.Bj. Alloway, Blackie, Glasgow, 100 (1990).
18. M.B. Mc Bride, *Jr. Environ. Qual.*, **24,** 5 (1995).
19. A.K. Pendias and H.Pendias, " Trace elements in soils and plants'', 2nd. ed. Boca Raton, Fl: CRC press, 365 (1992).
20. K.A. Smith, Manganese and Cobalt in heavy metals in soils, Ed.Bj. Alloway, Blackie, Glasgow, 197 (1990).
21. M.Radojevic and Vladimir, Practical environmental analysis, Royal society of chemistry, Cambridge, UK. 366 (1999).
22. L.Khan, N.Shafi, S.Farooq, S.N. Gilani, T. Mehmood and N. Ahmed, *Pak J. Sci.Ind. Res.* **46,** 307 (2003).
23. R. Lobinski and F.C. Adams, *Trends anal. Chem.*, **12,** 41 (1993).