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QUANTIFICATION OF VARIOUS METALS AND CYTOTOXIC PROFILE OF AERIAL PARTS OF *POLYGONATUM VERTICILLATUM*

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Abstract

The purpose of the current study was to ascertain the concentration of various micronutrients using atomic absorption spectrophotometer and macronutrients using flame photometry and evaluation of cytotoxicity of the aerial parts of the *Polygonatum verticillatum*. Based on the results, the crude extract and its various fractions contained marked concentrations of both micronutrients and macronutrients. The predominant micronutrients were Zn, Fe, Cu, Mn, Cr and Ni. It was noticeable that Ni concentration in hexane (1.80 ppm) and ethyl acetate (2.40 ppm) fractions were beyond the permissible limit (1.5 ppm) for plants and Zn concentration in butanol fraction (60 ppm) was also beyond the permissible limit for plants (50 ppm). Outstanding concentrations of macronutrients were possessed by all solvent fractions with Ca, Na and K ranges from 100–220 ppm, 120-560 ppm and 2500–3400 ppm respectively. There was no sign of brine shrimp cytotoxicity except in the chloroform fraction (LD_{50} was 1205.07 µg/mL). It is concluded that the aerial parts of the plant could be a significant source of micro and macro nutrients without significant cytotoxicity and thus this study validated the folkloric use of the plant as a tonic and energizer.

Introduction

The minerals are integral part of the normal physiology. The minerals essential to human nutrition are accumulated in different parts of plants. Generally, accumulation of a particular metal in plant is the function of its uptake capacity and intracellular binding states (Clemens *et al*., 2002). The plants also have the tendency to accumulate some of the metals which are not linked directly to their survival like Cd, Co and Ag (Ajasa *et al*., 2004). In human, trace elements play a pivotal role both as preventive and as curative agents against various diseases. However, the contaminations of heavy toxic metals in plants due to any factor could develop serious health problems because there is a narrow concentration range between the deficiency and toxicity levels of heavy metals in human (En *et al*., 2003).

Phyto-pharmaceuticals are extensively used for the therapeutic purposes through out the world in various forms (Anon., 2002). Similarly, the medicinal plants are also used in various herbal formulations. The popularity of herbal medicines is not affected by the recent developments in the field of synthetic drugs which are accompanied by potential side effects. The advocates of natural products have the opinion that the herbal medicines are safe enough and even if the desirable therapeutic response is not achieved, their use is not dangerous to health because of their natural origin. However, the clinical findings on the heavy metals poisoning from different parts of the world has ruled out this principle of safety (Ernst, 2002; Basgel *et al*., 2006; Garcia-Rico *et al*., 2007). The World Health Organization emphasized on the quality control parameters of plant based products.

Therefore, various standard analytical techniques are in practice for the analysis of toxic heavy metals and macronutrients in plants to ascertain their purity, safety and efficacy (Ajasa *et al*., 2004).

Polygonatum verticillatum [L.] All. (Nooreallam), a perennial rhizomatous herb belongs to the family *Convallariaceae* (Tamura, 1993; Monika *et al*., 2006). *Polygonatum* has been used in the treatment of various ailments for thousands of years. The rhizome is used in the treatment of pain, pyrexia, burning sensation and for phthisis (Amrit, 2006). It is also used in combination with other herbs to promote urine discharge (diuretic) and attenuate painful urination (Ballabh *et al*., 2008). Additionally the plant is also used as emollient, aphrodisiac, galactagogue (increases milk release), appetizer and tonic (Ghayur, 2004). Recently, we have proved the analgesic profile of the rhizomes of this plant in various animal models (Khan *et al*., 2010). Affinity chromatography has led to the purification of lectins from fresh rhizomes of the plant and was estimated 120 mg/kg (Antoniuk, 1993). While considering the folk uses of the plant in the treatment of different ailments and especially as tonic, the present study was designed to analyze the crude extract of the aerial parts of plant and its subsequent solvent fractions for various micro and macro nutrients and its preliminary safety profile in cytotoxic assay.

Materials and Methods

Plant material: The whole plant, *Polygonatum verticillatum* [L.] All. was collected from District Swat, N.W.F.P., Pakistan, in July-Aug 2007. The botanical identity of the plant material was done by the Taxonomy Department of PCSIR Laboratories Peshawar and a specimen with catalogue No: 9970 (PES) was deposited in the herbarium of PCSIR Laboratories, Peshawar.

Plant extraction and fractionation: The aerial parts of the plant (10 kg) were air dried in shade, chopped into small pieces and powdered. The extraction of plant material was carried out by soaking in methanol at ambient temperature for 14 days. The methanolic extract was filtered through filter paper and the marc obtained was again macerated with methanol. The same process of extraction was repeated three times and the combined filtrates were concentrated under vacuum at low temperature (40°C) using rotary evaporator (Khan *et al*., 2008). Finally, a crude methanolic extract (2.410 kg) was obtained. The crude extract (1.8 kg) was dissolved in distilled water and sequentially partitioned with various solvents to obtain *n*-hexane fraction (275 g), chloroform fraction (295 g), ethyl acetate fraction (210 g), *n*-butanol fraction (317g) and aqueous fraction (445 g).

Sample preparation for mineral analysis: Test sample (1 g) was taken in a conical flask and 10 mL of concentrated HNO_3 (67%) was added. The solution was kept overnight (12 h) at room temperature followed by the addition of 4 mL of $HClO_4$ (67%). The resulting solution was concentrated on hot plate at 60^oC until a clear solution of approximately 1 mL was left. The solution was supplemented with deionized/double distilled water, after cooling, filtered through Whatman (# 42) filter paper. Later on, final volume (100 mL) was made with deionized water that served as stock solution (Hussain *et al*., 2006). The sample was then analyzed in triplicate by flame atomic absorption spectrophotometer (Polarized Zeeman Hitachi 2000) and flame photometer (Jenway PFP7, UK). The materials of all the reference metals were obtained from Merck (Darmstadt, Germany). Calibration standard of each metal was prepared by appropriate dilution of the stock solutions. All chemicals used in the study were of analytical reagent grade.

Brine shrimp cytotoxic assay: Brine shrimp cytotoxic assay described in literature (Meyer *et al*., 1982; McLaghlin *et al.,* 1990) was used to ascertain the cytotoxic potential of the crude extract and its subsequent solvent fractions. Briefly, test samples were prepared in respective solvents in the concentrations of 10, 100, and 1000 µg/mL. Brine shrimp (*Artemia salina* Leach) nauplii were hatched in a specific tank at room temperature. From stock solutions, 5, 50 and 500 µg/mL were injected into 9 vials (3 vials for each dilution). Each vial contained ten shrimps and 5 mL of brine solution. The vials were supplemented with dry yeast suspension as their food and were incubated for 24 h under illumination. For analysis, the live nauplii were counted using a 3 x magnifying glass and the percent deaths at each dose were calculated. The resulting data were processed by using Finney programme on a simple computer to estimate LD_{50} values.

Statistical analysis: Results obtained from the experiments (n = 3) are expressed as Mean values ± SD.

Results and Discussion

Micronutrients: The results of micronutrients investigation of the crude extract and solvent fractions of aerial parts of *Polygonatum verticillatum* are presented in Table 1.

Iron status: Iron (Fe) is the most abundant essential trace element of human body tissues. Its optimal concentration is required for the survival of plants, animals and microorganisms (Arredondo & Nunez, 2005). The world health organization has reported that approximately 46% of the world's children and 48% of pregnant women are suffering from anemia. The Fe deficiency causes irreversible alterations of brain functions and affects immune response in many ways (Beard, 2001). Most of the body iron is taken by hemoglobin (57.6%) and non-heme iron complexes (33%) including ferritin and hemosiderin (James, 2005). In our findings, the samples had Fe concentration ranges from 63–204 ppm. None of the tested sample crossed the permissible limits (36–241 ppm) and therefore appeared as a significant source of iron. Food and Nutrition Board (Anon., 2001) has recommended the daily iron intake as 8 mg/day for male, 18 mg/day for female and 27 mg/day during pregnancy.

Copper status: Copper (Cu) is another essential micronutrient. Many human body proteins are dependent on copper. These include superoxide dismutase, ceruloplasmin, lysyl oxidase, cytochrome oxidase, tyrosinase and dopamine-β-hydroxylase. Cu is necessary for proper working of immune system (Huang & Failla, 2000). During infections, the generation of interleukin-2 by activated lymphocytic cells is dependent on Cu. Systemic decrease in Cu levels causes cellular iron deficiency (Arredondo & Nunez, 2005). Cu toxicity in infancy is based on improper liver functioning. Cu deficiency affects Fe transport in the body tissues and is responsible for a hypochromic microcytic anemia similar to that produced by Fe deficiency. The various fractions of the plant exhibited notable amount of Cu ranges from 0.6–7.43 ppm within permissible limit (10 ppm) for plants. The recommended dietary allowance (RDA) for Cu is 340–900 µg /day.

Table 1. Micronutrients status of the crude extract and subsequent solvent fractions of the aerial parts of the *Polygonatum verticillatum*.

Micro-elements (ppm)						
Minerals	**Crude**	**Hexane**	**Chloroform**	**Ethyl acetate**	**Butanol**	**Aqueous**
Zn	46.24±0.02	45.4±0.04	38.8±0.05	40.6±0.01	60±0.17	45.6±0.03
Cu	6.40±0.01	4.40±0.01	0.6±0.02	1.6±0.01	7.43±0.02	3.6±0.02
Cr	0.36±0.01	01±0.05	0.8±0.05	0.8±0.02	01±0.05	0.6±0.03
Fe	204±1.15	128.2±1.15	106.8±0.03	115.2±0.11	63±0.06	134±1.15
Pb	0.17±0.02	ND	ND	ND	ND	ND
Mn	7.91±0.11	7.20±0.05	5.6±0.02	6.4±0.02	5.20±0.05	7.20±0.11
Ni	0.54±0.02	1.80±0.01	1.20±0.01	2.40±0.01	1.2±00	0.2±0.01
Sb	ND	ND	ND	ND	ND	ND
Cd	ND	ND	ND	ND	ND	ND
Co	ND	ND	ND	ND	ND	ND

ND= Not detected. Data are expressed as the Mean ± SD (n = 3)

Zinc status: In human, Zinc (Zn) is classified as one of the most abundant essential nutrients. It is found in all body tissues mostly in muscles and bones (85%), 11% in the skin and the liver while the remaining Zn is distributed in all the other tissues (Tapiero & Tew, 2003). There are more than 300 Zn dependent body proteins. Zn acts as anti-inflammatory, antioxidant, bone resorptive, important for cell signaling, release of hormones and in apoptosis. Zinc deficiency in human mostly occurs in pregnancy (Moser-Veillon, 1990) and is characterized by growth failure, impaired parturition (dystocia), neuropathy, decreased cyclic food intake, diarrhoea, dermatitis, hair loss, bleeding tendency, hypotension, seizers and hypothermia. Acute Zinc toxicity causes abdominal pain, nausea, vomiting and diarrhoea. Chronic exposure of Zinc elicits copper deficiency (Anon., 2001). The aerial parts of the plants exhibited significant concentration of Zn in the range of 38.8–60 ppm. All the samples had values within the limit (50 ppm) except the *n*-butanol fraction (60 ppm).

Manganese status: Manganese (Mn) is an essential trace element. It plays a pivotal role in the normal growth, skeleton formation and normal reproductive function. Mn intoxication is responsible for Parkinsonism which usually becomes progressive and irreversible, reflecting to some extent the permanent damage of neurologic structures (Wang & Du, 2008). The permissible limit for plants is estimated as 200 ppm. In the present analysis, the crude extract had the highest concentration (7.91 ppm) while the overall range was from 5.20–7.91 ppm.

Chromium status: Chromium (Cr) is provisionally considered to be a nutrient because of its metabolic role and is one of the abundant elements on the earth (Emsley, 2001). It plays important role in the synthesis of fatty acids and cholesterols, metabolism of carbohydrates, proteins, lipids and has also been proved that it facilitates the action of insulin (Anon., 2001). Therefore, Chromium based supplements are used for weight loss (Lukaski *et al*., 2007). The estimated permissible limit for Cr in plants is 1.5 ppm. The results obtained in the present study showed the metal accumulation in the range of 0.36–01 ppm.

Table 2. Micronutrients status of the crude extract and subsequent solvent fractions of the aerial parts of the *Polygonatum verticillatum*.

Macro-element (ppm)						
Minerals	**Crude**	**Hexane**	**Chloroform**	**Ethyl acetate**	**Butanol**	**Aqueous**
Ca	129.42±0.04	100±0.56	140±1.15	140±1.73	140±00	220±1.15
Na	219.34±0.57	120±0.57	140±1.15	120±0.57	560±1.73	400±2.31
K	3250±2.31	2900±4.04	3400±6.92	2500±1.15	3120±2.88	3400±5.19

Data are expressed as the Mean ± SD (n = 3)

Nickel status: Nickel (Ni) is a metallic element that is naturally present in the earth's crust. Due to its abundance, natural nickel deficiency does not occur and dietary deficiency of nickel is rare because of nickel's abundance in all types of food. Health related hazards of nickel include skin allergies, lung fibrosis, variable degrees of kidney and cardiovascular system poisoning and stimulation of neoplastic transformation (Denkhaus & Salnikow, 2002). Nickel is mostly present in the pancreas and plays an important role in the production of insulin. Nickel deficiency is responsible for liver disorders (Cempel & Janicka, 2002). The permissible limit of Ni in plants is 1.5 ppm. Our data revealed that the plant accumulated reasonable concentration of Ni and was in the range of 0.54–2.40 ppm. The *n*-hexane fraction (1.80 ppm) and ethyl acetate fraction (2.40 ppm) exceeded the permissible limit.

Macronutrients: The results of macronutrient analysis in our investigation of the crude extract and its subsequent solvent fractions of aerial parts of the *Polygonatum verticillatum* are presented in Table 2.

Sodium status: Sodium (Na) is very important macronutrient of human body system. The most common dietary source of sodium is common table salt (NaCl). It has got the prime role in the maintenance of normal physiology in all living organisms. A lack of sodium intake is incompatible with survival. An adequate intake of sodium is required for optimal growth. Distribution of intracellular and extracellular fluid volumes are dictated by sodium and either a deficiency or excess of sodium will alter overall fluid balance and distribution (Morris *et al*., 2008). Na depletion is characterized by mood changes, muscle cramps, fatigue, hair loss, hypotension and dehydration (Harper *et al*., 1997). We observed marked concentration of Na in various fractions of the plant and were in the range of 120–560 ppm. There is no international limit which reflects concentration of Na in plants. However, the recommended daily intake of Na is 1–3.8 mg/day (Anon., 2004).

Potassium status: Potassium (K) represents a very important macronutrient of living organism. The concentration of K ions is most frequently associated with regulation of action potentials and intercellular signaling in electrically active cells. The K channels are involved in multiple functions in both excitable and non-excitable cells. These cellular regulations include regulation of membrane potential, signal transduction, insulin secretion, hormone release, regulation of vascular tone, cell volume and immune response (Curran, 1998). There is no international limit which reflects the concentration of potassium in plants. However, the average intake of Potassium is 2300 mg/day for adult women and 3100 mg/day for adult men (Anon., 2001). The results of our study showed a range of 2500–3400 ppm for tested samples which is absolutely within the permissible range.

Table 3. Brine shrimp cytotoxicity of the crude extract and subsequent solvent fractions of aerial of *Polygonatum verticillatum*.

Fractions	Conc (µg/mL)	No of shrimps taken	No. of shrimps survived	LD_{50} (µg/mL)
Crude extract	1000	30	19	
	100	30	20	-
	10	30	22	
Hexane	1000	30	26	
	100	30	27	-
	10	30	28	
Chloroform	1000	30	14	
	100	30	23	1205.07
	10	30	23	
Ethyl acetate	1000	30	23	
	100	30	26	-
	10	30	27	
Butanol	1000	30	23	
	100	30	25	-
	10	30	28	
Aqueous	1000	30	23	
	100	30	25	-
	10	30	25	
Etoposide	-	-	-	07.4625

Incubation at 28°C±1°C.

Calcium status: Calcium (Ca) is one of the most important macronutrients mostly obtained from various dietary sources. Apart from its crucial role in the body's metabolic process, Ca along with Phosphorus is a structural component of bones, teeth, and soft tissues (Shapiro & Heaney 2003). Binding of the Calcium ions on the surface of human growth hormone provides considerable thermodynamic stability to protein by changing the secondary structure of the protein (Saboury *et al*., 2005). Cellular Calcium is involved in various regulatory functions like regulation of muscle and nerve functions, glandular secretions, and blood vessel dilation and contraction. Ca deficiency is responsible for weakness of the bones and thus bones are more prone to fracture. It can produce skeletal muscles spasm and abnormality in heart beat and can even cease functioning of heart. Ca intoxication is rare but when occurs is characterized by hypercalcemia, which causes constipation, kidney stones, appetite loss, nausea, vomiting, abdominal pain, confusion, seizures, and even coma (Anon., 1997). The tested samples in our investigation accumulated significant concentration of Ca ranges from 100–220 ppm. According to the Food and Nutrition Board, the recommended daily intake of Ca is 1000 mg/day. Based on our results, aerial parts of the plant is a rich source of dietary Ca.

Cytotoxic assay: Brine shrimps cytotoxic assay was performed for the crude extract and subsequent solvent fractions of the aerial parts of the plant. It was demonstrated by the results that all the extracts were safe in the preliminary cytotoxic study while only the chloroform fraction had LD_{50} 1205.07 µg/mL.

It can be concluded on the base of our results that the aerial parts of *P. verticillatum* is an excellent source of micro and macro nutrients within the permissible limits for plants. Therefore, our study validated the ethno-botanical use of the aerial parts of the plant as tonic and energizer.

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