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Assessment of Heavy Metals in Dried *Stevia* Leaves by Atomic Absorption Spectrophotometer Grown under Various Soil Conditions

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Abstract: Stevia cultivation were conducted at different places of South India namely Ripponpet (Dist. Karnataka) and Aravavi (Dist. Gokak), under an acidic and basic soil zones (pH 6.10 and 8.20 respectively) to procure the high yield foliage production and to determine the accumulation of non essential heavy metal contents (Ni, Pb, Cr and Cd) in dried leaves of *Stevia* plant by applied of bio-fertilizers. Analyzed results revealed dried *Stevia* leaves contains very trace amount of Ni, Pb, Cr and Cd whereas the amount of leaf biomass increased up to six months of the study (341g) procured from acidic soil zone. The same trends were followed in case of basic soil samples, where dried biomass increased up to 325 g during total six months of study. The level of heavy metal content differed in the same *Stevia* plant collected from environmentally different sites and were found to be varied in both the soil zones but calculated lesser than that of toxic level. This may be due to nature of soil environment and intake of nutrients by supplied biofertilizers in soil.

Key words: Acid soil · Atomic Absorption Spectrophotometer · Basic soil · Heavy metals

INTRODUCTION

The use of medicinal plants is limited by the quality of active substances they contain and that's depends on many ecological factors especially the soil quality [1]. In view of renewed interest, herbal medicines have prominent role to play in the pharmaceutical and health markets of the 21st century [2]. It has been reported that food could cause metabolic disturbance subject to the allowed upper and lower limits of trace metals [3] because they plays an important role in the plant by formation of bioactive constituents in the medicinal plants. Both the deficiency and excess of essential micronutrients and trace of toxic metals may cause serious health hazards like hypertension, abdominal pain, skin eruptions, intestinal ulcer and different types of cancers [4, 5]. Cadmium is reported with its adversed effects as an esteomalacia and pyelonephritis and with Pb renal tumors. Thus, analytical data are necessary for referring plant as defective, if the element concentrations are toxic. World Health Organization (WHO) has recommend that medicinal plants which form the raw materials for the finished products may be checked for the presence of heavy metals, further it regulates maximum permissible limits of toxic metals like arsenic, cadmium and lead [6, 7].

Oflate, *Stevia*, the nature's sweetest gift belongs to the family asteraceae, is a native to South America (Paraguay, Brazil). It is extensively grow in places like Central America, Israel, Australia, Japan and China [8]. The plant having various sweet diterpene glycosides namely, rebaudioside A, rebaudioside C, stevioside and dulcoside in its leaf tissue [9], in which stevioside is about 350 times more sweeter than table sugar. The interest towards *Stevia* in World market is due to its versatile medicinal uses without any side effects. It is used for the treatment of various conditions such as cancer [10], diabetes [11], obesity, cavities, hypertension [12], fatigue, depression and in cosmetic and dental preparations [13]. It possesses hypoglycemic, hypotensive, vasodilating, taste improving, sweetening, anti-fungal, anti viral, anti inflammatory, anti bacterial [14] properties and increases urination function of the body. It is non toxic, non addictive, non carcinogenic, non mutagenic, non teratogenic and is devoid of genotoxic effect [15]. It does not affect blood sugar level hence safe for diabetics [16]. With these versatile applications, it is only in the past couple of years that is really started to capture attention in the India market as a healthy alternative bio sweetener to sugar. Raw *Stevia* leaves are 20-30 times sweeter than sugar and can be used

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in raw form. It can be used in the manufacture of chewing gums, mints, mouth refreshers and even in pan. The soft drink manufactures have introduced several health drinks and many food supplementary beverages. Looking at the importance of raw *Stevia* leaves, it was necessary to check the heavy metal contents in the same to avoid unwanted health toxicity and hence the present field investigation was carried out to determine the contents of heavy metals present in dried *Stevia* leaves and safety, from the different cultivated soil zones of South India.

MATERIALS AND METHODS

Cutting of *Stevia* plants, collected from Ankur Nursery, Ripponpet (Shimoga, Karnataka), India, were used as a test plant for the present study. As a part of Ph.D research study, a field experiment was conducted for the period of six months at the Ripponpet, Shimoga on acidic soil reaction (pH 6.10) and at Aravavi, Gokak on basic soil reaction (pH 8.20). After six month of field experiments the plant samples (leaves) were collected and oven dried at 60°C for 6 hours. Further the dried leaves were stored at 4°C and were used for further preparation of the herbal extract.

The weight of oven dried (60°C) plant biomass were recorded from the acidic and basic soil of Shimoga and Aravavi. The plant samples were powdered and the same were used for the analysis of different elements such as Cd, Cr, Pb and Ni contents. A ternary acid mixture was prepared by mixing 100 ml of concentrated nitric acid (HNO_3), 10 ml of concentrated sulphuric acid (H_2SO_4) and 40 ml of 60% perchloric acid ($HClO_4$) and allowed to cool and stored in a reagent bottle and kept for the use of oxidation of plant sample. Further a binary solution of two acids was prepared by mixing concentrated H_2SO_4 and $HClO_4$ at ratio of 6: 4. For correction readings, blank digestions (in duplicate) were run on the reagents added in the same amounts as employed in the determinations. Then pretreated sample with $HN0_3$ was placed in digestion flask and then mixed with appropriate amount of the ternary acid mixture, consisting of 5 ml for 1-2 gms of powdered plant samples. Digestion was carried out at 180°C to 200°C until dense white fumes of H_2SO_4: $HClO_4$ were evolved. The digestion was continued at 180°C to 200°C until the acid was largely volatilized and the residues in the flask were clear white and only slightly moist with H_2SO_4. The residue was diluted with glass distilled water (pure deionized water) and made up to definite volume in a volumetric flask. Then the solution was ready for the estimation of different toxic heavy metals like Cd, Cr, Pb and Ni with the help of Atomic Absorption Spectrophotometer (AAS) Perkin Elmer model AAnalyst 100.

RESULTS AND DISCUSSION

It was revealed that the higher concentration of Ni in plants may be due to anthropogenic activities and hence the most common ailment arising from Ni is an allergic dermatitis known as nickel itch, which usually occurs in moist skin. But, in this present investigation, Table 1 revealed that the amount of Ni content in leaves of *Stevia* plant growing in an acid soil has been found to be significantly very low and decreased with the progress of growth up to 6 months irrespective of treatments. As regards to the effect of different treatments, it was observed that the amount of Ni content was recorded a significantly very low when PSB and VAM was applied singly while the amount of the same recorded a trace value when PSB and VAM were applied in combination. However, the results suggested that the application of biofertilizers either applied singly or combinely did not show any significant accumulation of Ni in leaves of *Stevia* rather far below the level of its toxicity which was similar as reported earlier [17]. The same trend was followed by the *Stevia* leaves collected from basic soil zone where trace amount of Ni was reported in the treatment eight with combined application of three biofertilizers (Table 2).

Lead (Pb) is one of the non essential heavy metal that induced toxic effects in human in very low doses. It can cause colic, anemia, headache, convulsions and chronic nephritis of the kidneys, brain damage etc. Hence the determination of Pb was essential in the dried leaves of *Stevia*. The results (Table 3, 4) reveal that the amount of Pb content in leaves did not show any significant accumulation or increase during the growth period irrespective of treatments. However, the amount of Pb content in leaves of *Stevia* plant did not show any consistent trend of changes with the treatments and progress of growth. The combined application of PSB and VAM showed a trace amount of Pb in leaves while that of the same content showed a significant change with the application of three biofertilizers collected from both the soil zones. WHO (1998) prescribed limit for Pb contents in herbal medicine is 10 ppm while the dietary intake limit for Pb is 3 mg week^{-1} and the present results also were revealed the Pb content in *Stevia* leaves were mostly below detectable level [7].

Table 1: Ni content (mg kg^{-1}) in dry leaves of *Stevia* growing in acidic soil

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	0.08	0.05	0.08	0.10	Trace	Trace
PSB	0.31	0.20	Trace	0.08	0.18	Trace
AZO	0.20	Trace	Trace	Trace	Trace	Trace
VAM	0.15	Trace	0.47	Trace	0.45	0.14
V + A	Trace	0.08	0.15	0.12	0.21	Trace
P + A	0.43	Trace	0.28	Trace	Trace	0.37
P + V	Trace	Trace	Trace	Trace	Trace	Trace
P + A + V	0.06	0.16	0.02	0.14	0.21	0.13
CD value (p= 0.05)	0.018	0.010	0.011	0.010	0.010	0.012

*CD = Critical difference; V= VAM; A= AZO; P=PSB; Trace= negligible amount (Very low value)

Table 2: Ni content (mg kg^{-1}) in dry leaves of *Stevia* growing in basic soil

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	0.01	Trace	Trace	Trace	Trace	Trace
PSB	0.12	0.10	0.05	0.03	0.02	0.02
AZO	0.11	0.12	0.03	0.03	0.02	0.02
VAM	0.16	Trace	Trace	Trace	0.02	Trace
V + A	0.14	Trace	0.02	Trace	Trace	Trace
P + A	0.14	0.02	Trace	Trace	0.02	Trace
P + V	Trace	0.04	0.02	0.03	Trace	Trace
P + A + V	Trace	Trace	0.04	Trace	Trace	Trace
CD value (p= 0.05)	0.03	0.05	NS	NS	NS	NS

*CD = Critical difference; V= VAM; A= AZO; P=PSB; Trace= negligible amount (Very low value)

Table 3: Pb content (mg kg^{-1}) in dry leaves of *Stevia* growing in acidic soil

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	0.34	0.32	Trace	0.04	0.20	Trace
PSB	0.48	0.46	0.25	0.33	0.42	Trace
AZO	0.35	Trace	Trace	Trace	Trace	Trace
VAM	0.04	Trace	0.76	Trace	0.62	0.14
V + A	Trace	0.52	0.23	0.72	0.39	Trace
P + A	1.07	Trace	0.32	Trace	Trace	0.07
P + V	Trace	Trace	Trace	Trace	Trace	Trace
P + A + V	0.02	0.43	0.37	0.14	0.79	0.67
CD value (p= 0.05)	0.033	0.012	0.018	0.015	0.094	0.044

*CD = Critical difference; V= VAM; A= AZO; P=PSB; Trace= negligible amount (Very low value)

Table 4: Pb content (mg kg^{-1}) in dry leaves of *Stevia* growing in basic soil

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	Trace	0.02	0.02	0.01	Trace	Trace
PSB	0.19	0.03	0.03	0.02	Trace	Trace
AZO	0.12	0.02	0.02	0.02	Trace	Trace
VAM	Trace	Trace	Trace	Trace	0.02	Trace
V + A	Trace	Trace	0.03	Trace	0.02	Trace
P + A	Trace	0.03	0.02	Trace	Trace	Trace
P + V	0.10	0.09	0.05	0.02	0.02	Trace
P + A + V	0.14	0.15	0.06	0.03	0.02	Trace
CD value (p= 0.05)	0.03	0.05	NS	NS	NS	NS

*CD = Critical difference; V= VAM; A= AZO; P=PSB; Trace= negligible amount (Very low value)

Table 5: Cr content (mg kg^{-1}) in dry leaves of *Stevia* growing in acidic soil

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	Trace	Trace	Trace	0.88	0.23	Trace
PSB	Trace	Trace	0.33	Trace	0.43	Trace
AZO	Trace	Trace	Trace	Trace	Trace	Trace
VAM	Trace	Trace	Trace	Trace	Trace	0.47
V + A	Trace	0.02	0.53	Trace	0.75	Trace
P + A	0.35	Trace	0.08	Trace	Trace	0.16
P + V	Trace	Trace	Trace	Trace	Trace	Trace
P + A + V	0.15	0.76	0.18	0.01	Trace	0.12
CD value (p= 0.05)	0.016	0.011	0.016	0.010	0.026	0.013

*CD = Critical difference; V= VAM; A= AZO; P=PSB; Trace= negligible amount (Very low value)

Table 6: Cr content (mg kg^{-1}) in dry leaves of *Stevia* growing in basic soil

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	0.03	Trace	Trace	Trace	Trace	Trace
PSB	0.02	0.02	0.03	Trace	Trace	Trace
AZO	0.04	Trace	0.02	0.03	Trace	0.02
VAM	Trace	0.03	Trace	Trace	Trace	Trace
V + A	Trace	Trace	0.02	Trace	Trace	Trace
P + A	0.02	Trace	0.03	0.02	0.02	Trace
P + V	Trace	0.02	0.02	Trace	Trace	Trace
P + A + V	0.02	0.03	Trace	Trace	Trace	Trace
CD value (p= 0.05)	NS	NS	NS	NS	NS	NS

*CD = Critical difference; V= VAM; A= AZO; P=PSB; Trace= negligible amount (Very low value)

Table 7: Cd content (mg kg^{-1}) in dry leaves of *Stevia* growing in acidic soil

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	0.21	0.07	Trace	Trace	Trace	Trace
PSB	Trace	Trace	0.11	0.14	0.19	Trace
AZO	0.01	Trace	Trace	Trace	Trace	Trace
VAM	Trace	Trace	Trace	Trace	0.16	Trace
V + A	Trace	Trace	Trace	Trace	0.37	Trace
P + A	0.34	Trace	0.18	Trace	Trace	0.07
P + V	Trace	Trace	Trace	Trace	Trace	Trace
P + A + V	0.03	0.18	0.06	Trace	Trace	Trace
CD value (p= 0.05)	0.021	0.004	0.017	0.007	0.014	0.003

*CD = Critical difference; V= VAM; A= AZO; P=PSB; Trace= negligible amount (Very low value)

Table 8: Cd content (mg kg^{-1}) in dry leaves of *Stevia* growing in basic soil

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	0.04	Trace	Trace	Trace	Trace	Trace
PSB	0.05	0.03	0.02	0.03	0.02	Trace
AZO	0.03	Trace	0.03	0.03	Trace	Trace
VAM	0.05	Trace	Trace	Trace	Trace	Trace
V + A	0.03	Trace	0.02	Trace	Trace	Trace
P + A	0.02	0.02	0.02	0.02	Trace	Trace
P + V	Trace	0.03	0.02	Trace	0.02	Trace
P + A + V	Trace	0.02	0.03	Trace	Trace	Trace
CD value (p= 0.05)	0.02	NS	NS	NS	NS	NS

CD = Critical difference; V= VAM; A= AZO; P=PSB; Trace= negligible amount (Very low value)

Table 9: Changes in dried biomass yield of *Stevia* (g) plant growing in acidic soil

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	50.00	125.00	157.00	175.00	179.00	183.00
PSB	53.00	142.00	167.00	187.00	194.00	210.00
AZO	55.00	152.00	170.00	172.00	180.00	198.00
VAM	30.00	115.00	162.00	176.00	185.00	204.00
V + A	55.00	165.00	175.00	195.00	205.00	216.00
P + A	64.00	160.00	185.00	215.00	224.00	238.00
P + V	50.00	145.00	190.00	210.00	230.00	229.00
P + A + V	42.00	180.00	240.00	296.00	310.00	341.00
CD value (p= 0.05)	11.02	20.39	24.43	13.44	22.65	19.05

*CD = Critical difference; V= VAM; A= AZO; P=PSB

Table 10: Changes in dried biomass of *Stevia* (g) plant growing in basic soil

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	62.00	148.00	149.00	158.00	160.00	182.00
PSB	66.00	162.00	155.00	168.00	174.00	201.00
AZO	71.00	168.00	164.00	175.00	183.00	192.00
VAM	46.00	132.00	160.00	172.00	192.00	209.00
V + A	73.00	194.00	171.00	188.00	203.00	225.00
P + A	76.00	183.00	169.00	181.00	190.00	252.00
P + V	71.00	171.00	176.00	192.00	211.00	278.00
P + A + V	61.00	208.00	208.00	214.00	239.00	325.00
CD value (p= 0.05)	6.12	16.44	18.56	21.33	25.68	28.96

*CD = Critical difference; V= VAM; A= AZO; P=PSB

Table 11: Coefficient of correlations (r) between biomass yield *Stevia* and different heavy metal contents in leaves growing under acidic soil

	Ni	Pb	Cr	Cd	Bio mass
Ni	1.000				
Pb	0.556**	1.000			
Cr	0.218**	0.187*	1.000		
Cd	0.465**	0.350**	0.478**	1.000	
Bio mass	-0.066	0.131	0.076	-0.119	1.000

** significant at 1% level; *Significant at 5% level

Table 12: Coefficient of correlations (r) between biomass yield *Stevia* and different heavy metal contents in leaves growing under basic soil

	Ni	Pb	Cr	Cd	Bio mass
Ni	1.000				
Pb	0.204*	1.000			
Cr	0.160	0.363**	1.000		
Cd	0.525**	0.312**	0.401**	1.000	
Bio mass	-0.546**	-0.344**	-0.322**	-0.421**	1.000

** significant at 1% level; *Significant at 5% level

The toxic effects of Chromium (Cr) intake is skin rash, nose irritations, bleeds, upset stomach, kidney and liver damage, nasal itch and lungs cancer, chromium deficiency is characterized by disturbance in glucose lipids and protein metabolism [17]. Hence the determination of Cr in dried leaves of *Stevia* can't be ignored. The results (Table 5, 6) show that the amount of content in leaves of *Stevia* plant did not show any significant increase or accumulation during the growth period of plant irrespective of treatments. All the treatments either applied singly or combinedly showed a trace amount of Cr which suggested that the application of biofertilizers namely, PSB, Azotobacter and VAM did not have any significant role for the accumulation of Cr in the plant that were collected from both the fields. It was reported that the daily intake of Cr 50-200 ìg has been recommended for adults by US National Academy of Sciences [18] and our results showed below detectable limit which were safe for human use.

Cadmium (Cd) accumulates in human body damages mainly the kidneys and liver. Hence the accumulation of the same can determine. The results (Table 7, 8) from both the soil zones show that the amount of Cd content in leaves was recorded a significantly low or trace amount in almost all the treatments during the growth period of the plant. It was suggested that the application of biofertilizers viz. PSB, Azotobacter and VAM have favorable effect by preventing accumulation of toxic heavy metals especially Cd by the *Stevia* plant when applied in sole or in combinations. The lowest level of Cd which can cause yield reduction is 5- 30 ppm, while the maximum acceptable concentration for food stuff is around 1 ppm [19]. Interestingly, no Cd was detected in plant samples (Below detection limit) collected from both the soil samples.

The results (Table 9) show that the yield of dried biomass of *Stevia* has been found to be increased progressively and significantly irrespective of treatments. The magnitude of such increase in biomass yield, however, varied with treatments, being significantly highest increase (341 g) in the treatment where PSB, Azotobacter and VAM were applied togetherly at 6 months of plant growth. As regards to the individual applications of biofertilizers, the application of PSB exhibits a greater increase, being about 15 % over control during 6 months period of growth, while that of the same biomass yield was further increased to about 30 % over control when PSB and Azotobacter was applied combinedly which might be due to greater availability of N and P in soils enhancing growth of *Stevia* plant. However, such increase in biomass yield has been found to be further enhanced when PSB, Azotobacter and VAM were applied simultaneously, being about 86 % increase over control during 6 month period of growth. Such highest increase might be explained by the greater availability of plant nutrients especially of N and P in soil solution influencing growth of the plant favourably. Same trend has followed by the *Stevia* plants collected from the basic soil zone and showed higher (325 g) with the treatment eight where combined three biofertilizers were applied together (Table 10).

Correlation between all the heavy metals with biomass yields were drawn and reported. The results (Table 11) show that the biomass yield of *Stevia* plant showed a non-significant positive and negative correlations with Ni, Pb, Cr and Cd contents in leaves of *Stevia* growing under acidic soil with Ni (r = -0.066), Pb (r = 0.131), Cr (r = 0.076) and Cd (r = -0.119). This co-efficient of correlation has suggested that with an increase in biomass yield of *Stevia*, the above heavy metal contents are very low and vice-versa. Where as significant negative correlation were reported with heavy metals and biomass yields that were collected from basic soil zones with Ni (r = -0.546**), Pb (r = -0.344**), Cr (r = -0.322**) and Cd (r = -0.421**) (Table 12).

CONCLUSIONS

Concentration of non-essential heavy metals in medicinal plants beyond permissible limit is a matter of great concern to public safety all over the world. The overall results reveal that the amount of heavy metal contents (Ni, Pb, Cr and Cd) were recorded significantly far below the toxic level with the simultaneous increase in biomass yield of *Stevia* growing under both acidic and basic soils. The results further suggested that the production of biomass of *Stevia* was found to be free from toxic heavy metals when it was grown with the applications of biofertilizers either singly or combinedly or togetherly.

The implication of findings may be taken into consideration whilst using the raw leaves of *Stevia* herb for human consumption and safe. *Stevia* plant may not be considered as a hyperaccumulator toxic plant for most of the heavy metals including Ni, Pb, Cr and Cd.

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