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Research Article

Biochemical Alterations Induced by Sodium Arsenate in *Momordica charantia* L. Seeds *In vitro*

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Abstract

Momordica charantia L. (Family: Cucurbitaceae) is a well known medicinal plant and widely distributed and cultivated in many parts of the world. *Momordica* is a powerful, nutrient-dense plant, composed of a complex array of beneficial compounds. Seeds were collected from arsenic free area and were propagated in tissue culture media in presence of different concentration of sodium arsenate along with control one. The aim of the present study was to investigate some biochemical parameters of extracts of *Momordica* under the influence of arsenic. Chlorophyll and carotenoid content was affected by arsenic maximum (150 µM) in *Momordica*. Arsenic induced alteration in free radical scavenging activity, total polyphenolic content, lipid peroxidation, antioxidant activity and flavonoid content were determined in this study.

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- Chlorophyll
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- Free radical scavenging activity

INTRODUCTION

Momordica charantia L. (bitter melon) is a vegetable crop plant of the family Cucurbitaceae [28,29,38] widely grown in India, South Asia, China, Africa and the Caribbean [2]. It is a medicinally important species which is also used as foodstuff [37]. Popularity of *M. charantia* in various systems of traditional medicine for several ailments focused the investigator's attention on this plant [23].

Arsenic (As) ranks twentieth most abundant of elements in the earth's crust and fourteenth in sea water. As is the twelfth of most abundant element usually found in human body [18]. Arsenic is an element that is nonessential for and toxic to plants [42]. Large areas of Bangladesh, West Bengal and other states in India and Vietnam rely on arsenic contaminated ground-water for irrigation [1,3]. Arsenic originates from geochemical and anthropogenic sources. Arsenic is known to induce oxidative stress [8] resulting a wide range of responses in plants, including readjustment of transport and metabolic processes, growth inhibition [12] and biochemical changes in plants. The present study was designed to specifically investigate biochemical alteration such as chlorophyll, carotenoid, free radical scavenging activity, total polyphenolic activity, lipid peroxidation, total antioxidant, total flavonoid content etc.

MATERIALS AND METHODS

Collection of seeds and *in vitro* propagation

Seeds of *M. charantia* were collected from Burdwan town, District Burdwan, West Bengal, India. Seeds were propagated by proper tissue culture technique [23]. Seeds were germinated in sodium arsenate heptahydrate ($Na_2HAsO_4.7H_2O$) (Himedia, Mumbai, India) supplemented agar sucrose media. Different concentration of sodium arsenate i.e. 10 µM, 25 µM, 50 µM, 75 µM, 100 µM, 125 µM, 150 µM, 175 µM and 200 µM were chosen for the experiment along with control one (no treatment) *in vitro* (LD_{50} was determined as 200 µM).

Preparation of extracts

Plant extracts were prepared by taking 100 mg of roots and shoots from each of the samples (control and sodium arsenate treated). The tissues were finely crushed and dissolved in 1 ml of ethanol (Merck, Germany) using mortar and pestle. The mixture was then ultrasonicated for 20 min, followed by centrifugation. The supernatant was collected and prepared for the experiments. The plant extracts were used for determination of 1, 1-diphenyl-2-picryl hydroxyl (DPPH) radical scavenging activity, polyphenol content, total antioxidant activity, and total flavonoid assay.

Estimation of chlorophyll and carotenoid content

Chlorophyll and carotenoid (carotene + xanthophyll) contents

were determined following the methods of Lichtenthaler [15] with minor modifications.1 mg leaves from seedlings (arsenic treated and control seedlings of *M. charantia*) were taken and were crushed in a mortar and pestle with acetone and adjusted to 5ml. After homogenization the extract is centrifuged and the clear supernatant was diluted and used for spectrophotometric analysis. Absorbance for Chlorophyll a and Chlorophyll b was taken at 662 nm and 645 nm wavelengths respectively. Absorbance for carotenoids (Carotene and Xanthophyll) was measured at 470 nm. Chlorophyll and carotenoid content was calculated using the following equations:

Chlorophyll a (µg/ml) = 11.24(A_{662}) – 2.04 (A_{645})

Chlorophyll b (µg/ml) = 20.13 (A_{645}) – 4.19 (A_{662})

Total Chlorophyll (µg/ml) = 7.15 (A_{663}) + 18.71 (A_{646})

Carotenoids (Carotene+Xanthophyll) (µg/ml) =

(1000 A_{470}-1.90 Chlorophyll (a)-63.14 Chlorophyll (b)

214

The chlorophyll and carotenoid content was finally expressed as mg/g fresh weight (FW).

Free radical scavenging assay

Free radical scavenging assay was done using the method of Brand-Williams et al. [4], with some modifications. Antioxidants react with DPPH and reduce it to DPPH-H which is yellow in color where as the original color of free radical, DPPH is red in color. Thus the degree of decolorization indicates scavenging activity of the antioxidants using their hydrogen-donating ability. 150 µl extract of each sample was allowed to react with 2.850 ml DPPH solution and kept for 1h in the dark. DPPH is a stable free radical with a characteristic absorption at 517 nm, was used to study the radical-scavenging effects of *Momordica* grown in different concentration of arsenic *in vitro*. The antioxidant capacity was calculated using the following formula:

DPPH radical scavenging activity = $[(A_{control} - A_{sample})/A_{control}] \times 100\%$.

$A_{control}$ is the absorbance of the control (without any extract) and A_{sample} is the absorbance of extract.

Estimation of total polyphenol

Total polyphenol content was determined by Folin-Ciocalteu's phenol reagent (FCR) using the method of Singleton et al. [33], Plant extract (shoots and roots) of each of the samples was mixed with 250µl FCR and 750µl of 10% sodium carbonate solution. The mixture was mixed well and incubated at room temperature for 30 min in dark condition. The absorbance was then measured at 760 nm in a UV–visible spectrophotometer. Total polyphenol content was expressed as grams of gallic acid equivalents (GAE) per kg FW of tissue by comparing with the gallic acid (Sigma Aldrich, StLouis, MO, USA) standard curve. Standard curve was prepared with a range of standard gallic acid concentrations from 0 to 100 µg ml^{-1}.

Lipid peroxidation assay

The level of lipid peroxidation in samples was expressed as malondialdehyde (MDA) content and measured [10]. Root and shoot tissue samples were crushed 0.1 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged. The supernatant was collected and mixed with 4 ml TCA–thiobarbituric acid (TBA) solution. The mixture was heated at 95°C for 30 min and then quickly cooled on ice. The absorbance of the supernatant was read at 532 nm. The concentration of lipid peroxides together with oxidative modified proteins of plants were thus quantified in terms of MDA level using an extinction coefficient of 155 mmol L^{-1} cm^{-1} and expressed as µmol $L^{-1}g^{-1}$ FW.

Estimation of total antioxidant

Antioxidant activity of the plant extracts was determined using phosphomolybdenum assay method [26]. The assay is based on the reduction of Mo (VI)-Mo (V) by the plant extracts and then formation of a green phosphate-Mo(V) complex at acidic pH. Best results were obtained at 695 nm absorbance. 0.3 ml of each samples were mixed with 3ml reagent solution. The reaction mixture was incubated at 95^0C for 90 min. Absorbance was taken at 695 nm in UV–visible spectrophotometer .The antioxidant activity was expressed as grams of ascorbic acid equivalents (AAE) per Kg FW tissue by comparing with the ascorbic acid calibration curve. Standard curve was prepared with a range of standard ascorbic acid (Sigma Aldrich, StLouis, MO, USA) concentrations from 0 to 100 µg ml^{-1}.

Estimation of total flavonoids content

The total flavonoid content was determined using aluminium chloride ($AlCl_3$) method [16]. 1 ml root and 1 ml shoot extract of each sample with control one was taken. Samples were mixed with 0.1 ml $AlCl_3$, 0.1 ml of potassium acetate and 1.8 ml deionized water. The flavonoid contact was expressed as grams of rutin equivalent (RE) per kilogram of FW of tissue by comparing with the rutin (Sigma Aldrich, StLouis, MO, USA) standard curve. Standard curve was prepared with rutin concentration from 0-100 µg mL^{-1}.

Data analysis

The data were presented as the mean ± standard error of mean (SE). The experiments were performed with three replicates. The statistical analysis was carried out by using one-way analysis of variance (ANOVA). Data represent the mean ± SE. Asterisks indicate significant differences at $p < 0.05$ (*) or $p < 0.01$ (**) or $p < 0.001$ (***) compared to respective controls.

RESULTS AND DISCUSSION

To evaluate the impact of abiotic stress induced by sodium arsenate chlorophyll content was measured in *Momordica*. Changes in pigment content are associated with photosynthetic efficiency [6]. The thylakoids of the chloroplast may be damaged by the effect of arsenic toxicity. As a result, decrease of chlorophyll content was observed with increasing doses of sodium arsenate. Chlorophyll-a was more badly affected than chlorophyll-b *in vitro* by exposure to inorganic arsenic. A highest reduction in chlorophyll-b (38%) was observed at 150 µM sodium arsenate treated sample as compared to control (Figure 1). Amounts of carotenoids decreased as the concentration of arsenic in the media increased (Figure 2). Highest carotenoid content was

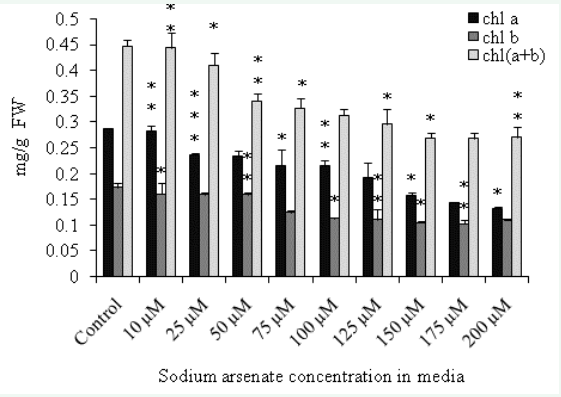

Figure 1 Histogram of amount of chlorophyll in *M. charantia* grown in different sodium arsenate concentration in media *in vitro*.

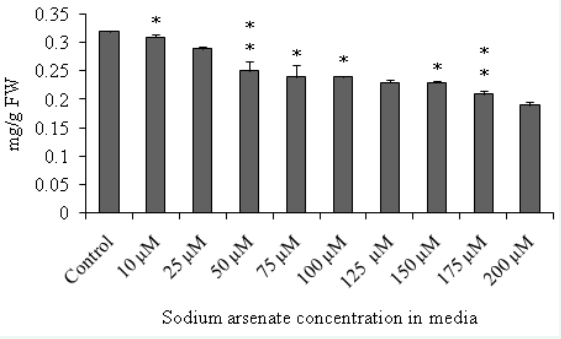

Figure 2 Histogram of amount of carotenoids in *M. charantia* grown in different sodium arsenate concentration in media *in vitro*.

found in control plant, i,e. 0.32 mg/g FW. Srivastava et al. [35], reported a similar decline of chl a and chl b content in *Hydrilla verticillata* at higher doses of arsenic treatment. High arsenic concentrations in soils and water are regularly connected with negative effects in the physiological state of plants [36]. Thus photosynthetic pigments content acts as potential indicators of arsenic toxicity.

The decrease in chlorophyll [22] and carotenoid contents appears to be one of the first clear biomarkers of arsenic toxicity. Decrease in total chlorophyll content is one of the parameter used as a bioindicator of oxidative stress caused by heavy metals [17]. It was found that arsenic induced oxidative stress in *M. charantia* and changes in chlorophyll content signifies the result. Decrease of growth in *Momordica* may be related to the decrease in the photosynthetic pigments and consequently in the photosynthetic process [19]. The significant decrease of pigment contents in arsenic-treated plants is a sign of absence of adaptive adjustments of pigment synthesis to high arsenic levels.

DPPH is a free radical compound and has been widely used to test the free radical-scavenging ability in plant system [27]. Highest (37.08% ± 1.8%) of DPPH radical scavenging activity was found in the sample which was exposed to 100µM sodium arsenate (Figure 3). DPPH radical scavenging activity was lower in the control (shoot 23.38% ±1.9% and root 23.29 ± 1.56%) as it contained lower amounts of polyphenols. To mitigate the harmful effects of free radicals, plant cells have developed antioxidant defense mechanism which is composed of enzymatic antioxidants and nonenzymatic antioxidants (eg. carotenoids) and phenolic compounds (eg. flavonoids) that act as the scavengers of free radicals [20,29].

In the present study, As treatment caused significant alterations in total antioxidant, reflected in free radical-scavenging machinery of **M. charantia** seedlings. Arsenic-induced generation of free radicals can cause cell damage through activation of oxidative sensitive signaling pathways and antioxidant systems which protect the plants against reactive oxygen species. Tiwari et al. [38], found arsenic induced antioxidant response in Pteris *vittata* and *Vetiveria zizanioides*.

Total polyphenol content was determined by Folin-Ciocalteu's phenol reagent method using gallic acid standard curve (Figure 4a). Gallic acid being the most important polyphenol in natural product was used to determine the polyphenol content in experiments. Poonam et al. [25], found increase in various phenols like catechin, chlorogenic acid, caffeic acid, coumaric acid, rutin and quercetin in *Brassica juncea* under 0.5 mM Cu stress. Hosseinzadeh et al. [11], found changes in galic acid, catechin, epi-catechin, chlorogenic acid, cumaric acid, fluoridizin and quersetin in effect of surfactants on extraction of apple extracts.

The amount of polyphenol increased up to the concentration of 100 µM sodium arsenate in media (Figure 4b). Highest polyphenol content was found in100 µM sodium arsenate treated sample and the value was 0.335 ± 0.01 g GAE Kg^{-1} FW in shoot and 0.29 ± 0.015 g GAE Kg^{-1} FW in root. In this study, it was observed that polyphenol content during the initial phase of arsenic stress was less but as the dose of arsenic increased, polyphenol content of *Momordica* was found to be enhanced.

However when we introduced more than 100 µM sodium arsenate in the media, polyphenol content of *M. charantia* was decreased. This result indicates the positive role of polyphenols in free radical scavenging activity. We observed that there was greater accumulation of polyphenols under stress in *Momordica*. An increase of phenolics correlated to the increase in activity of enzymes involved in phenolic compounds metabolism was reported, suggesting de novo synthesis of phenolics under

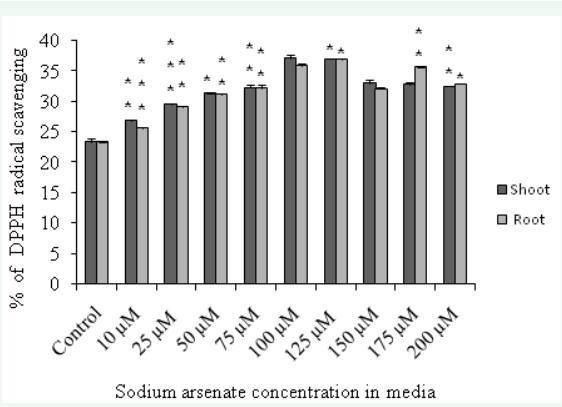

Figure 3 Histogram of Free radical scavenging assay of *M. charantia* grown in different concentration of sodium arsenate in media *in vitro*.

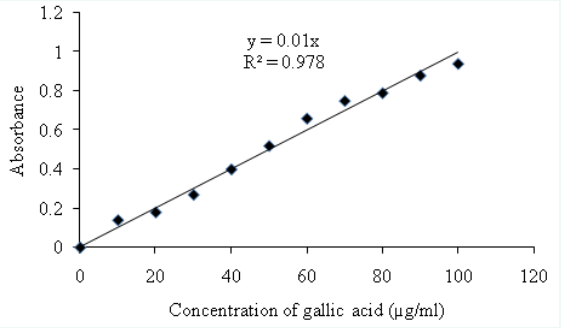

Figure 4a Standard curve of Gallic acid for the determination of total polyphenolic content.

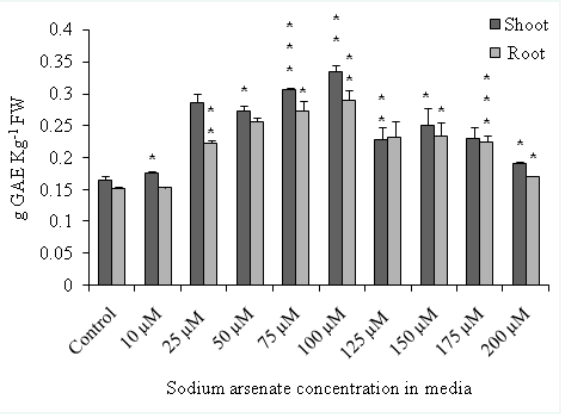

Figure 4b Histogram of total polyphenol assay of *M. charantia* grown in different concentration of sodium arsenate *in vitro*.

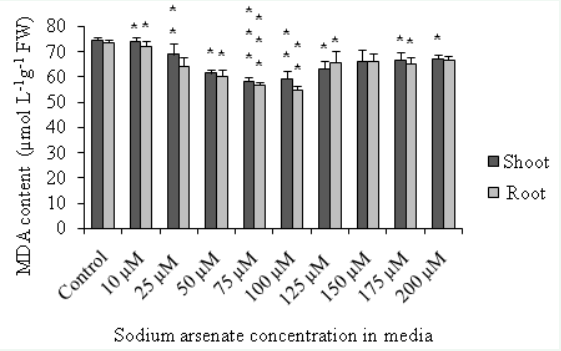

Figure 5 Histogram of lipid peroxidation activity of *M. charantia* grown in different concentration of sodium arsenate *in vitro*.

heavy metal stress [14]. This result showed that the enhanced accumulation of polyphenol with the arsenic stress. Total polyphenol content suddenly decreased at higher arsenic stress, which may be due to tissue damage of *Momordica*.

In growing seedlings of *Momordica* in arsenic added media, the level of lipid peroxidation was measured in terms of MDA content [9]. MDA contents in both the roots and shoots are presented in (Figure 5). Under As treatments initially decreased level of lipid peroxides was observed in roots and shoots as compared to control-grown seedlings. In this experiment arsenic exposure significantly changes the MDA content compared with control. De Oliveira et al. [5], also observed same trend in P. vittata under As stress. Lipid peroxidation was found to decline with moderate arsenic stress. The high antioxidant activity and low lipid peroxidation in the moderate arsenic stress can be correlated with the high phenolic and flavonoid content.

They may have contributed to the inhibition of lipid peroxidation and thereby employed antioxidant capacity. Lipid peroxidation has been thought to be a toxicological phenomenon that can lead to various physiological consequences. MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in the free radicals may causes overproduction of it. Thus MDA level is commonly known as marker of oxidative stress [7]. To cope with the arsenic-induced oxidative stress in plant system, antioxidant defense system plays a vital role [30].

Metals can induce lipid peroxidation and disrupt the membrane lipid bilayer arrangement that may inactivate membrane-bound receptors and enzymes and increase tissue permeability. The phenolic compounds in plants are found to be effective antioxidants due to their redox properties. They act as reducing agents i.e., free radical terminators, hydrogen donors, singlet oxygen quenchers and metal chelators. Increased lipid peroxidation in response to arsenic toxicity demonstrates increased generation of ROS in respect of oxidative stress. This is in accordance with previous reports that arsenic caused severe lipid peroxidation in bean [31,36] and Pteris sp. [32, 34].

The total antioxidant capacity of Momordica roots and shoots significantly increased up to the concentration of 75µM of As exposed to media (Figure 6b), indicating the activation of antioxidant mechanisms in plant sample. At concentrations higher than 75 µM, however, concentration of As is likely to become toxic, affecting the antioxidant capacity and consequently the uptake of nutrients. It was observed that in control plant antioxidant activity was quite high. The increased antioxidant activity could be related to the enhanced production of polyphenols. Ascorbic acid standard curve was prepared (Figure 6a).

Flavonoid is also considered as polyphenolic compound having antioxidant activity. Flavonoid aggregation in arsenic induced *Momordica* seedlings followed a similar trend to that of total polyphenols content. Flavonoids are organic molecule that

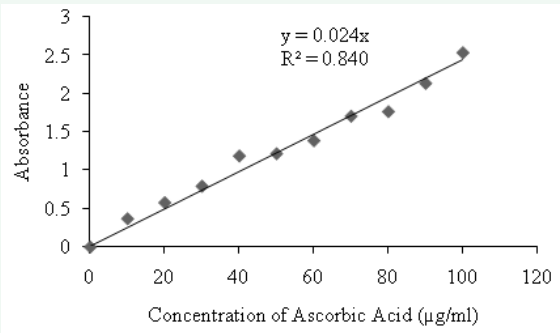

Figure 6a Standard curve of ascorbic acid (µg/ml) for the determination of total antioxidant activity.

has been shown to have a protective role against several stresses [13], both by themselves and in conjugation with peroxidases [21]. Flavanols have protein-binding capacity thus they can interact with membrane phospholipids through hydrogen bonding to the polar head groups of phospholipids [39]. As a consequence, these compounds can be accumulated at the membranes' surface, both outside and inside the cells.

Flavonoid content also increased with dose of arsenic exposure in culture media (Figure 7b) but after 100 µM arsenic stress the amount of flavonoid dropped (0.706 ± 0.036 g RE kg^{-1} FW in shoot and 0.695 ± 0.025 g RE kg^{-1} FW in root portion). During initial stages of stress, the amount of flavonoid increased. Flavonoid aggregation in plants followed a similar trend to that of polyphenols. Rut in standard curve was prepared (Figure 7a). Flavonoid is also considered as polyphenolic compound having antioxidant activity. Flavonoid aggregation in arsenic induced *Momordica* seedlings followed a similar trend to that of total polyphenols content. Flavonoids are organic molecule that has been shown to have a protective role against several stresses [13], both by themselves and in conjugation with peroxidases [21]. An increase in radical scavenging was observed with subsequent arsenic stress indicates the positive role of polyphenols in free radical scavenging. Yesil-Celiktas et al. [40], showed that radical scavenging activity increases with the increase in polyphenol content. Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind with metals. Polyphenols are used for possessing antioxidant properties in plants. Plant flavonoids also play a role as antioxidant. High polyphenol and flavonoid content might have played an important role as antioxidant in this experimental system. Plants possess an antioxidant defensive machinery to protect against stress damage. Plants have antioxidant enzymes to scavenge the toxic effects metal stress. The phytotoxicity of arsenic, including a major damage in the membrane caused by metalloid like arsenic, can also be the cause of this decrease in nutrients uptake [24]. We observed that there was greater accumulation of polyphenols in the stress condition in *Momordica*. During stress, polyphenols might be involved in prevention of oxidative damage in plant and therefore could be an essential index for the adaptive mechanism in adverse circumstances. An increase of phenolics correlated to the increase in activity of enzymes involved in phenolic compounds metabolism was reported, suggesting de novo synthesis of phenolics under heavy metal stress [14]. Total polyphenol content suddenly decreased at higher arsenic stress, which may be due to tissue damage of *Momordica*.

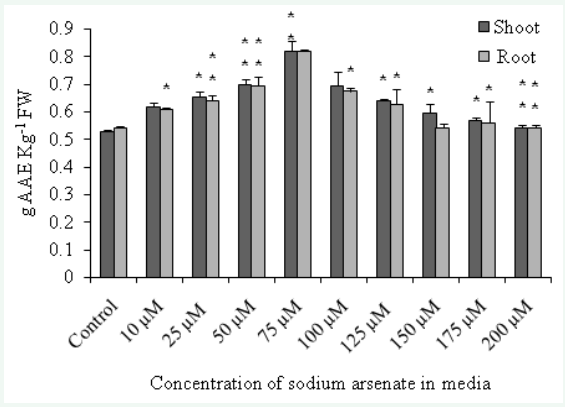

Figure 6b Histogram of total antioxidant activity of *M. charantia* grown in different concentration of sodium arsenate *in vitro*.

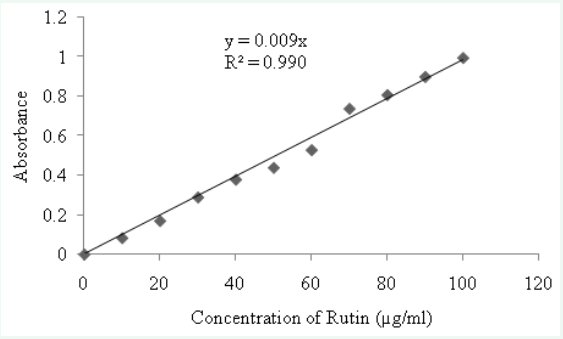

Figure 7a Standard curve of rutin (µg/ml) for the determination of total flavonoid content.

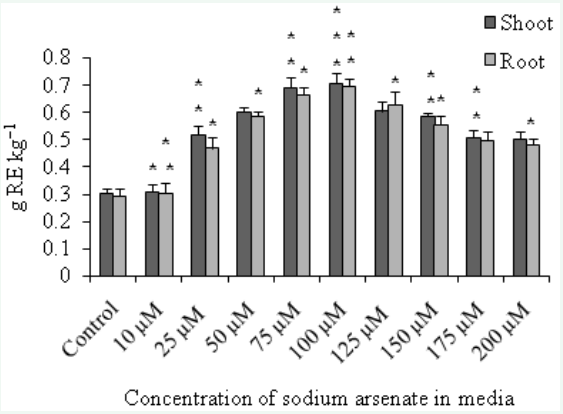

Figure 7b Histogram of total flavonoid content of *M. charantia* grown in different concentration of sodium arsenate *in vitro*.

CONCLUSION

The present investigation demonstrated that under arsenic stress *M. charantia* underwent different biochemical changes to cope up with the environment. On the basis of the results following conclusion can be drown: 1) the treatment of arsenic in *Momordica in vitro* had a negative effect on pigment (chlorophyll and carotenoids) content 2) Metal induced changes in biochemical characteristics of *Momordica* such as free radical scavenging, lipid peroxidation, antioxidant activity, flavonoid content etc. The i*n vitro* grown seeds procured from arsenic free zone but grown in presence of sodium arsenate had a tremendous negative impact on *M. charantia*.

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