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AMELIORATIVE POTENTIAL OF *GLYCOSMIS PENTAPHYLLA* (RETZ.) DC IN INDUCED ARSENICOSIS IN RATS

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ABSTRACT

Arsenic poisoning is one of the major causes of chronic human illness and mortality in Bengal Delta plain with limited treatment options.The present study was conducted to evaluate the ameliorating effect of *Glycosmis pentaphylla* (Rutaceae) leaf extract against sodium arsenite ($NaAsO_2$) induced toxicity. Forty eight adult albino rats were divided into four groups viz.,G_0,G_1,G_2 and G_3.Sodium arsenite was administered @ 4mg/kg daily in drinking water in groups G_1, G_2 and G_3 for 90 days; the control group(G_0) received water for 120 days.The G_1 group of animals received water, instead of arsenic during 91-120 days , while rats of group G_2 and G_3 were orally treated with plant leaf extract at 320 mg/kg ($^1/_{10}^{th}$ LD_{50}) and 160 mg/kg ($^1/_{20}^{th}$ LD_{50}) respectively daily during the period. Analysis of arsenic concentrations in tissue samples,hair and faeces showed that treatment with *G. pentaphylla* leaf extract significantly ($p<0.05$) reduced arsenic accumulation in tissues, hair and in faeces. A significantly ($p<0.05$) higher organo-arsenic fraction and lower arsenite and arsenate fraction was also observed in *G. pentaphylla* leaf extract treated group(G_2 &G*3*). The study showed that oral treatment with *G.pentaphylla* leaf extract could ameliorate induced arsenicosis.

KEY WORDS: **Arsenic,speciation, rats, *Glycosmis pentaphylla*, amelioration.**

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INTRODUCTION

Arsenic is one of the toxic metalloids abundantly found in soil and water, and is a potential carcinogen[1]. Humans are exposed to arsenic predominantly through contaminated drinking water and foods; inhalation and skin absorption may be minor entry routes[2]. According to the WHO intake of drinking water containing arsenic more than 10µg/l is harmful to the human .Chronic arsenic exposure in human leads to skin diseases including hyper- pigmentation hyperkeratosis leading to cancers of skin and cardiovascular ,respiratory, central nervous system, gastrointestinal and reproductive complications increasing morbidity and mortality.[3,4] Arsenic is known to be a potent sulfhydryl- reactive chemical capable of binding and cross linking cellular proteins,[5] there by altering multiple cellular pathways including expression of growth factors, suppression of cell cycle cheek point proteins, promotion of apoptosis, inhibition of DNA repair ,decreasing immunocompetence and increasing oxidative stress. Various medicinal plants with high antioxidant properties have generated increased interest for their therapeutic potential in reducing free radical –induced tissue injury.[6,7] Many plant products exert their protective effects against oxidative –stress-mediated diseases by scavenging free radicals. Generally, arsenic undergoes methylation to mono - methylarsonic acid and dimethylarsenic acids which are potent inhibitors of GSH reductase and causes hepatotoxicity in humans and animals.[8]Chronic arsenic exposure in animals can also produce liver endotheliial cell damage, which subsequently damages parenchymal cells. [9] Numerous plant products contain antioxidants, vitamins, flavonoids and polyphenolic compounds that have been demonstrated as scavengers of free radicals and inhibitors of lipid per oxidation.[10] Medicinal plants having patent anti-oxidant property can help to reduce oxidative stress and hepatotoxicity caused by metals Gora I[11]. *Glycosmis pentaphylla* commonly known as toothbrush plant or orange berry, is used in fever, liver disorders, cough and jaundice as a tonic and appetizer to women after delivery.[12,13] Chitra et al [14] reported that carbazole is one of the active constituent of leaf extract of *G. pentaphylla*. But the effects of *Glycosmis pentaphylla* against heavy metal toxicity in general and arsenic toxicity particular is scarcely available in literature..Therefore the present work was undertaken to examine beneficial effects of *G.pentaphylla* in arsenicosis .

MATERIALS AND METHODS

1. Experimental animals

Forty eight adult albino rats of either sexes or having 150-200 gm body weight were procured from registered animal breeder. They were housed in polypropylene cages and were acclimatized in experimental animal room for seven days before the experiment. The animals were fed standard pellet feed and provided drinking water *ad libitum*. The Institutional Animal Ethics Committee approved the study protocol vide no.EC/235/2013/CPCSEA.

2. Preparation of G. pentaphylla extract

The plant under study was authentically identified by Dr. Subir Bandyopadhyay, Botanist of BSI(Botanical Survey of India, Howrah, Kolkata) and the voucher no. of *G.pentaphylla* was WBUAFS/KOL/1 and the voucher specimen was kept at the Dept. of Pharmacology and Toxicology, West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal, India.Fresh plant leaves of *G. pentaphylla* were collected from local areas,leaves were washed with water, cut into pieces and dried in shade for 7 days. The dried leaves were pulverized into a coarse powder in a grinding machine, and methanol extract was prepared in soxhlet apparatus using methanol (99%). The condensed solution was kept at room temperature for 2 to 3 days, dissolved in millipore grade water and analysed for carbazole, one of the active principles of the plant , before administration to rats.

3. Analysis of carbazole in G. pentaphylla

A stock solution of 100 ppm of carbazole (purity 98.7%) procured from Sigma Aldrich,was prepared in methanol as standard. Agilent Technologies 1200 Series , coupled with PDA detector was used for carbazole estimation. The I- acetonitrile : ammonium acetate 1mmol (60:40); flow rate - 1ml/min., column - reversed phase C_{18} [5µ Zorbax-SB C_{18}, USA; 250x46mm(RP)], detection wave length - 232nm. The retention time of carbazole was 8.1(Fig.1) and limit of detection was 0.2ppm. The retention time of the external standard and the data was recorded in Chemstation version. To 100gm of leaf powder of *G. pentaphylla* , 400 ml of methanol (99%) was added in soxhlet apparatus and heated for 18 hrs. A semisolid solution was prepared and reduced the volume by using rotary vacuum evaporator at 40◦c and the residue was measured. The obtained 1.0gm of leaf extract was dissolved in 50 ml of HPLC grades methanol, the mixture is taken in a syringe filter(.2 micron filter paper) and filtered. A 20µl of mixture was an injected in HPLC instrument. It was found that 0.17 gm of carbazole is present in the 100gm leaf extract of *G.pentaphylla.*[15]

4. Determination of LD_{50} of GP leave extract

LD_{50} of *G.pentaphylla* leaf extract in rats was determined following the method described by Ghosh [15] which was found to be 3200mg/kg.

5. Experimental Design

Forty eight animals were randomly divided into four groups viz., G_0,G_1, G_2,and G_3 having twelve rats in each . Rats in group G_0 were offered feed and water *ad libitum*.On the basis of the mean total body weight of the rats, animals in groups G_1, G_2 and G_3 were treated with sodium arsenite at 4mg/kg[5] daily in drinking water for 90 days and methanolic extract of *G. pentaphylla,*dissolved in distilled water, was administered at 320 and 160mg/kg b.w (1/10th and 1/20 of LD_{50}).(equivalent to 0.544 and 0.272 mg Carbazole per kg.) to animals of groups G_2 and G_3 respectively from 91 to 120 days. G_1 group of animals were not treated with the plant extract and was considered as experimental control.

Tissue samples, hair and faeces were collected on day 0,90 and 120 after sacrificing four animals in each group.

6.Estimation of total arsenic

Sample preparation for estimation of arsenic

Faeces

Faeces samples of rats were collected from cages where the animals were maintained .Total amount of faeces excreted were also recorded on respective days. To 2 gm of faeces, 15 ml of methanol and water mixture (1:1) was added and homogenized for 5 min in Remi homogenizer. Homogenized faeces were kept in a100 ml conical flask.

Hair

Similarly hairs (1gm) was collected in polythin bag on the same days as above,cut into pieces and homogenized in 15 ml of methanol-water mixture(1:1).

Tissue

The rats were sacrificed on stipulated days maintaining standard protocol. Two grams of each liver, lung, kidney, spleen, heart, muscles and intestine were collected and processed as above for estimation of total arsenic. Total arsenic contents in lung, heart, intestine, kidney,muscle, liver,spleen,hair and faeces were estimated as per standard method Datta *et al*, [16] using atomic absorption spectrometer (Varian AA240, model VGA77) equipped with vapor generation accessories. Reducing agent (aqueous solution of 0.6% sodium borohydride in 0.5% sodium hydroxide) and acid (40% hydrochloric acid) were prepared before use. Estimation conditions followed were:arsenic hollow cathode lamp, wavelength 193.7nm; slit width- 0.5nm, lamp current- 10.0 mA, vapor type- air/acetylene, air flow- 10.00 l/min, inert gas for hydride generation-argon[16].

7. Arsenic speciation study

Speciation of arsenic in faeces, liver and hair was done accordingly the method of Datta *et al* [16]. Briefly, to every 2 gm of faeces or liver and 1 gm of hair samples, 15 ml of methanol - water mixture (1:1) was added and homogenized for 5 min. Homogenized samples were transferred to an ultra sound bath at 60° C for 3 hr for extraction of inorganic arsenic, final volume was made up to 50 ml by Millipore water and then filtered .Care was taken to ensure that the pH of the resulting solution remains between 4 and 9. A 20 ml syringe was filled with the above solution connected to an arsenic speciation cartridge (Metal Soft Centre, High land, Park, NJ) and by pushing the plunger the solution of the syringe was emptied into glass graduated tube. The first 5ml of the solution coming out from the syringe was discarded. The solution which was filled in the syringe represent total inorganic arsenic fraction (arsenite + arsenate); the solution coming out from syringe represent organo arsenic fraction, which was analysed by hydride generation method.

The organic and inorganic arsenic fractions in different samples were calculated as follows:

Total arsenic analysis by wet ashing acid digestion procedure: A

Total inorganic arsenic analysis: B

Total arsenite analysis: C

Arsenate fraction: B - C

Organic arsenic fraction: A – B

Percentage of arsenite: $\frac{C}{A} \times 100$

Percentage of arsenate: $\frac{B - C}{A} \times 100$

Percentage of organoarsenic: $\frac{A - B}{A} \times 100$

Statistical analysis

The total arsenic contents and various fractions in different experimental groups were analysed statistically (F test) by using univariant General Linear Model with two way Anova in SPSS10 software.

RESULTS AND DISCUSSION

The carbazole content in *G.pentaphylla* leaf extract was estimated at 8.1 min(Fig.1) The leaf extract of *G.pentaphylla* (320 and 160 mg/kg) significantly reduced the total arsenic content in different tissues (Fig.2), hair (Fig.3) and faeces (Fig.4) on the day 120. Also G.pentaphylla leaf extract increased (320 and 160 mg/kg) the organo-arsenic fraction and decreased the arsenate and arsenite fraction (Table1,2,3)in faeces, liver and hair on 120th day.

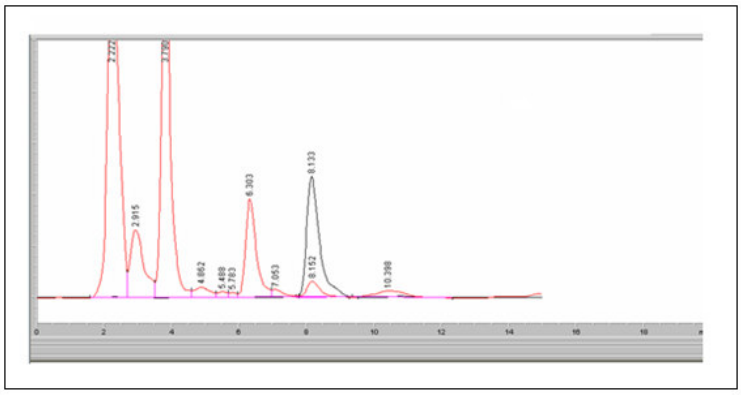

Figure 1

Chromatogram of carbazole in standard and in plant leaf extract

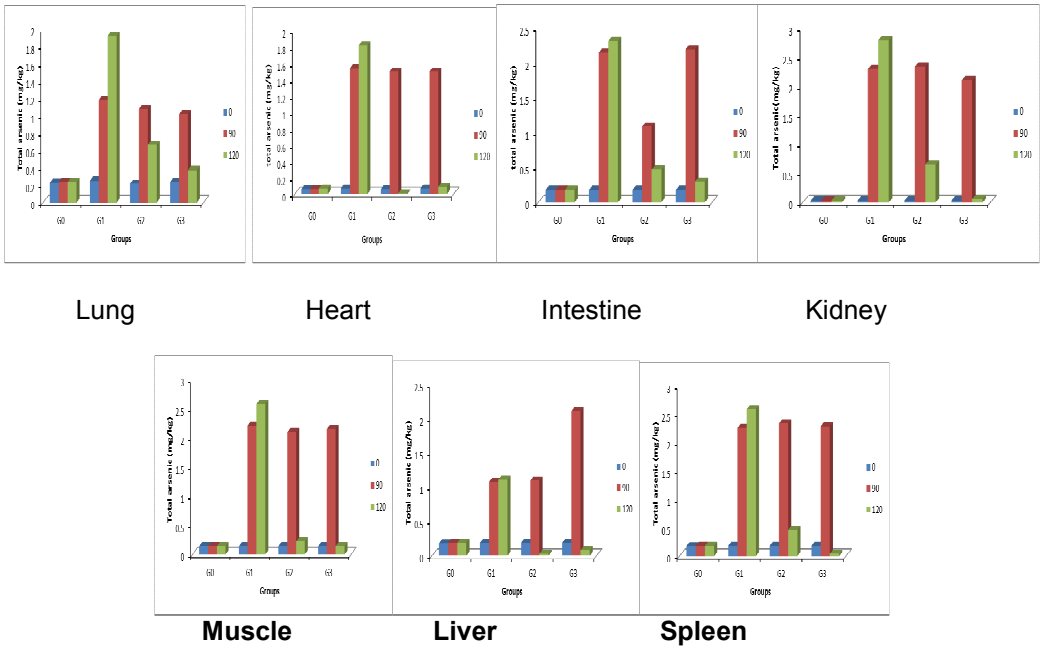

Figure 2
Concentration of total arsenic (mg/kg) in different tissues of rat of different groups and days (n=4)

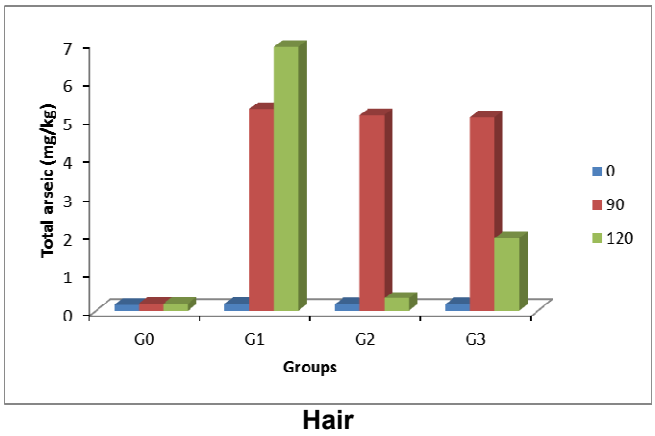

Figure 3
Concentration of total arsenic (mg/kg) in hair of rat of different groups and days (n=4)

Each group (12 animals) was again subdivided into 3, each containing 4 animals for 0, 90 and 120 days respectively.

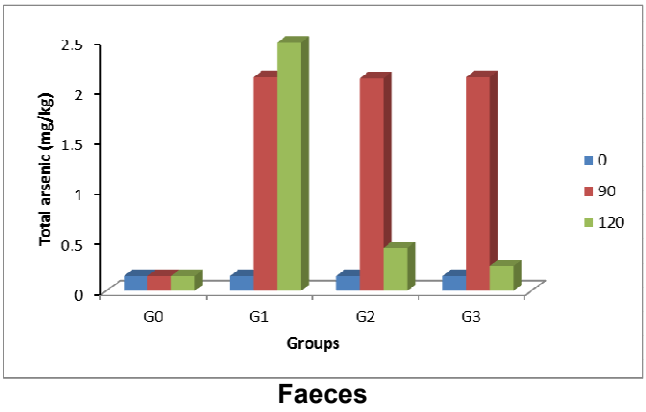

Figure 4
Concentration of total arsenic (mg/kg) in faeces of rat of different groups and days (n=4)

Table 1

Speciation of arsenic(%) in faeces after daily single oral administration of sodium arsenite @ 4mg/kg for consecutive 90 days and ameliorative effect by G.pentaphylla leaf extract after 90 days onwards. (Mean±SE, n=4)

Groups	Arsenite (As^{+3})(%)			Arsenate (As^{+5})(%)			Organo arsenic(%)		
	0	90	120	0	90	120	0	90	120
G0	26.18 ± 0.59^{y}	28.25 ± 0.61^{xyb}	29.66 ± 0.74^{xb}	9.64 ± 01.86^{xa}	10.44 ± 0.13^{xb}	11.0 ± 0.09^{xb}	64.18 ± 0.40^{x}	61.31 ± 0.74^{ya}	59.34 ± 0.70^{ya}
G1	29.82 ± 1.47^{y}	33.88 ± 0.85^{yab}	47.59 ± 1.16^{xa}	9.69 ± 0.12^{y}	29.81 ± 4.93^{xa}	38.18 ± 1.86^{xa}	60.49 ± 1.58^{x}	36.97 ± 5.71^{yb}	14.23 ± 2.49^{zb}
G2	27.04 ± 2.40^{y}	36.17 ± 1.54^{xa}	31.14 ± 1.32^{xyb}	9.32 ± 0.29^{y}	28.62 ± 3.85^{xa}	15.91 ± 1.78^{yb}	63.64 ± 2.38^{x}	35.22 ± 4.67^{yb}	52.96 ± 2.58^{xa}
G3	26.83 ± 0.88^{z}	38.55 ± 1.21^{xa}	32.83 ± 1.37^{xb}	8.94 ± 0.37^{z}	26.12 ± 1.70^{xa}	16.43 ± 1.53^{yb}	64.23 ± 0.61^{x}	35.33 ± 2.91^{zb}	52.25 ± 1.70^{ya}

***Values are mean with SE**

Rows bearing different superscript (x – z) differ significantly (p<0.05).

Columns bearing different superscript (a – d) differ significantly (p<0.05).

Table 2

Speciation of arsenic(%) in liver after daily single oral administration of sodium arsenite @ 4mg/kg for consecutive 90 days and ameliorative effect by G.pentaphylla leaf extract after 90 days on wards (Mean±SE, n=4)

Groups	Arsenite (As^{+3})(%)			Arsenate (As^{+5})(%)			Organo arsenic(%)		
	0	90	120	0	90	120	0	90	120
G0	21.0 ± 0.58^{z}	23.97 ± 0.85^{yc}	26.50 ± 0.35^{xb}	2.20 ± 0.15^{y}	2.33 ± 0.09^{yb}	2.80 ± 0.09^{xc}	76.80 ± 0.72^{x}	73.74 ± 0.86^{ya}	70.70 ± 0.42^{zb}
G1	21.10 ± 0.55^{y}	45.33 ± 1.76^{xab}	52.30 ± 1.96^{xa}	3.23 ± 0.15^{z}	10.25 ± 0.38^{ya}	12.08 ± 0.31^{xa}	76.00 ± 0.76^{x}	44.42 ± 2.13^{yb}	35.63 ± 2.24^{zb}
G2	20.18 ± 0.55^{z}	43.40 ± 1.29^{xb}	29.10 ± 1.02^{yb}	2.83 ± 0.32^{y}	11.40 ± 0.62^{xa}	4.40 ± 0.29^{yb}	76.98 ± 0.69^{x}	46.84 ± 3.43^{zb}	66.50 ± 1.07^{ya}
G3	20.72 ± 0.23^{z}	45.40 ± 0.59^{xab}	30.59 ± 1.57^{yb}	2.05 ± 0.10^{z}	10.60 ± 0.43^{xa}	5.10 ± 0.13^{yb}	77.98 ± 0.69^{x}	44.00 ± 0.58^{zb}	64.83 ± 1.94^{ya}

Rows bearing different superscript (x – z) differ significantly (p<0.05).

Columns bearing different superscript (a – d) differ significantly (p<0.05).

Table 3

Speciation of arsenic(%) in hair after daily single oral administration of sodium arsenite @ 4mg/kg for consecutive 90 days and ameliorative effect by *G.pentaphylla* leaf extract after 90 days onwards (Mean±SE, n=4)

Groups	Arsenite (As^{+3})(%)			Arsenate (As^{+5})(%)			Organo arsenic(%)		
	0	90	120	0	90	120	0	90	120
G0	25.85 ± 1.16^{y}	28.20 ± 0.55^{yb}	32.79 ± 1.04^{xb}	8.46 ± 0.27^{y}	9.57 ± 0.23^{yb}	11.98 ± 0.48^{xb}	65.59 ± 1.43^{x}	62.23 ± 0.78^{xa}	55.29 ± 1.35^{yb}
G1	27.23 ± 0.58^{y}	37.62 ± 0.88^{xa}	42.30 ± 1.82^{xa}	8.73 ± 0.27^{y}	15.80 ± 2.53^{yab}	25.89 ± 2.04^{xa}	64.03 ± 0.31^{x}	46.58 ± 3.40^{yb}	31.82 ± 3.75^{zb}
G2	25.00 ± 1.61^{z}	35.83 ± 0.91^{xa}	30.83 ± 1.32^{yb}	8.78 ± 0.49^{y}	16.58 ± 1.65^{xab}	10.61 ± 0.68^{yb}	66.22 ± 1.34^{x}	47.58 ± 2.19^{yb}	58.56 ± 1.95^{xa}
G3	24.45 ± 1.34^{y}	34.53 ± 1.90^{xa}	31.86 ± 1.44^{xb}	9.17 ± 0.38^{y}	17.61 ± 1.78^{xab}	13.02 ± 1.72^{xyb}	66.98 ± 1.51^{x}	47.85 ± 3.54^{yb}	55.12 ± 1.62^{ya}

Rows bearing different superscript (x – z) differ significantly (p<0.05).

Columns bearing different superscript (a – d) differ significantly (p<0.05).

The study detected presence of traces of arsenic in control (G_0) group as well as on beginning of the experimentation, possible from arsenic contamination in feed items which is common in Bengal delta[17].The total arsenic contents in vital organs, hair and faeces increased significantly ($p<0.05$)in G_1,G_2 and G_3 groups of animals on 90th day, compared to "0 day" of respective groups and control (G_0) animals as a result of daily arsenic administration and its accumulation in different tissues. As expected, the level of arsenic was not reduced in G_1 group of animals (not receiving the plant extract) on 120 day,rather increased progressively. Arsenic content in the internal organ were significantly higher in all arsenicosis rats. Hair is

one of the major routes of elimination of arsenic, besides lungs, kidneys and faeces. Bertolero *et al*[18] reported that in most animal species exposure to either arsenite or arsenate leads to an initial accumulation in the liver, kidneys,and lungs which was similar to the present findings. We estimated slightly higher load of arsenic in spleen, intestine and kidney, compared to that in lung, and highest accumulation in hair. The total arsenic content in vital organs, hair and faeces of G_2 and G_3 groups of animals decreased signinificantly on 120^{th} day compared to that on 90^{th} day following treatment with extract of *G.pentaphylla* at both the dose levels suggesting an ameliorating effect of the plant extract against arsenicosis. The animals were fed with arsenite, however, speciation study recovered arsenic as arsenite, arsenate and organic-arsenic complexes in faeces, liver and hair indicating *in vivo* transformation of the metalloid to other forms. From the results , it is apparent that about 26-30% and 35-36% of administered arsenite is transformed and excreted as arsenate and organic-arsenic complex respectively after 3 months of administration; the proportion of arsenate was lower and organic-arsenic fraction was higher in liver and in hair, compared to that in faeces . Treatment of G_2 and G_3 groups of animals with *G.pentaphylla* extract enhanced organic-arsenic transformation up to the level of 53% in faeces, 65-66% in liver and 55-59% in hair at the expense of most toxic inorganic forms, particularly the arsenite. Compared to arsenite, organic arsenic complexes are less toxic[19].Arsenic undergoes methylation into monomethyl arsenic acid (MMA^{III}) or even tri methyl arsenic acid in liver faciliating its excretion. In the present study, it was not possible to separate to all organo-arsenic species, hence we estimated the total organo-arsenic forms. *G. pentaphylla* contained active principles like Glycolone[20]. The component having three methyl groups in its structure is likely to donate methyl groups to inorganic arsenic through methylation resulting in higher transformation of arsenite to organo-arsenic forms.[21,22] Thus the results showed that treatment of arsenicosis animals with *G.pentaphylla* at both the dose levels caused a marked decline in total arsenic accumulation , combined with arsenic transformation in favour of less toxic and excretable forms, which might greatly favour the arsenicosis animals.

CONCLUSION

The results of the present study suggest that, treatment with leaf extract of *G. pentaphylla* could ameliorate induced arsenicosis in rats at both the doses of 320 and 160 mg/kg. As both the doses are equally effective to reduce arsenic burden in the body , the lower dose of 160 mg/kg daily orally in rats ,may be suggested..

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