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Hepatoprotective Efficacy of Cadamba Fruit Extract against Arsenic Trioxide Toxicity

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Abstract – Objective: ***To study the medicinal utility of cadamba fruit, in reducing the toxic effects of arsenic on the liver in Swiss albino mice and the importance of cadamba fruit for the people as a tonic in reducing the effect of arsenic by giving simultaneously both cadamba ,arsenic to the albino mice.***

Method: Oxidative stress was induced by oral administration 4 mg/kg b. wt of arsenic trioxide (As2O3,) for 45 days in experimental rats. The level of liver arsenic concentration, lipid peroxidation, reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione-S-transferase (GST), and glutathione peroxidase (GPx) were determined in adult male Wistar rats. Hepatotoxicity was assessed by quantifying the aspa alanine transaminase (ALT) and alkaline phophatase (ALP). Hepatoprotective efficacy of cadamba fruit extract (100 mg/kg b.wt) was evaluated by combination treatment with As2O3.

Results: As2O3 administration leads to the generation of reactive oxygen species (ROS), arsenic accumulation, serum marker enzymes release and decrease in antioxidant enzymes in liver. Retention of arsenic in liver caused increased level of lipid per oxidation with a concomitant decline in the glutathione dependant antioxidant enzymes and antiperoxidative enzymes. Cadamba fruit extract treatment protected the liver from arsenic induced deterioration of antioxidant levels as well as oxidative stress. And also a significant decrease in hepatic arsenic accumulation and serum marker enzymes was observed. Histopathological examination revealed a curative improvement in liver tissue.

Conclusions: These findings lead to the conclusion that cadamba fruit extract may have the potential to protect the liver from arsenic induced toxic effects.

Keywords: Hepatoprotective, arsenic trioxide, cadamba fruit extract, oxidative stress, arsenic accumulation.

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INTRODUCTION

Arsenic is one of the most important global environmental toxicants and its exposure in humans comes mainly from consumption of drinking water contaminated with inorganic arsenic . In clinical trials it is considered as a first choice cancer chemotherapeutic against certain leukemia and has potential against a variety of other cancers, including solid tumors . Specifically, arsenic trioxide (As_2O_3,) is used in the treatment of acute promyelocyticleukemia and it greatly improves the clinical outcome even in refractory or multiple relapsed cases. But, toxic side effects of arsenicals are often a major concern; including the potential for fatal hepatotoxicity .The liver is a major target organ for both arsenic metabolism and toxicity. Arsenic induced hepatic injury is known to be exerted through excess production of reactive oxygen species, namely superoxide (O_2), hydroxyl OH), and peroxy (ROO) radicals and hydrogen peroxide (H_2O_2) . The harmful expressions of arsenic are primarily due to an imbalance between pro-oxidant and antioxidant homeostasis in physiological system and also due to its fascination to bind sulfhydryl groups of proteins and thiols of glutathione (GSH). Thus, an agent able to reduce the toxic potential of arsenic in liver cells would clearly to be useful ccompounds for arsenical chemotherapy.

Neolamarckiacadamba primarily consist of indole alkaloids, terpenoids, sapogenins, saponins,terpenes, steroids, fats and reducing sugars. The bark also consists of tannins and an astringent principle; which is due to the presence of an acid similar to cincho-tannic acid. A new pentacyclictriterpenic acid isolated from the stem bark Neolamarckiacadambanamedcadambagenic

acid (18α-olean-12ene-3β-hydroxy 27, 28-dioic acid) (Fig. 1), along with this acid quinovic acid (Fig. 2) and β- sitosterol (Fig. 3) have also been isolated

The isolation and structure of 3β-dihydrocadambine and 3β-isodihydrocadambine (Fig. 8) alkaloids reported from the leaves of Neolamarckiacadamba with molecular formula ($C_{37}H_{44}N_{15}O_2$). A new saponin named saponin B ($C_{48}H_{76}O_{17}$) reported from Neolamarckiacadamba (Miq.). Neolamarckiacadamba also contain an acid called chlorogenic acid (CGA) (Fig. 9) . Recently some worker isolated two novel triterpenoidsaponins, phelasin A and phelasin B from the bark of Neolamarckiacadamba (Roxb.) Miq. Two novel monoterpenoidindole alkaloids, aminocadambine A ($C_{24}H_{27}N_30_5$) (fig. 10) and aminocadambine B $C_{25}H_{29}N_3O_5$(fig. 11) obtained from the leaves of Neolamarckiacadamba, previously named Anthocephaluschinensis whereas some worker biosynthetically synthesized glucosidicindole alkaloid cadambine from its biological precursor secologanin which is the main precursor of various indole alkaloids

Three monoterpenoidgluco-indole alkaloids, 3β-isodihydrocadambine, cadambine and 3α-dihydrocadambineisolated from Naucleacadamba (Roxb.). The flowers of Neolamarckiacadamba yield an essential oil and the main constituents of oils are linalool, geraniol, geranyl acetate, linalyl acetate, α-selinene, 2-nonanol, β-phellandrene, α-bergamottin, p-cymol, curcumene, terpinolene, camphene and myrcene. Two triterpenoid glycosides, glycosides A and B were isolated from the bark of Neolamarckiacadamba and defined as 3-o-(α-L-rhamnopyranosyl)-quinovic acid-28-o-(β-D-glucopyranosyl) ester and 3-o-(β-D- glucopyranosyl)-quinovic acid-28-o-(β-D- glucopyranosyl) ester respectively and eight different alkaloids also obtained from Neolamarckiacadamba named cadambine, CFJ 83, isomalindan, cadamine, 2 derivs. HFP34, GZM28, malindan, dihydrocadambine (Fig. 12), 2derivs. GPX71, GPX73, isomalindan, isodihydrocadambine, 2 derivs. GPX51, GPX53, malindan. The seeds of Anthocephalusindicus composed of water-soluble polysaccharides D-xylose, D-mannose and D-glucose in the molar ratio 1:3:5. Almost all parts of the plant Neolamarckiacadambais used in the treatment of various diseases. Decoction of leaves is used as gargle in aphthae or stomatitis and in the treatment of ulcers, wounds, and metorrhea.Bark of the plant is used in fever, inflammation, cough, vomiting, diarrhoea, diabetes, burning sensation, diuresis, wounds, ulcers and in the treatment of snake-bite.

Arsenic exposure leads to the incidence of hepatotoxicity as manifested by increase in the levelsof total bilirubin, alanine aminotransferase, aspartate.

Cadamba fruit extract is a naturally occurring antioxidant that plays an important role by inactivating harmful free radicals produced through normal cellular activity and from various stressors thus terminating lipid peroxidation and stabilizing the molecular composition of cellular membranes, preventing the harmful effects of reactive oxygen species (ROS). Therefore cadamba fruit extract is used to ameliorate the toxic effects of arsenic.

MATERIAL AND METHOD :

Preparation of aqueous extract of cadamba fruit:

In the present study,fresh fruit of cadamba were collected from plant of Neolamarckiacadamba from premises of Mahavir Cancer Institute $Research Centre, Patna, India. The identity of the medicinal plant was confirmed by Dr. S.K RAY . The collected fruit of cadamba were shade dried and were grinded to fine powder. The aqueous extract dose was calculated after LD50 estimation which was found to be 1500mg kg-1 body weight and the final dose was fixed to 100mg kg-1 body weight.

Animals: Thirty male swiss albino mice (28g to 32g) were obtained from animal house of Mahavir Cancer Institute & Research Centre, Patna, India . Food and water to mice were provided ad libitum (prepared mixed formulated feed by the laboratory itself). Animals were maintained in colony rooms with 12 hrs light/dark cycle at 22 ± 2^{o}C.

Experimental protocol

The mice were divided into four groups of six rats each, a normal control group, a cadamba fruit extract control which received 100 mg/kg b.wt of cadmba fruit extract, one As2O3 (4 mg/kg b.wt) administered group and a combination group treated with 4 mg/kg b.wt of As2O3 and 100 mg/kg b.wt of cadamba fruit extract. 0.2% DMSO solution was used as vehicle for cadamba fruit extract administration. Experimental groups received this via oral intubation daily for a period of 80 days. At the end of the experimental period animals were decapitated, blood was collected and centrifuged at 3 000 rpm for 20 minutes; the clear serum obtained was used for the determination of marker enzymes. Liver was removed immediately, washed in ice cold 0.15M NaCl and blotted on a filter paper. Then the tissue was weighed and homogenized by using Teflon glass homogenizer (1/10th weight/volume) in ice cold tris-HCl buffer (0.2M, pH 7.4). The homogenate wascentrifuged at 10 000g for 20 min at 4 C and the supernatant was used for the estimation of lipid peroxidation and various enzymatic and non enzymatic assays.

Estimation of total protein content

The total protein content was measured by the method of Lowry et al. (1951). The assay mixture contained 0.1 ml of liver homogenate, 0.9 ml of NaOH (0.1 N) and 5.0 ml of alkaline copper sulphate reagent. The reaction mixture was incubated for 15 min at room temperature and then 0.5 ml of FolinCiocalteau reagent (1 N) was added. The reaction mixture was further incubated for 30 min at room temperature. The absorbance was measured at 660 nm. Bovine serum albumin was used as standard.

Estimation of lipid peroxidation assay

Thiobarbituric acid reactive substance, the last product in lipid peroxidation pathways was estimated by the methods of Niehaus and Samuelsson (1968). 0.2 ml of liver homogenate was treated with 4 ml of TBA-TCA-HCl reagent (1:1:1ratio, 0.37% TBA, 0.25 N HCl and 15% TCA) and placed in boiling water bath for 15 min, cooled and centrifuged for 5 min at 5000 rpm. The absorbance of clear supernatant was measured against blank at 535 nm. The values were calculated using molar extinction coefficient of chromophore (1.56 X 105 M-1cm-1).

Estimation of catalase activity

The catalase was colorimetrically assayed as described by Sinha (1972). The reaction mixture (1.5 ml) contained 1.0 ml phosphate buffer (10 mM, pH 7.0), 0.1 ml liver homogenate and the reaction was started by addition of 0.4 ml H2O2 (2000 mM). The reaction mixture was incubated for 3 min at room temperature. The reaction was stopped by addition of 2.0 ml dichromate-acetic acid reagent (5 % potassium dichromate and glacial acetic acid were mixed in 1:3 ratio), was incubated at 100 °C for 2 min. The absorbance was measured at 620 nm. Catalase activity is expressed as µM H2O2 consumed / min / mg protein. For control, 0.1 ml Tris-HCl buffer (25 mM, pH 7.4) was used in place of the liver homogenate.

Estimation of glutathione peroxidase activity

The glutathione peroxidase (GPx) activity was measured by the method described by Rotruck et al. (1973) and Mills (1959). The reaction mixture contained 1.0 ml TrisHCl buffer (400 mM, pH 7.0), 0.5 ml sodium azide (10 mM), 0.5 ml liver homogenate, 0.5 ml glutathione (4mM) and the reaction was started by addition of 1.0 ml H2O2 (1.25 mM). The reaction mixture was incubated for 3 min at 37 ºC. The reaction was stopped by addition of 1.5 ml TCA (10 %) and centrifuged for 5 min at 5000 rpm. GSH in the protein free filtrates was determined by mixing 1.0 ml supernatant with 2.0 ml phosphate buffer (200 mM, pH 8.0) and 1.0 ml DTNB solution (0.02 % DTNB in 1 % tri sodium citrate). The absorbance was read at 412 nm. Glutathione peroxidase activity is expressed as µM GSH utilized / min / mg protein. For control, 0.5 ml of Tris-HCl buffer (25 mM, pH 7.4) was used in place of the liver homogenate.

Estimation of superoxide dismutase activity

The superoxide dismutase (SOD) activity was analysed by the method described by Kakkar et al (1984). The assay mixture contained 0.1 ml liver homogenate, 1.2 ml sodium pyrophosphate buffer (52 mM, pH 8.3), 0.15 ml PMS (0.186 mM), 0.35 ml of NBT (0.3 mM), 1.0 ml of distilled water, 0.25 ml of NADH (0.75 mM). Reaction was started by addition of NADH. After incubation at 30 ºC for 90s, the reaction was stopped by addition of 1.0 ml of glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4.0 ml n-butanol. The mixture was centrifuged for 5 min at 5000 rpm and butanol layer was taken out. Color intensity of chromogen in butanol layer was measured at 560 nm. A single unit of SOD is expressed as 50 % inhibition of nitro blue tetrazolium reduction / min / mg protein. For control, 0.1 ml of Tris-HCl buffer (0.025 M, pH 7.4) was used in place of the liver homogenate.

The quantitative determination of uric acid concentration inSerum and urine

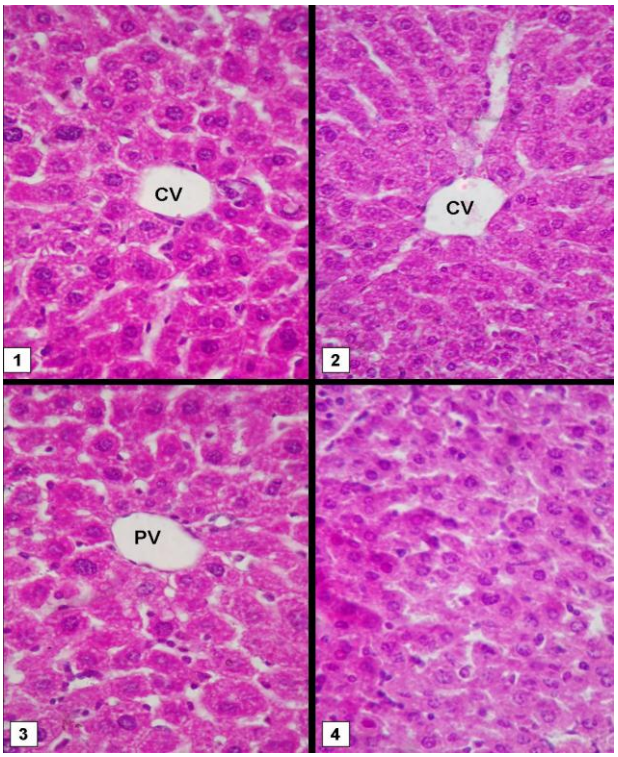
In the human body uric acid is the end-product of purine metabolism. It is excreted by the kidney. Increases of uric acid in the serum plasma or urine can be due to the overproduction of purine containing molecules or to insufficient excretion. Theconcentration is increased in various renal diseases, with increased cell lysis in thepresence of tumors, leukemia, toxemia of pregnancy. Prolonged elevation of theConcentration leads to gout.

Histopathology

Small sections of liver, fixed in 10% buffered formalin were processed for embedding in paraffin. Sections of 5-6 µm were cut and stained with hematoxylin and eosin and examined for histopathological changes under the microscope (Motic AE 21, Germany). The microphotographs were taken using Moticam-1000 camera at original magnification of 100X.

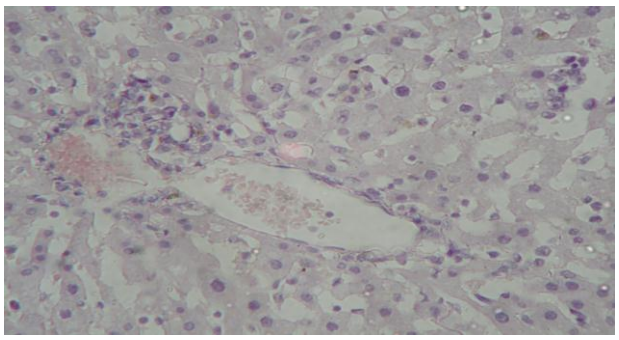
RESULT:

Histopathology of liver :



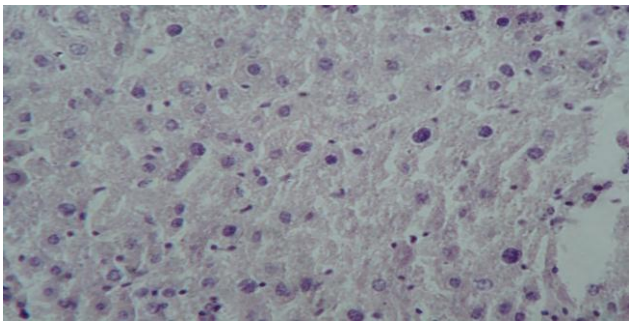
- **Plate 1st, 2nd, 3rd& 4th:-**

Hepatic plates of control mice consisting of the Hepatocyte are radially arranged around the Central Vein (CV). Uniformly spaced Sinusoids are well observed. Hepatocytes may contain one or two nuclei with well-defined cytoplasm. Portal Vein (PV) distinctly visible



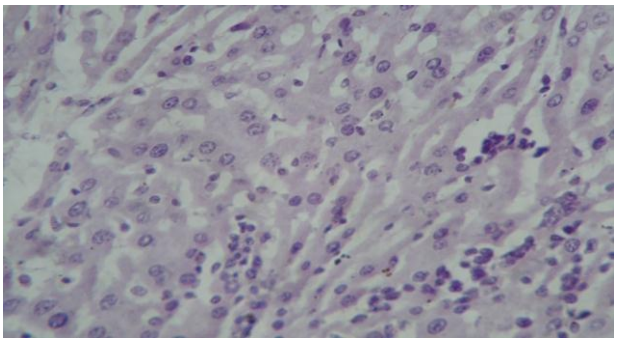
- **Plate 5th:-**

Sections of Arsenic treated liver showing degenerated Portal Vein with degenerated Hepatocytes in sinusoids is well observed. Haemorrhage Portal Vein is prominently observed. Vasculations in sinusoids are also observed.



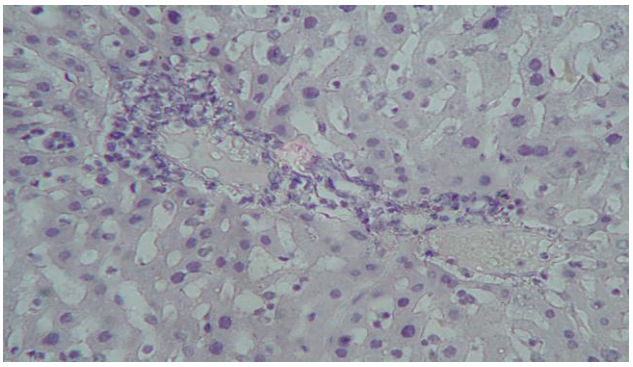
- **Plate 6th:-**

Section of Arsenic treated liver showing very few Hepatocytes in the sinusoids with polymorphic nucleus. Many degenerated Hepatocytes are are also observed. Kuffer cells are clearly observed in the sinusoids



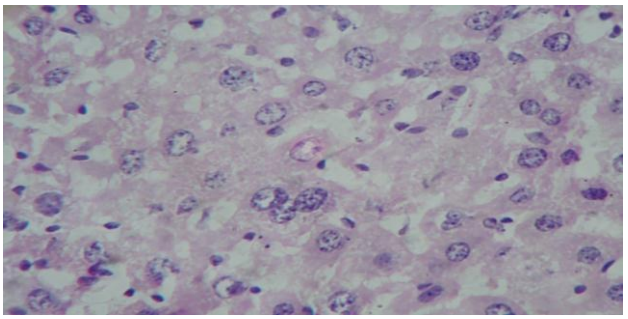
- **Plate 7th:-**

Sections of Arsenic treated liver showing agglutination of Kuffer cells at one spot of sinusoids are clearly visible. Polymorphic nuclei are clearly visible.



- **Plate 8th:-**

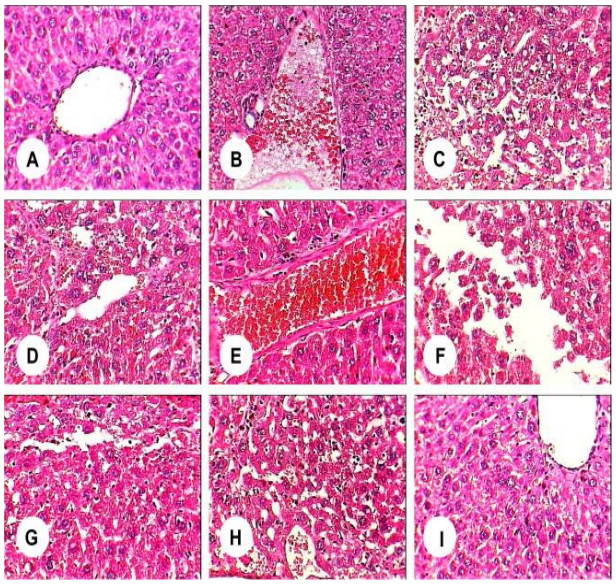
Sections of Arsenic treated liver showing degenerated Portal Vein with degenerated Hepatocytes in sinusoids are well observed. Haemorrhage Portal Vein is prominently observed. Vasculations in sinusoids are also observed. Kuffer cells morphologically changed.



- **Plate 9th:-**

Sections of Arsenic treated liver showing many degenerated Hepatocytes. Kuffer cells are clearly observed in the sinusoids &hetro-chromaization in the nucleus. Few hepatocytes showing fusion of the nuclei.

Parameters	Control	Arsenic Trioxide	Cadamb Extract	Arsenic Trioxide + Cadamba Extract
TBARS(nM/mg protein)	3.19 +/- 0.19	5.49 +/- 0.28	2.15 +/- 0.14	3.94 +/- 0.16
SOD(U/mg protein)	9.13 +/- 0.13	7.64 +/- 0.16	9.77+/- 0.14	8.74 +/- 0.14
GST (µM of CDNB – GSH conjugate formed/min/mg protein)	1.25 +/- 0.10	0.54 +/- 0.10	1.44 +/- 0.10	1.06 +/- 0.11
GPx (µg of GSH consumed/min/mg protein	11.29 +/- 0.29	8.19 +/- 0.35	11.67 +/- 0.44	10.88 +/- 0.47
CAT (µ moles of H_2O_2 consumed/min/mg protein)	32.94 +/- 2.37	23.07 +/- 2.14	32.55 +/- 4.25	29.94 +/- 2.17
GSH (µ M/g tissue)	53.77 +/- 7.47	34.88 +/- 5.84	59.39 +/- 5.63	47.34 +/- 4.77
GR (nmol of NADPH oxidized/min/mg protein)	0.69 +/- 0.03	0.48 +/- 0.03	0.79 +/- 0.04	0.59 +/- 0.04



(A–I) Liver micrographs of control (A), arsenic trioxide (B–F),arsenic trioxide plus cadamba flower extract (G and H) and cadamba flower extract (I) treated mice. Original magnification ×400.

Effect of Cadamba extract on arsenic trioxide induced changes in Lipid peroxidation and antioxidant status.

Data represented as mean +/- SD, n = 6. aP< 0.05 versus Control, bP< 0.05 versus As_2O_3 treated groups.

The effect of cadamba on lipid peroxidation and the activity of antioxidant enzymes were outlined in table 1. Thiobarbituric acid assay was used to measure the extent of lipid peroxidation induced by arsenic trioxide in liver of mice. Results indicated that TBARS level was significantly (P < 5) increased in liver of mice treated with arsenic trioxide .Cotreatment with cadamba caused significant (P < 0.05) decrease in liver TBARS compared to the arsenic trioxide treated mice.

A significant (P < 0.05) decline in the level of GSH was noticed in liver tissue of arsenic treated mice as compared to controls. Arsenic trioxide administered mice showed significant decrease (P < 0.05) in the activity of SOD, CAT, GPx, GST and GR in hepatic tissue. Co – treatment with cadamba extract exhibited significant increase in the GSH levels compared to arsenic trioxide mice. The cadamba extract treatment showed significant (P < 0.05) recovery in the antioxidant enzymes activity.

DISCUSSION :

There are three parts of the study. First part is to know the effect of arsenic trioxide on the testes, liver and kidney of the swiss albino mice. In second phase of our study, these arsenic treated mice were treated with cadamb fruit extract to find out the remedial action of cadamba fruit on the arsenic affected organs of the mice. This part of the study confirmed that cadamb fruit can be used in treatment of arsenic induced toxicity. The final part of the study comprises the determination of combine effect of arsenic trioxide and cadamb extract on the organs of Albino mice. The study concludes that when the people who are bound to intake arsenic trioxide due to its presence in water given cadamb fruit, can get relief from the toxic effects of arsenic.

In most of the areas of Bihar, Uttarpardesh and Madhyapardesh, arsenic trioxide is present in significant amount in groundwater. People living in these areas are bound to drink this water. Although there are a number of techniques to purify the drinking water, but these are too costly to be availed by the economically weaker section of the population living in these areas. Cadamba fruit is available in these areas in abundant and is in reach

of every person. Therefore, the study was oriented in the direction of finding the most economical remedy for the most common and severe health problems of Bihar, Uttar Pradesh and Madhya Pardesh etc. Now we discuss about the major cause of sufferings for the people of these states i.e. arsenic and the diseases caused by this.

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