Abdul Mohamed Jalaludeen et al /J. Pharm. Sci. & Res. Vol.3(9), 2011,1447-1455 ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Studies on the antioxidant and free radical-scavenging effect of sinapic acid: An *in vivo* and *in vitro* model

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Abstract:
The aim of the present study was to evaluate the circulating antioxidants such as vitamin C, vitamin E and GSH in arsenic induced toxicity in rats and *in vitro* free radical scavenging assay. In this investigation, arsenic (5 mg/kg body weight (b.w) was administered orally for 30 days to induce toxicity. Sinapic acid was administered orally (40 mg/kg body weight) for 30 days with oral administration of arsenic. The toxic effect of arsenic was indicated by significantly decreased levels of non-enzymatic antioxidants like vitamin C, vitamin E and reduced glutathione. Treatment with sinapic acid exhibited a significant ($P < 0.05$) reversal of arsenic induced toxicity in rats. The free radical scavenging properties of sinapic acid at different concentrations (10-50uM) were investigated with various *in vitro* methods such as 2, 2′-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$), hydroxyl radical, superoxide anion scavenging activity and reducing power. Among the different concentrations, 50 µM of sinapic acid was more effective compared to other concentrations in all *in vitro* assays. Based on these findings, sinapic acid possesses potent *in vivo* and *in vitro* antioxidant activity and also effective free radical scavenger, augmenting its therapeutic value.
Keywords: Antioxidants, Arsenic, Free radical, Hydroxyl radical, Reducing power, Sinapic acid.

Introduction:

Arsenic is a member of the nitrogen family with both metallic and nonmetallic properties, and is ubiquitous in the environment [1]. Many countries, especially Taiwan, Argentina, India, Bangladesh, Mexico, Hungary, and Chile, have reported extensive arsenic contamination of their groundwater supplies [2, 3]. The worst affected countries in the World in recent years include India and Bangladesh [4]. Arsenic exerts its toxicity by generating reactive oxygen species (ROS) during its metabolism which results in the oxidative damage and depresses the antioxidant defense system [5].

Reactive oxygen species (ROS) including superoxide anion radical, hydroxyl radical, and hydrogen peroxide, are formed and degraded by all aerobic organism, can cause oxidative damage of all major groups of biomolecules (DNA, protein, lipids and small cellular molecules), which in turn leads to cardiovascular and neurodegenerative diseases [6]. However, humans and other organisms possess antioxidant defenses (enzymes, such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherols and glutathione) and repair systems that protect them against oxidative damage, [7]. These protective mechanisms are disrupted by various pathological phenomenon and antioxidant supplements are essential to conflict the oxidative damage. Moreover, antioxidants may play a role in helping to prevent diseases such as cancer, cardiovascular disease, Alzheimer's disease and muscular degeneration by scavenging free radicals [8].

Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are extensively used as antioxidants in order to reduce the damage caused by free radicals. However, the possible toxicity as well as general consumer rejection led to decreasing use of these synthetic antioxidants. Against this background, the evaluation of the antioxidant properties of specific chemical scavengers is of particular value for their potential use in preventing or limiting the damage induced by free radicals. Recently an intensive search for novel

types of antioxidants has been carried out from numerous plant materials [9, 10]. The antioxidant properties of various plants have been reported by various studies [11, 12]. The phenolic compounds in plants are found to be effective antioxidants due to their redox properties. They act as reducing agents (free radical terminators), hydrogen donors, singlet oxygen quenchers and metal chelators [13].

Sinapic acid is a cinnamic acid derivative, which possesses 3, 5-dimethoxyl and 4-hydroxyl substitutions in the phenyl group of cinnamic acid [Figure.1].

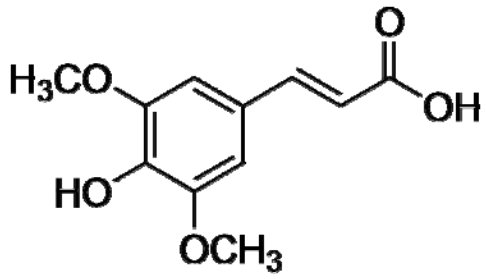

Fig. 1: Structure of sinapic acid

Sinapic acid is a widely prevalent substance in the plant kingdom and is obtained from various sources such as rye, fruits, and vegetables [14, 15]. Sinapic acid has already been pharmacologically evaluated as potent anxiolytic [16], anti-inflammatory [17] and peroxynitrite scavenging effect [18]. Moreover, sinapic acid attenuates kainic acid-induced hippocampal neuronal damage in mice [19]. The objective of this study is to find antioxidant and free radical-scavenging activities of sinapic acid using various *in vivo* and *in vitro* models.

Materials and Methods:

Chemicals:

Sinapic acid, arsenic, reduced glutathione (GSH), 2,2′-dipyridyl, 2,4-dinitro phenylhydrazine (DNPH), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS) and butylated hydroxytoulene were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The rest of the chemicals utilized were obtained from a local firm (Himedia Laboratories Ltd., Mumbai, India) and were of analytical grade.

Animals

Adult male albino rats of Wistar strain (190 – 220 g) were used for the experiment. The animals were housed in polypropylene cages and maintained in 12-h light/12-h dark cycle, 50% humidity and 25±2 °C. The animals had free access to standard pellet diet (M/S. Pranav Agro Industries Ltd., Bangalore, India) and water *ad libitum.* This study was approved (Vide.No.740, 2010) by Institutional Animal Ethics Committee of Annamalai University and the study conducted in accordance with the "Guide for the Care and Use of Laboratory Animals".

Experimental design:

The animals were randomly divided into four groups of six rats in each group.

Group 1: Normal control rats (vehicle treated)

Group 2: Normal rats received sinapic acid (40 mg/kg b.w/day) dissolved in corn oil using intragastric tube for 30 days

Group 3: Normal rats received arsenic as sodium arsenite (5 mg/kg b.w/day) dissolved in drinking water using intragastric tube for 30 days

Group 4: Normal rats received arsenic with co-administration of sinapic acid using intragastric tube for 30 days

At the end of experimental period, animals in different groups were sacrificed by cervical decapitation under ketamine hydrochloride (35 mg/kg body

weight) anesthesia. Blood was collected in a tube, heparinised for plasma. Plasma separated by centrifugation and used for various biochemical estimations.

Biochemical assays

Determination of plasma non-enzymatic antioxidants:

Vitamin C concentration was measured by Omaye et al [20]. To 0.5 mL of plasma, 1.5 mL of 6% TCA was added and centrifuged (3500 ×g, 20 min). To 0.5 mL of supernatant, 0.5 mL of DNPH reagent (2% DNPH and 4% thiourea in 9N sulfuric acid) was added and incubated for 3 h at room temperature. After incubation, 2.5 mL of 85% sulfuric acid was added and colour developed was read at 530 nm after 30 min.

Vitamin E was estimated by the method of Desai [21]. Vitamin E was extracted from plasma by addition of 1.6 mL ethanol and 2.0 mL petroleum ether to 0.5 mL plasma and centrifuged. The supernatant was separated and evaporated on air. To the residue, 0.2 mL of 0.2% 2, 2-dipyridyl, 0.2 mL of 0.5% ferric chloride was added and kept in dark for 5 min.An intense red coloured layer obtained on addition of 4 mL butanol was read at 520 nm.

Reduced glutathione (GSH) was determined by the method of Ellman [22]. 1 mL of supernatant was treated with 0.5 mL of Ellman's reagent (19.8 mg of 5, 5-dithiobisnitro benzoic acid in 100 mL of 0.1% sodium citrate) and 3.0 mL of phosphate buffer (0.2 M, pH 8.0) was added and the absorbance was read at 412 nm in spectrophotometer.

***In vitro* antioxidant activity:**

Free radical scavenging activity: The ability to scavenging the stable free radical, DPPH was measured as a decrease in absorbance at 517 nm by the method of Mensor et al [23]. To a methanolic solution of DPPH (90.25 mmol), an equal volume of sinapic acid (10-50 µmol) was added and made up to 1.0 mL with methanolic DPPH. An equal amount of methanol was added to the control. After 20 min, the absorbance was recorded at 517 nm in a Systronics UV-visible Spectrophotometer. The inhibition of free radicals by DPPH in percentage terms (%) was calculated by using the following equation.

% = [(A control-A sample)/A blank] x 100

Where A control is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound.

Total Antioxidant activity assay: Total antioxidant potential of sinapic acid was determined by the ABTS assay, as described by Miller et al [24]. The reaction mixture contained ABTS (0.002 M), sinapic acid (10-50 µmol) and buffer in a total volume of 3.5 mL. The absorbance was measured at 734 nm in a Systronics UV- visible Spectrophotometer. The percentage of inhibition was calculated.

Super oxide anion scavenging activity: Superoxide anion scavenging activity of sinapic acid was determined by the method of Nishmiki et al [25] with modification. 1 mL of NBT (100 µmol of NBT in 100mM phosphate buffer, pH 7.4), 1 mL of NADH solution (14.68 µmol of NADH in 100 mmol phosphate buffer, pH 7.4) and varying volumes of sinapic acid (10-50 µmol) were mixed well. The reaction was started by the addition of 100 µmol of PMS (60 µmol/100 mmol of phosphate buffer pH 7.4). The reaction mixture was incubated at 30ºC for 15 min. The absorbance was measured at 560 nm in a spectrophotometer. Incubation without sinapic acid was used as blank.

Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging. The % of inhibition was calculated.

Hydroxyl radical-scavenging activity: The hydroxyl radical scavenging activity was determined by the method of Halliwell et al [26]. The following reagents were added in the order stated below. The incubation mixture in a total volume of 1 mL contained 0.1 mL of 100 mmol of potassium dihydrogen phosphate-KOH buffer, varying volumes of sinapic acid (10-50 µmol), 0.2 mL of 500 mmol of ferric chloride, 0.1 mL of 1 mmol of ascorbic acid, 0.1mL of 10 mmol of H_2O_2 and 0.2 mL of 2-deoxy ribose. The contents were mixed thoroughly and incubated at room temperature for 60 min. Then 1 mL of 1% TBA (1 gm in 100 mL of 0.05 N NaOH) and 1 mL of 28% TCA were added. All the tubes were kept in a boiling water bath for 30 min. The absorbance was read in a spectrophotometer at 532 nm with reagent blank containing distilled water in a place of sinapic acid. The percentage scavenging activity was determined. Decreased absorbance of the reaction mixture indicated increased hydroxyl radical scavenging activity.

Reducing power: The reducing power was determined according to the method of Oyaizu [27]. Different concentrations of sinapic acid (10-50 µmol) were prepared in methanol mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (K_3Fe (CN) (2.5 mL, 1%). The mixture was incubated at 50^{o}C for 20 min and 2.5 mL of TCA (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a standard.

Statistical Analysis

The data for various biochemical parameters were analysed using analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). Values were considered statistically significant when $p < 0.05$.

Results:

Effect of sinapic acid on plasma antioxidants: Table 1 depicts the changes in vitamin C, vitamin E and GSH in plasma of normal control and experimental animals. Vitamin C, vitamin E and GSH levels were significantly lower in arsenic intoxicated rats than in normal control rats. In contrast arsenic intoxicated rats treated with sinapic acid led to significant increase in the plasma antioxidant levels of vitamin C, vitamin E and GSH.

In vitro antioxidant activity: Several concentrations ranging from 10-50 µM of the sinapic acid was tested for their antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner in all the models.

Figure 2 shows the percentage scavenging action of sinapic acid on free radical generation. Sinapic acid scavenges DPPH radical in a dose-dependent manner (10-50 µM). The DPPH radical scavenging activity was detected and compared with ascorbic acid. However, the highest percentage (51.68%) scavenging activity of sinapic acid was observed at 50 µM.

Figure 3 shows the total antioxidant activity was measured using the ABTS assay.

Table 1: Changes in the levels of vitamin - C, vitamin - E and GSH in plasma of normal control and experimental rats

Groups	Normal control	Normal + Sinapic acid (40mg/kg)	Normal + Arsenic (5mg/kg)	Arsenic + Sinapic acid (40mg/kg)
Vitamin - C (mg/dl)	1.72 ± 0.16^{a}	1.74 ± 0.18^{a}	0.51 ± 0.04^{b}	1.53 ± 0.13^{c}
Vitamin - E (mg/dl)	1.49 ± 0.15^{a}	1.52 ± 0.15^{a}	0.56 ± 0.05^{b}	1.29 ± 0.14^{c}
GSH (mg/dl)	24.76 ± 1.41^{a}	25.53 ± 1.46^{a}	12.34 ± 0.83^{b}	20.48 ± 1.29^{c}

Values are given as mean ± S.D. from six rats in each group. Values not sharing a common letter (a-c) differ significantly at P<0.05 (DMRT).

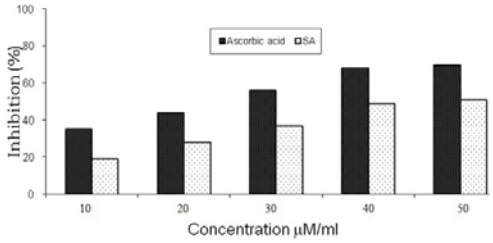

Fig. 2: DPPH free radical scavenging activity of sinapic acid. The bars represent sinapic acid and a positive control ascorbic acid. Each bar represents % inhibition.

Inhibition of the ABTS radical showed dose-dependent (10-50 µM) scavenging activity. The percentage scavenging activity of sinapic acid increases with increasing concentration. However, the highest percentage (55.42%) scavenging activity was observed at 50 µM and compared with butylated hydroxytoulene.

Figure 4 shows the percentage *in vitro* scavenging effects of sinapic acid on superoxide radical. Sinapic acid scavenges the above mentioned radicals *in vitro* in a dose-dependent manner. The percentage scavenging activity of sinapic acid increases with increasing concentration. The highest percentage (35.52%) scavenging activity was observed at 50 µM and compared with ascorbic acid.

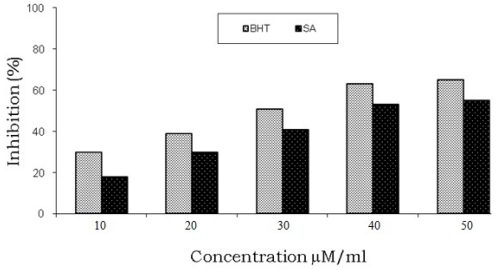

Fig.3: ABTS - Total antioxidant scavenging assay of sinapic acid. The bars represent sinapic acid and a positive control butylated hydroxytoulene. Each bar represents % inhibition.

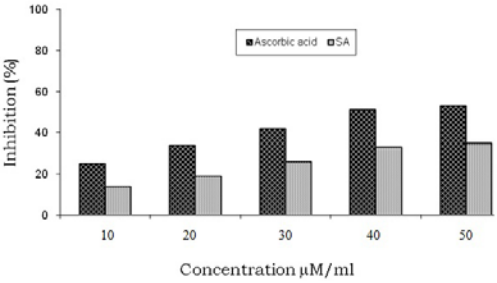

Fig. 4: Superoxide radical scavenging effect of sinapic acid. The bars represent sinapic acid and a positive control ascorbic acid. Each bar represents % inhibition.

Figure 5 shows the percentage *in vitro* scavenging effects of sinapic acid on hydroxyl radical. Sinapic acid scavenges

the above mentioned radicals *in vitro* in a dose-dependent manner. The percentage scavenging activity of sinapic acid increases with increasing concentration. The highest percentage (33.66%) scavenging activity was observed at 50 µM and compared with ascorbic acid.

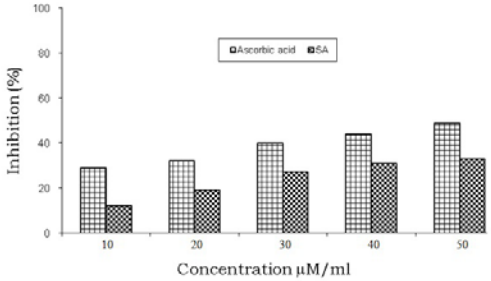

Fig. 5: Hydroxyl radical scavenging assay of sinapic acid. The bars represent sinapic acid and a positive control ascorbic acid. Each bar represents % inhibition.

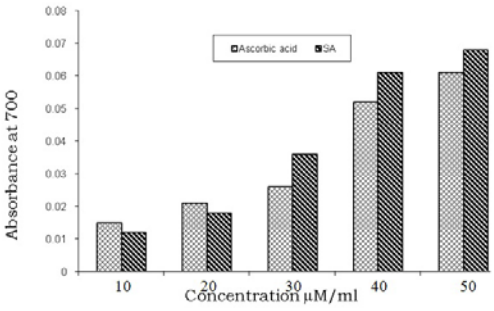

Fig. 6: Reducing power of sinapic acid. The bars represent sinapic acid and a positive control ascorbic acid. Each bar represents absorbance at 700 nm.Figure 6 shows the reducing power of sinapic acid and the reference compound, ascorbic acid increased steadily with increasing concentration. Increased absorbance with the increased concentrations of the reaction mixture indicated the increased reducing power. However, the highest (0.061) scavenging activity was observed at 50 µM.

Discussion:

Free radicals and other reactive species are thought to play an important role in many human diseases [28]. In recent studies provided experimental evidence that arsenic-induced generation of free radicals can cause cell damage and death through activation of oxidative sensitive signaling pathways [29]. Antioxidant systems protect the organism against reactive oxygen species (ROS). These defense mechanisms stop the radical chain reaction and direct the resultant ROS to target where it would cause less injury [30]. Major non-enzymatic defense is provided by vitamin C, vitamin E and glutathione [31].

Vitamin C is a hydrophilic antioxidant in plasma, because it disappears faster than other antioxidants when plasma is exposed to reactive oxygen species. The observed significant decrease in the level of plasma vitamin C could be caused by increased utilization of vitamin C as an antioxidant defense against ROS or by a decrease in GSH, which is required for the recycling of vitamin C.

Vitamin E is a well known physiological antioxidant and membrane stabilizer. It interrupts the chain reaction of LPO by reacting with lipid peroxy radicals, thus protecting the cell structures against damage [32]. The decreased level of vitamin E observed in the toxic rats is compatible with the hypothesis that plasma vitamin E excess plays a protective role against increased peroxidation in toxicity.

GSH is a very effective cellular antioxidant and plays an important role in maintaining cellular redox status. In addition, GSH level is a good marker of oxidative stress of an organism [33]. It acts as a substrate for GPx and GST that are involved in preventing the deleterious effect of oxygen radicals [34]. Several papers have reported decreased levels of GSH after exposure to arsenic. Treatment with sinapic acid brought vitamin C, vitamin E and

reduced glutathione to near normal levels which could be as a result of decreased membrane damage as evidenced by the antioxidant nature [35]. Radical scavenging activities are very important due to the deleterious role of free radicals in biological systems. The $DPPH^{\bullet}$ radical is relatively stable and has been widely used to test ability of natural compounds to act as free radical scavengers or hydrogen donors as a means of evaluating their antioxidant potentials [36]. Further, it is well accepted that the $DPPH^{\bullet}$ free radical scavenging by antioxidants is due to their hydrogen-donating ability [37]. The reduction capability of $DPPH^{\bullet}$ radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. Ascorbic acid is the reagents used as standards. In this study, sinapic acid *in vitro* scavenges $DPPH^{\bullet}$ in dose dependently. The highest percentage scavenging effect of sinapic acid on $DPPH^{\bullet}$ at the concentration of 50 µM was 39.68%.

$ABTS^{\bullet+}$ radical-scavenging method is common spectrophotometric procedures for determining the antioxidant capacity of compound. $ABTS^{\bullet+}$ radicals are more reactive than DPPH radicals and unlike the reactions with DPPH radical which involve H atom transfer, the reactions with $ABTS^{\bullet+}$ radicals involve electron transfer process [38]. When an antioxidant is added to the radicals there is a degree of decolourization owing to the presence of the antioxidants which reverses the formation of the $ABTS^{\bullet+}$ cation. The reaction of the preformed radical with free-radical scavengers can be easily monitored by following the decay of the sample absorbance at 734 nm. The highest percentage scavenging effect of sinapic acid on $ABTS^{\bullet+}$ at the concentration of 50 µM was 33.42%.

Scavenging of hydroxyl radical is an important antioxidant activity because of very high reactivity of the $OH^{\bullet}$ radical, enabling it to react with a wide range of molecules found in living cells, such as sugars, amino acids, lipids, and nucleotides [39]. Thus, removing $OH^{\bullet}$ is very important for the protection of living systems. As is the case for many other free radicals, $OH^{\bullet}$ can be neutralized if it is provided with a hydrogen atom. Hydroxyl radical scavenging activity was quantified by measuring the inhibition of the degradation of deoxyribose by the free radicals generated by the Fenton reaction. In this process, the ferric iron is reduced by superoxide, with subsequent oxidation of ferrous iron by H_2O_2 forming hydroxyl radical thereby initiating the series of oxidative reactions. The results obtained in the present study may be attributed to a number of reasons including, the scavenging of hydroxyl or superoxide radical, by changing the ratio of Fe^{3+}/Fe^{2+}, reducing the rate of conversion of ferrous to ferric or by chelating iron [40]. Sinapic acid exhibited hydroxyl radical scavenging activity in a dose dependent manner in the range of 10-50 µM in the reaction mixture.

Numerous biological reactions generate superoxide radical which is a highly toxic species. Although they cannot directly initiate lipid oxidation, superoxide radical anions are potential precursors of a highly reactive species, such as hydroxyl radical, and thus the study of the scavenging of this radical is important [41]. Therefore, superoxide anion scavenging capacity in the living organisms is the first line of defense against oxidative stress [42]. Superoxide anion is an oxygen-centred radical with a

selective reactivity. Although relatively weak oxidants, superoxides exhibit only limited chemical reactivity, but can generate more dangerous species, including singlet oxygen and hydroxyl radicals, which cause the peroxidation of lipids [43]. Superoxide anion plays an important role in the formation of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA [44]. It has also been reported that antioxidant properties of some phenolic compounds are effective mainly via scavenging of superoxide anion radical [45]. The highest percentage scavenging effect of sinapic acid on superoxide at the concentration of 50 μM was 37.52 %.

The antioxidant activity has been reported to have a direct, positive correlation with the reducing power [46]. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [47]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation [48]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Our data on the reducing power of the tested compound suggest that it is likely to contribute significantly towards the observed antioxidant effect. The reducing power of sinapic acid increases with increasing amount of sample.

Conclusion:

According to data obtained from the present study, sinapic acid was found to be an effective *in vivo* antioxidant effect such as vitamin C, vitamin E and GSH along with *in vitro* assays including DPPH, ABTS radical, superoxide anion radical, hydroxyl radical scavenging and reducing power. Further understand of the mechanism of sinapic acid on antioxidant action may ultimately be useful in the design of drugs that improve arsenic induced toxicity.

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