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Syzygium cumini Seed Extract Ameliorates Arsenic-Induced Blood Cell Genotoxicity and Hepatotoxicity in Wistar Albino Rats

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Abstract

Background: Arsenic is a well-documented human carcinogen widely distributed in the environment. Chronic exposure of humans to inorganic arsenicals causes many adverse health effects. The present work was conducted to evaluate the protective effect of *Syzygium cumini* seed extract (SCE) on arsenic-induced genotoxicity and hepatotoxicity in Wistar albino rats.

Methods: Rats were randomly divided into five groups of six animals each. Group 1 served as normal control, Group 2 received SCE, 200 mg/kg daily, and Group 3 received arsenic, 100 ppm in drinking water. Groups 4 and 5 received SCE, 200 mg/kg and 400 mg/kg, respectively, daily, simultaneously with 100 ppm arsenic in drinking water. After 60 days, blood samples were collected and comet assay was performed using isolated lymphocytes. Activities of serum marker enzymes were assayed and lipid peroxidation (LPO) levels were estimated. Serum catalase (CAT) and superoxide dismutase (SOD) activities, and blood reduced glutathione (GSH) were measured.

Results: Exposure to arsenic caused a significant increase in serum aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and bilirubin, accompanied by a decrease in total protein levels as well as CAT and SOD activities, and GSH. Enhanced LPO and lymphocyte DNA damage was also observed in arsenic-administered rats. The arsenic-induced toxicity was significantly reversed by the simultaneous administration of SCE at both the lower and higher dosages.

Conclusions: This investigation offers strong evidence for the hepato-protective and antioxidative effects of SCE against arsenic-induced oxidative stress.

Keywords: Antioxidants, Arsenic, Comet assay, Reactive oxygen species, *Syzygium cumini*.

Introduction

Arsenic is a toxic metalloid widely distributed in nature (1). It is released into the environment by many processes including weathering of rocks, the pesticide and smelting industries, coal-fired power plants, glassware manufacturing, and release of wood preservatives, among others (2, 3). Arsenic poisoning due to arsenic-contaminated water consumption is reported to affect more than 200 million people worldwide, of whom approximately 38 million live in the Indo-Bangladesh region (4). Arsenic concentrations in drinking water in Mexico, Taiwan, Argentina, and the Indo-Bangladesh region have been reported to be well above the 10 µg/l standard adopted by the US Environmental Protection Agency (USEPA) in 2001 (5). Arsenic exists in the environment in both organic and inorganic forms (1). Inorganic arsenicals include both trivalent (arsenite or As III) and pentavalent (arsenate or As V) species, both of which are highly toxic and considered as class I carcinogens (1, 6). Chronic human exposure to these compounds is associated with many toxic effects including peripheral neuropathy, liver injury, and increased incidences of cancers of the bladder, skin, lung, and

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liver (7). These compounds are genotoxic to both humans and animals (10).

It is reported that arsenic induces its toxicity via formation of excess reactive oxygen species (ROS) and disruption of the prooxidant/antioxidant balance in the body (4, 10). Metabolic processing of arsenic is related to the production of free radicals and ROS, which further results in DNA single-strand breakage, deletion mutations, micronuclei formation, sister chromatid exchange, and DNA-protein cross-linking (10).

Presently, the most effective treatment for arsenic toxicity is chelation therapy in which drugs such as British anti-Lewisite (dimercaprol) and dimercaptosuccinic acid are used; however, these compounds have several moderate to severe side effects including itching, nausea, abdominal pain, hypertension, and changes in body temperature (8, 9). Some studies reported the administration of antioxidant-enriched plant extracts to treat arsenic poisoning-induced toxicity. Dietary antioxidants present in many plants have been quite effective in reducing genotoxic and other toxic arsenic effects (10). Resveratrol, an antioxidant found in grapes, peanuts, berries, and pines (11), potentially minimized sodium arsenite-induced ROS level and DNA damage in human bronchial epithelial cells (12). Tetrahydrocurcumin, a powerful antioxidant derived from curcumin present in turmeric, decreased arsenic-induced oxidative DNA damage in rat hepatic cells (13). Green tea (*Camellia sinensis*) extract significantly ameliorated arsenic-induced genotoxicity and damage to intestinal tissues in rats, and this protection was attributed to polyphenols in green tea (14, 15).

Syzygium cumini is a tropical plant of the Myrtaceae family widely distributed in India, Sri Lanka, Malaysia, and Australia. The fruit of this plant is commonly known as jamun (Hindi), black plum, java plum, jambul, and Indian blackberry (16, 19). The seed, leaf and bark extracts of this plant have been shown to have hypoglycemic (17), anti-inflammatory, antipyretic (18), antidiarrheal (19), and antibacterial (20) properties. *Syzygium cumini* has been reported to contain a variety of active phytochemicals with potent antioxidant and free radical-scavenging activities (21, 22).

Here we report the effect of *S. cumini* seed extract (SCE) at two different dosages on sodium arsenite-induced blood cell genotoxicity and hepatotoxicity in Wistar albino rats.

Materials and methods

Plant material

Syzygium cumini seeds were purchased from the local market from Hisar district, Haryana, India. Seeds were identified and authenticated by Raw Materials Herbarium and Museum, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi.

Preparation of seed extract

The seeds were freed of pericarp, dried at room temperature, and finely powdered. The seed extract was prepared by soxhlet extraction using methanol as the solvent. The liquid extract was cooled and concentrated by evaporating its liquid contents in vacuum and then freeze dried. The yield of SCE was 10.8% of the dried powdered seeds. The seed extract was stored at –20 °C until further use.

Experimental animals

Both male and female Wistar albino rats weighing between 100 and 125 g were used in the study. The animals were obtained from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana. All rats were housed in the departmental animal house on a 12:12 hr light: dark cycle at 26 ± 2 °C and 44–56% relative humidity. Animals were provided with a balanced rat diet. The experimental protocol was approved by the Institutional Animals Ethics Committee (IAEC) of Guru Jambheshwar University of Science and Technology, Hisar (CPCSEA/0436), and the animals were cared in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, on animal experimentation. The animals were allowed to acclimate for one week before the commencement of the experiment.

Experimental Protocol

Rats were randomly divided into following five groups of six animals each as follows:

Group I: Received only drinking water *ad libitum*, and served as control.

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Group II: Received SCE (200 mg/kg) daily for 60 days by oral gavage.
Group III: Received arsenic (100 ppm) in drinking water *ad libitum* for 60 days.
Group IV: Received arsenic (100 ppm) in drinking water *ad libitum* and SCE (200 mg/kg) daily for 60 days by oral gavage.
Group V: Received arsenic (100 ppm) in drinking water *ad libitum* and SCE (400 mg/kg) daily for 60 days by oral gavage.
Food, water intake, and animal body weights were monitored throughout the experiment. On completion of the 60-day experimental period, the final body weights of all the experimental rats were recorded. Rats were anaesthetized with diethyl ether and blood samples were collected from the eye retro-orbital plexus.

Lymphocyte isolation

Freshly-collected blood was diluted with phosphate-buffered saline (PBS) in 1:1 ratio and layered on top of Lymphocyte Separation Medium (LSM) 1084 media and centrifuged at 400 x *g* for 30 min. The buffy coat interface, which contained the lymphocytes, was carefully aspirated and washed 2 x with PBS by centrifugation at 250 x *g* for 10 min. The supernatant was discarded and pelleted lymphocytes were immediately used for the comet assay.

Comet assay

The comet assay was performed as previously described by Singh *et al*., 1988 (23), with minor modifications. 150 µl of 0.5% normal melting agarose (NMA) was coated on microscope slides and dried for 10 min at 65 °C. The isolated lymphocytes were carefully resuspended in 75 µl of 0.5% LMA, layered onto slides precoated with 0.5% NMA, and spread with a coverslip. After solidifying for 10 min at 4 °C, the coverslip was removed and third layer of 0.5% LMA was added to slides. The slides were covered and stored for 15-20 min at 4 °C. The coverslips were then removed and the slides were immersed in cold fresh lysing solution containing 100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Tris, 10% DMSO, and 1% Triton X-100, pH 10-10.5, for 2 h at 4 °C in the dark. To avoid additional DNA damage, the following steps were performed under dim light. After lysis, the slides were placed for 20-25 min in electrophoretic buffer containing 300 mM NaOH and 1 mM Na_2EDTA, pH 13.5, at 0 °C. Then the slides were electrophoresed using the same buffer in an ice bath for 20 min at 24 volts and 300 mA. The slides were neutralized 2 x 5 min with 0.4 M Tris, pH 7.5, fixed with 3 ml of absolute ethanol for 3 min, and stained with 50 µl of 0.4 µg/ml EtBr immediately before analysis. Comet images were examined on an Olympus CX 41 fluorescence microscope at 400× magnification. Images of 50 randomly-selected cells were analyzed from each sample. Tail percent DNA, tail lengths, and tail moments were measured using Open Comet software. Data were analyzed applying a one-way ANOVA and post-hoc Tukey's test. Probability values of $P < 0.05$ were considered to be statistically significant.

Biochemical assays

Activities of serum markers

The activities of serum aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and total protein and bilirubin concentrations were assayed using commercially available diagnostic kits.

Estimation of lipid peroxidation (LPO)

Lipid peroxidation in the serum was measured in terms of thiobarbituric acid reactive substances (TBARS) by the method of Ohkawa et al. (24). The absorbance was read at 532 nm.

Reduced Glutathione (GSH)

Reduced glutathione concentration in blood was measured by the method of Beutler *et al*. (25). This method is based on the reaction of GSH with 5, 5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) which results in the development of yellow color. The absorbance was read at 412 nm.

Superoxide dismutase (SOD)

Superoxide dismutase activity in serum was estimated as described by Madesh and Balasubramanian (26), with some modifications. This method involves the generation of superoxide by pyrogallol autooxidation and inhibition of superoxide-dependent reduction of the tetrazolium dye MTT to its formazan, which is measured at 570 nm.

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Catalase (CAT)

Serum CAT activity was measured as described by Goth (27). Serum samples were incubated in substrate containing 65 µmol/ml hydrogen peroxide in 60 mmol/l sodium-potassium phosphate buffer, pH 7.4, at 37 °C for 60 s. Under these conditions, one unit of CAT decomposes 1 µmol of hydrogen peroxide per minute. The enzymatic reaction was stopped with 32.4 mM ammonium molybdate and the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm against a blank containing all the components except the enzyme.

Data were analyzed applying a one-way ANOVA and post-hoc Tukey's test. Probability values of $P < 0.05$ were considered to be statistically significant.

Results

Weight gain differences

The body weight gain was less in the arsenic-treated group (III) than in the control (I) and SCE-treated (II) groups, and greater in the groups receiving SCE and arsenic together (IV and V) than in the arsenic-only group (III) (Table 1).

Table 1. Body weight gains in control and experimental rats

Groups	Initial weight (g)	Final weight (g)	Body weight gain (%)
I	105.33 ± 5.47	145.67 ± 5.89	38.51
II	103.50 ± 6.50	147.33 ± 5.72	42.66
III	102.17 ± 9.20	138.17± 4.58	35.93
IV	102.83 ± 6.05	142.17± 6.49	38.38
V	104.33 ± 7.31	145.67± 7.23	39.81

Values are given as means ± SDs for six rats in each group

Groups: I - Control, II – SCE, III – Arsenic, IV – Arsenic + SCE (200 mg/kg), V – Arsenic + SCE (400 mg/kg)

Serum biomarkers

Serum bilirubin was significantly greater in the arsenic-only group (III) than in controls (I), and significantly less in the SCE (II) and arsenic plus SCE-treated (IV and V) groups than in the arsenic-only group (III). Activities of ALT, AST, and ALP were all significantly greater in the arsenic-only group (III) than in controls (I), while the same activities were all less in arsenic plus SCE groups (IV and V) than the arsenic-only group (III). AST and ALP activities were significantly lower in arsenic plus 400 mg SCE group (V) than the arsenic-only group (III). Serum total protein was significantly less in the arsenic-only group (III) than in controls (I) and significantly greater in SCE (II) and arsenic plus SCE groups (IV and V) than in the arsenic-only group (III). No serum biomarker values in the SCE (II) or arsenic plus 400 mg SCE (V) groups differed significantly from controls (I) (Fig. 1).

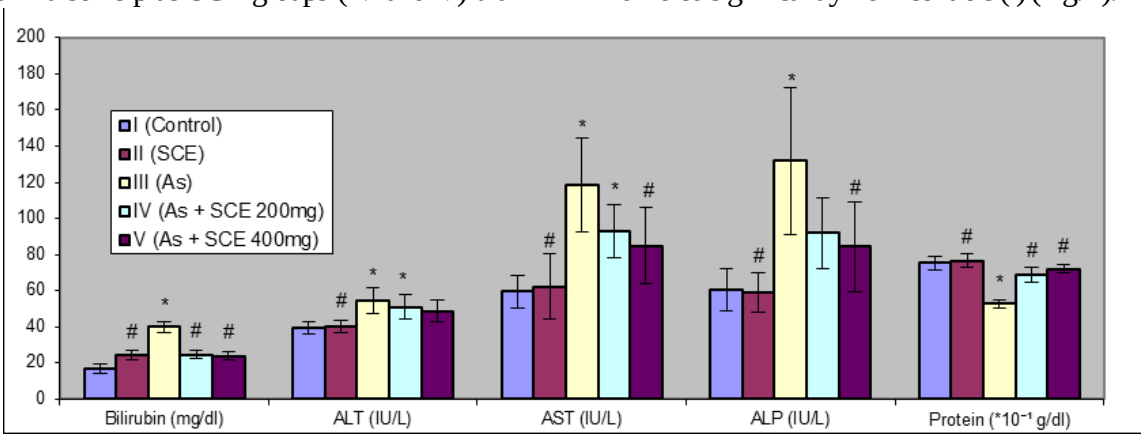

Fig. 1. Serum biomarkers activity in control and experimental rats. Values are given as means ± SDs for six rats in each group; * $p<0.05$ compared with controls (I), # $p<0.05$ compared with the arsenic group (III)

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Catalase, SOD, LPO, and GSH differences

Catalase and SOD activities in sera, and GSH concentration in blood, were all significantly less in arsenic-treated (III) than in control (I) groups, while the same activities and concentrations were all significantly greater in SCE (II) and both arsenic plus SCE (IV and V) groups than in the arsenic-only group (III). The level of lipid peroxidation in sera was significantly increased in arsenic-treated (III) animals when compared to control (I) group. Significant decrease in LPO level was observed in SCE (II) and both arsenic plus SCE (IV and V) groups than in the arsenic-only group (III) (Fig. 2).

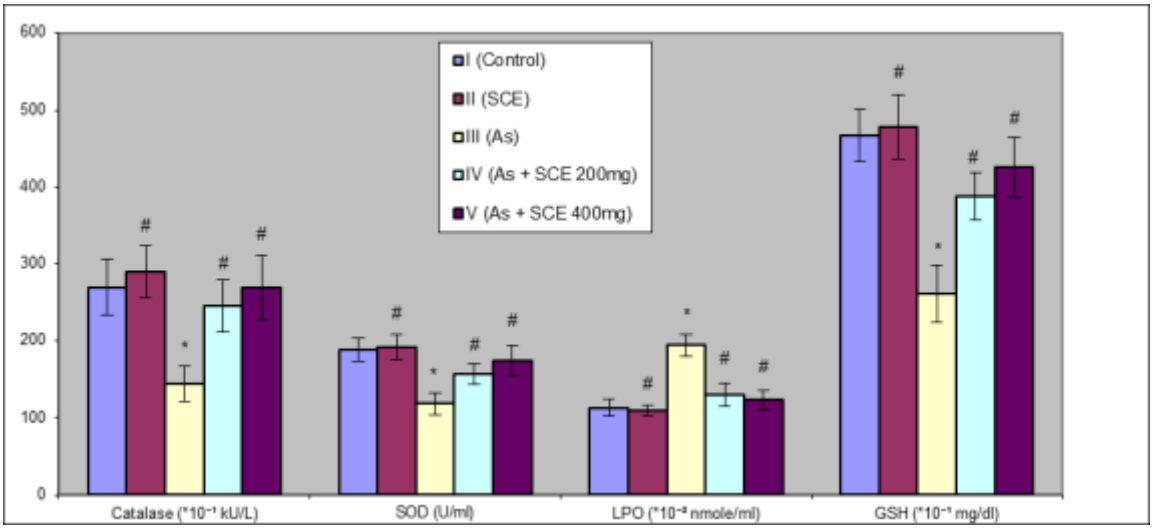

Fig. 2. Catalase and SOD activities and LPO concentrations in sera and GSH concentration in blood of control and experimental rats. Values are given as means ± SDs for six rats in each group; * $p<0.05$ compared with control group (I), # $p<0.05$ compared with arsenic group (III).

Comet assays were performed to evaluate DNA damage in lymphocytes. The mean values of tail percent DNA, tail lengths, and tail moments were significantly greater in the arsenic-only group (III) than in controls (I). Tail percent DNA was significantly less in the SCE (II) and 400 mg/kg arsenic plus SCE (V) groups, while tail length and tail moment were significantly less in the SCE (II) and both arsenic plus SCE (IV and V) groups than in the arsenic group (III). The DNA damage indices values in the SCE-only group (II) were not significantly different from controls (I) (Table 2).

Table 2. Alkaline comet assay results from control and experimental rat lymphocytes

Groups	Parameters		
	Tail % DNA	Tail length (μM)	Tail Moment
I	15.38±5.19	5.63±2.87	3.46±2.09
II	14.39±4.24**	5.45±1.31**	3.08±1.38**
III	33.17±14.44*	15.19±6.36*	12.04±5.63*
IV	20.36±12.88	7.74±4.13**	5.39±4.52**
V	16.54±4.42**	6.93±1.80**	4.16±1.46**

Values are given as means ± SDs for six rats in each group; * $p<0.05$ compared with control group (I),
** $p<0.05$ compared with arsenic group (III)
Groups: I - Control, II – SCE, III – Arsenic, IV – Arsenic + SCE (200 mg/kg), V – Arsenic + SCE (400 mg/kg)

Discussion

The results of this study suggest a beneficial role for SCE in reducing arsenic toxicity in Wistar albino rats. Our results agree with previous findings in which administration of antioxidants from plant sources such as green tea (*Camellia sinensis*), amla (*Emblica officinalis)* and *Moringa oleifera* resulted in decreased arsenic toxicity in arsenic-exposed animals (14, 28, 29). *Syzygium cumini* seeds also

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are known to possess bioactive compounds including triterpenoids, ellagic acid, quercetin, acetyl oleanolic acid, myricetin, and kaempferol (21, 22).

Body weight is a nonspecific indicator of an animal's general well-being. Reduced body weight gain implies impaired growth. In this study, the body weight gain in arsenic-intoxicated rats was lower than that of control rats; however, co-administration of SCE with arsenic restored the body weight similar to that of controls, which indicates beneficial effects of SCE against impaired body growth caused by arsenic intoxication.

The liver is the principal target organ of arsenic toxicity (30, 31). Arsenic-mediated increase in bilirubin and the activities of serum AST, ALT, and ALP indicate hepatic toxicity, and these findings agree with previous studies (32, 33). Administration of SCE in arsenic-exposed rats significantly restored these altered biochemical variables. Serum total protein was significantly less in arsenic-treated than in untreated rats and this difference might be due to the damaging effect of arsenic on hepatic cells or alterations in protein synthesis and/or metabolism (32, 34). Treatment with SCE significantly increased serum total protein content toward normal in arsenic-treated rats.

Lipid peroxidation is regarded as a molecular mechanism of lipid-based biomolecule oxidation. Reactive oxygen species overproduction enhances LPO, which subsequently increases malondialdehyde (MDA) and other TBARS levels. Enhanced LPO thus leads to increased cellular macromolecule degradation (35). In our study LPO was significantly greater in arsenic-treated rats than in controls; however, LPO was significantly less in both SCE plus arsenic groups than in the arsenic group alone, and the reduction in LPO was greater in rats receiving the higher dose of SCE than in those receiving the lower dose. Enzymatic antioxidants such as SOD and CAT are the first line of defense against arsenic-induced oxidative stress. Superoxide dismutase catalyzes the reduction of superoxide to hydrogen peroxide while CAT converts these hydroxyl radicals to water. Increased ROS decreases the activity of these enzymes (28, 36). Our results demonstrated a significant decrease in SOD and CAT activity in arsenic-exposed rats while co-administration of SCE with arsenic restored the activity of these enzymes to a great extent.

Reduced glutathione, a tripeptide non-enzymatic antioxidant, protects cells against increased ROS (37) and also inhibits the oxidation of sulfhydryl groups (38). Depleted levels of GSH have been observed in the livers of arsenic-intoxicated animals, which might be due to the oxidation of GSH by free radicals (39), or by formation of an arsenic-GSH complex (40) or its electron-donor ability (41). Antioxidant treatments have resulted in elevated levels of GSH in arsenic-exposed animals (42). In our study, GSH was significantly less in arsenic-treated than in untreated rats, and treatment of arsenic-treated rats with SCE markedly increased the GSH activity in a dose-dependent manner.

To measure DNA damage in the individual cells, the comet assay is considered a rapid, perceptive, and versatile method (43). The DNA-damaging effect of sodium arsenite has been detected in rat liver, blood, kidney, and bone marrow cells in this way. However, supplementation with the antioxidant vitamins C and E in these arsenic-intoxicated rats reduced the extent of DNA damage (44). It is suggested that excess free radicals are involved in arsenic-induced genotoxicity. Earlier studies show that ROS are involved in arsenic-induced cell signaling and transcription factor activation (45) leading to chromosomal aberrations, gene mutations, DNA strand breakage, generation of micronuclei, and apoptosis (10, 46). Nitric oxide is also known to be involved in arsenic-induced DNA strand breakage (47). Moreover, arsenic metabolism generates oxygen radicals that may damage cell macromolecules and decrease cytP450 biotransformation enzymes involved in metabolism of xenobiotics (48, 49). Previous findings indicate that antioxidants reduce arsenic-induced DNA damage by decreasing arsenic-induced oxidative stress via increasing the activities of cellular antioxidants (14) and decreasing oxidation processes such as LPO and protein oxidation (28, 50). In our study, the DNA-damage indices of tail percent DNA, tail length, and tail moment in lymphocytes of arsenic-exposed rats indicated greater DNA damage in these cells than in those of controls; however, co-administration of SCE and arsenic significantly decreased the lymphocyte DNA damage in a dose-dependent manner. The results agree with previous studies as discussed

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above. *Syzygium cumini* seed extract possesses potent antioxidant and free radical-scavenging activity due to the presence of active ingredients, especially the flavonoids myricetin, quercetin, kaempferol, and polyphenol ellagic acid (21, 22), which might be responsible for its protective effect against arsenic-induced genotoxicity and hepatotoxicity.

In conclusion, oral administration of SCE to arsenic-treated rats led to prominent normalization in altered biochemical variables, and significantly restored the activity of cellular enzymatic, as well as non-enzymatic, antioxidants. Arsenic-induced DNA damage in lymphocytes was also alleviated by SCE; therefore, SCE represents a potential therapeutic option to ameliorate arsenic toxicity.

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