AMORPHOPHALLUS CAMPANULATUS (ROXB.) BLUME. TUBER AMELIORATES HEPATIC OXIDATIVE STRESS DURING COLON CARCINOGENESIS INDUCED BY 1, 2 DIMETHYLDIAZINE

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ABSTRACT

Objective: To evaluate the ameliorative effect of Amorphophallus campanulatus tuber methanolic extract (ACME) on DMH induced toxic damage and oxidative stress in rat liver.

Methods: Five groups of six rats each were selected for the study. Group I animals treated as vehicle control received 5% Tween 80 and normal saline instead of ACME and DMH respectively. Group II rats received ACME (250 mg/kg/day; p.o.) for 15 weeks. Group III rats received DMH (20 mg/kg body weight) injections subcutaneously once a week for 4 consecutive weeks and then kept without any treatment till the end of the experimental period. Groups IV - V (DMH+ACME 125; DMH+ACME 250) animals received subcutaneous injections of DMH as in group III along with ACME at dose of 125 and 250 mg/kg body weight/day; peroral respectively for the entire experimental period of 15 weeks.

Results: ACME supplementation significantly (p ≤ 0.05) prevented the elevation of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and hepatic malondialdehyde levels (MDA). When compared to the DMH alone treated group, the rats receiving DMH plus ACME exhibited significant (p ≤ 0.05) increase in hepatic antioxidant activities including reduced glutathione (GSH), glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GPx), and catalase (CAT) levels. Histopathological examination also supported the dose dependent protective effects of ACME.

Conclusion: These results suggest that the extract of A. campanulatus tuber has the potential to ameliorate carcinogen (DMH) induced hepatotoxicity by markedly enhancing the antioxidant enzyme systems of the liver.

Keywords: Amorphophallus campanulatus; Antioxidants; Chemoprevention; 1, 2-Dimethylhydrazine; Hepatoprotection.

INTRODUCTION

Liver is a major organ in which most of the chemicals, drugs and carcinogens undergo metabolism [1]. Several environmental carcinogens have been reported to elicit hepatic oxidative stress and alter the activities of detoxification and antioxidant enzymes during extra hepatic tumorigenesis [2]. Dimethylhydrazine has been shown to be present in tobacco, commercial and wild mushrooms and other food items also [3-5]. Studies have shown that the colon-specific carcinogen 1, 2-dimethylhydrazine (DMH) induce oxidative stress to both liver and colon tissues [6]. It is a procarcinogen metabolized in the liver and produces highly reactive electrophiles i.e., carbonium ions and alkyl free radicals which severely damage the liver causing necrosis and fatty infiltration, methylate nucleobases and disrupt the polysomal assembly [7-10]. The oxidative stress elicited by DMH is due to the damage brought about by free radical attack on cellular macromolecules such as lipids and DNA. It occurs when the production of free radicals increases, scavenging of free radicals or repair of oxidatively damaged macromolecules decreases, or both [11-12].

Epidemiological studies have shown that many phytonutrients of fruits and vegetables might protect the human body against damage by reactive oxygen species (ROS). The consumption of natural antioxidant phytochemicals were reported to have potential health benefits [13]. Amorphophallus campanulatus (Roxb.) Blume belonging to the family of Araceae is a perennial herb commonly known as elephant foot yam. It is basically a tuber crop of south East Asian origin and is largely cultivated throughout the plains of India for its corn (bulb) as food [14]. This plant is also valuable as medicine especially the corn has been used traditionally for the treatment of liver diseases, abdominal pain, abdominal tumours, piles, enlargement of spleen, asthma and rheumatism [15]. Besides, the corn has been reported to possess antioxidant, antibacterial, antifungal, cytotoxic and hepatoprotective activities [16-17]. In the present study, an attempt has been made to evaluate the ameliorative effect of A. campanulatus tuber on DMH induced toxic damage and oxidative stress in rat liver.

MATERIALS AND METHODS

Chemicals

1, 2-Dimethylhydrazine dihydrochloride (DMH) was purchased from Sigma – Aldrich, St. Louis, MO, USA 5, 5’-di-thiobis-(2-nitrobenzoic acid) (DTNB), 1-chloro-2, 4-dinitrobenzene (CDNB) and nitroblue tetrazolium (NBT) were purchased from Sisco Research Laboratories (SRL), Mumbai, India. Assay kits for serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase were purchased from Agappe Diagnost, India. All other chemicals were of analytical grade.

Animals and diets

Animal studies were followed according to Institute Animal Ethics Committee regulations approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg. No. B/442009/6). Male Wistar rats weighing 160 ± 5.3gm (Mean ± S.D, n = 30) were used in this study. Commercial pellet diet containing 4.2% fat was powdered and mixed with 15.6% peanut oil making a total of 20% fat in the diet [18]. This modified feed was fed to rats in all groups throughout the experimental period of 15 weeks and water was given ad libitum.

Collection and preparation of plant extract

A. campanulatus tubers were collected from the local market (Kottayam, Kerala, India) and authenticated. A voucher specimen (SIBSRL02) is maintained in the institute. The powdered tubers were soaked extracted with methanol and were concentrated using rotary evaporator. The yield of methanolic extract was 9.3% (w/w). Two different doses (125 and 250 mg/kg) of A. campanulatus tuber methanolic extract (ACME) suspended in 5% Tween 80 were prepared for oral administration to the animals. It is reported that the extracts of A. campanulatus tubers are safe up to the dose of 2000 mg/kg [19].

Carcinogen administration

DMH was dissolved in 1mM EDTA just prior to use and the pH adjusted to 6.5 with 1mM NaOH and administered subcutaneously in
the right thigh of rats at a dose of 20 mg/kg body weight once a week for the first 4 consecutive weeks.

Treatment schedule
A total of 30 rats were randomly divided into 5 groups with six rats in each group and that were treated as follows:

- **Group I**: Control (Rats treated as vehicle control received 5% Tween 80 and normal saline instead of ACME and DMH respectively).
- **Group II**: Drug control (Rats received ACME (250 mg/kg/day; p.o.) for 15 weeks).
- **Group III**: DMH control (Rats received DMH (20 mg/kg body weight) injections subcutaneously once a week for 4 consecutive weeks and then kept without any treatment till 15 weeks).
- **Group IV**: Animals received subcutaneous injections of DMH as in group III along with ACME at dose of 125 mg/kg body weight/day; peroral for the entire experimental period of 15 weeks.
- **Group V**: Rats received subcutaneous injections of DMH as in group III along with ACME at dose of 250 mg/kg body weight/day; peroral for the entire experimental period of 15 weeks.

Serum enzyme analysis
The blood collected from each animal was allowed to clot for 45 min at room temperature and the serum was separated by centrifugation at 2000 rpm at 4°C for 15 min. The hepatic oxidative damage was assessed by quantifying the serum levels of AST (EC 2.6.1.1), ALT (EC 2.6.1.2) and ALP (EC 3.1.3.1) by kinetic method using the kit of Agappe Diagnostic Ltd., India. Activities of these serum enzymes were measured using semi autoanalyzer (RMS, India).

Percentage protection was calculated using the formula,

\[
\text{Percentage protection} = \frac{\text{Toxic control} - \text{Extract treated} \times 100}{\text{Toxic control}}
\]

Tissue analysis
Biochemical analysis of hepatic tissue
At the end of the experimental period, the animals were anesthetized with pentothal sodium followed by neck decapitation. Liver tissue were immediately excised and washed with ice-cold saline. 10% tissue homogenate was prepared in 0.1M Tris HCl buffer (pH – 7.4) and centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was used for the following biochemical estimations.

The level of lipid peroxidation was measured as malondialdehyde (MDA), a thiobarbituric acid reacting substance (TBARS), using 1'1'3'3'tetramethoxypropane as standard [20]. Reduced glutathione (GSH) was determined based on the formation of a yellow colored complex with DTNB [21]. Glutathione-S-transferase (GST) (EC 2.5.1.18) activity was determined from the rate of increase in conjugate formation between reduced glutathione and CDNB [22]. Glutathione reductase (GR) (EC 1.6.4.2) activity was assayed at 37 °C and 340 nm by following the oxidation of NADPH by GSSG [23]. Glutathione peroxidase (GPx) (EC 1.11.1.9) activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of \( \text{H}_2\text{O}_2 \) and \( \text{NaN}_3 \) [24]. Tissue catalase (CAT) (EC 1.11.1.6) activity was determined from the rate of decomposition of \( \text{H}_2\text{O}_2 \) [25]. Protein content in the tissue was determined using bovine serum albumin (BSA) as the standard [26].

Histopathological examination
Small pieces of liver tissues fixed in ten percent 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.

Statistical analysis
Results are expressed as mean ± S.D and all statistical comparisons were made by means of one-way ANOVA test followed by Tukey’s post hoc analysis. The level of statistical significance was set at \( p \leq 0.05 \).

RESULTS
Rats treated alone with DMH (group III) showed significantly \( (p \leq 0.05) \) elevated levels of liver specific serum marker enzymes such as AST, ALT and ALP (Fig. 1), when compared to control group of animals. However the supplementation of ACME along with DMH significantly \( (p \leq 0.05) \) and dose dependently lowered the increased levels of serum enzymes. Treatment with 125 and 250 mg/kg of ACME exhibited a protection of 47.0 and 83.0% in AST levels, 62.2 and 79.1% in ALT levels and 61.5 and 83.8% in ALP levels respectively. In other words, these DMH dependent increases in serum AST, ALT and ALP levels were clearly inhibited by ACME (125 and 250 mg/kg) treatments, suggesting protective properties of ACME against DMH induced hepatic damage.

When compared to the control group of animals (group I), a significant increase \( (p \leq 0.05) \) in hepatic MDA level was observed in DMH treated rats (group III). Administration of ACME to the rats injected with DMH (group IV and V) significantly \( (p \leq 0.05) \) prevented the elevation of hepatic MDA level (Table 1). But the supplementation of ACME to the drug control rats (group II) did not show significant alteration in hepatic MDA level.

The effect of DMH administration and ACME supplementation on the level of hepatic reduced glutathione and the activities of glutathione dependent enzymes are also shown in Table 1. In group III rats, subcutaneous injection of DMH significantly decreased \( (p < 0.05) \) the level of GSH and activities of GSH dependent enzymes such as GR, GPx and GST as compared to the control group of animals (group I). Administration of ACME at two different doses (125 mg/kg and 250 mg/kg) markedly \( (p < 0.05) \) increased the reduced glutathione level as well as glutathione dependent enzymes activities, compared to rats treated with DMH alone. A more pronounced effect was observed at a dose of 250 mg/kg body weight (group V).

![Graph showing Aspartate aminotransferase activity](image)
Fig. 1: Effect of ACME on serum enzyme levels of rats intoxicated with DMH. (A) Aspartate aminotransferase (B) Alanine aminotransferase and (C) Alkaline phosphatase.

N - Normal control; DC - Drug control (ACME - 250 mg/kg); T - Toxic control (DMH); D1 - DMH + ACME - 125 mg/kg; D2 - DMH + ACME - 250 mg/kg.

Values are expressed as mean ± S.D. (n = 6). Error bar indicating the standard deviation. Statistical significance: p < 0.05. * DMH group differs significantly from control group. ** DMH + ACME - 125 mg/kg and DMH + ACME - 250 mg/kg groups differs significantly from DMH alone treated group. *** DMH + ACME - 250 mg/kg group differs significantly from DMH + ACME - 125 mg/kg.

Table 1: Effect of ACME on the levels of MDA, GSH and activities of antioxidant enzymes (GR, GPx, GST and CAT) in the liver of control and experimental rats.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>MDA (nmol/g tissue)</th>
<th>GSH (nmol/mg protein)</th>
<th>GR (nmol of GSSG utilized/min/mg protein)</th>
<th>GPx (nmol of GSH oxidized/min/mg protein)</th>
<th>GST (µmol CDNB-GSH conjugate formed/min/mg protein)</th>
<th>CAT (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>45.29 ± 0.32</td>
<td>25.22 ± 0.46</td>
<td>24.35 ± 0.70</td>
<td>296.23 ± 3.12</td>
<td>72.98 ± 0.36</td>
<td>51.86 ± 0.94</td>
</tr>
<tr>
<td>Control + ACME 250 mg/kg</td>
<td>46.2 ± 0.13</td>
<td>26.07 ± 0.70</td>
<td>22.26 ± 1.18</td>
<td>298.15 ± 2.59</td>
<td>74.05 ± 0.26</td>
<td>51.25 ± 0.66</td>
</tr>
<tr>
<td>DMH</td>
<td>80.42 ± 0.29</td>
<td>19.03 ± 0.32</td>
<td>15.98 ± 0.99</td>
<td>151.35 ± 2.12</td>
<td>42.21 ± 0.45</td>
<td>40.91 ± 1.04</td>
</tr>
<tr>
<td>DMH + ACME 125 mg/kg</td>
<td>72.48 ± 0.35</td>
<td>22.08 ± 0.36</td>
<td>19.03 ± 0.56</td>
<td>240.46 ± 1.82</td>
<td>56.06 ± 0.54</td>
<td>46.05 ± 1.09</td>
</tr>
<tr>
<td>DMH + ACME 250 mg/kg</td>
<td>54.4 ± 0.28</td>
<td>23.51 ± 0.47</td>
<td>20.13 ± 0.75</td>
<td>260.51 ± 2.49</td>
<td>68.06 ± 0.29</td>
<td>48.96 ± 0.77</td>
</tr>
</tbody>
</table>

Values are the mean ± S.D from 6 rats in each group. Statistical significance: p ≤ 0.05. * DMH group differs significantly from control group. ** DMH + ACME - 125 mg/kg and DMH + ACME - 250 mg/kg groups differs significantly from DMH alone treated group. *** DMH + ACME - 250 mg/kg group differs significantly from DMH + ACME - 125 mg/kg.

The activity of CAT in hepatic tissue of control and experimental rats are also shown in Table 1. The activity of CAT in DMH alone treated rats was significantly (p < 0.05) lower than that of control group of animals. However, treatment with ACME at a dose of 125 mg/kg and 250 mg/kg significantly (p < 0.05) increased the hepatic CAT activity. The effect was more evident when ACME supplemented at a dose of 250 mg/kg body weight.

Histopathological examinations revealed that the normal architecture of the liver was completely lost in rats treated alone with DMH (group III) (Fig. 2C) with the appearance of cell necrosis, haemorrhage, fatty infiltration and pleomorphic nuclei. But the administration of ACME (125 and 250 mg/kg) to DMH treated animals (Fig. 2D and 2E) seems to prevent such hepatic changes as evident from hepatic architectural pattern with mild to moderate hepatitis.
DISCUSSION

1, 2 dimethylhydrazine is not only a colon carcinogen but also a potent necrogenic hepatic carcinogen that alkylates hepatocellular DNA [10]. DMH is metabolized in the liver into methylhydrazine (MAM), which is catalyzed by the enzyme cytochrome P450IE1 [27]. Metabolic activation of MAM to highly reactive carcinogenic electrophiles (methylazoxymethanol and carbonium ion) occurs in the liver and colon, which is known to elicit oxidative stress [28]. Oxidative stress is an important element of mutagenesis and carcinogenesis [29]. Antioxidant and detoxification enzymes can block carcinogenesis by acting as inhibitors of environmental carcinogens and/or mutagens. Hence, enhancement of these enzymes by a natural or synthetic component may result in the amelioration of carcinogen induced hepatotoxicity and inhibition of extra hepatic tumorigenesis [30].

In the present study, subcutaneous injection of DMH (20 mg/kg body weight) once a week for 4 consecutive weeks led to hepatic damage, which has been proven by the significant difference in biochemical markers between the DMH control and normal control rats. The increase in the activities of AST, ALT and ALP in serum of toxic control rats might be due to the increased permeability of plasma membrane or cellular necrosis leading to leakage of the enzymes to the blood stream [31]. Treatment with ACME at a dose of 250 mg/kg produced a better restoration of serum enzyme levels than 125 mg/kg, shows the dose response action of the extract against DMH induced increase of serum enzymes. This may be related to the activity of ACME in stabilization of the plasma membranes as well as the repair of hepatic tissue damage due to DMH.

MDA is a major oxidation product of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation [32]. In the present study we observed that the MDA formation was significantly (p ≤ 0.05) increased in the liver of DMH alone exposed group (group III) of animals. The elevation of hepatic lipid peroxidation as evidenced by the increased levels of MDA could be due to the reactive oxygen metabolites released during the metabolism of DMH in the liver. ACME supplementation...
potentially reduced MDA level, suggesting that ACME might have antioxidant principles to produce such response.

The biochemical evidence from our study suggests that the hepatic reduced glutathione (GSH) level and GR, GPx and GST activities were lower in rats treated alone with DMH as compared to control group of animals. This may be due to the inhibition of its action by the DMH metabolites or decreased synthesis of GSH and glutathione dependent enzymes in the liver due to increased free radicals. GSH - an important non-protein thiol, plays a vital role in the detoxification of many environmental carcinogens and free radicals [33]. GR is a glutathione regenerating enzyme that permits the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) by the oxidation of NADH to NAD + [34]. GST and GPx are biotransformation enzymes involved in the detoxification of xenobiotics, carcinogens, free radicals and peroxides by conjugating these toxic substances with GSH, ultimately protecting cells and organs against carcinogen induced toxicity [35]. Since, the reactive ultimate carcinogenic form of DMH is electrophilic diazonium ions, these enzymes may an important role in its detoxification. A significant (p ≤ 0.05) increase in reduced glutathione level and other glutathione dependent enzyme activities observed in ACME supplemented groups (group IV and V) might be due to the reduced oxidative stress and possibly enhanced repair mechanisms in the liver.

CAT is a priority enzyme of the antioxidant system in defense for oxidative stress occurring in many pathological conditions including cancer. CAT deficiency may lead to accumulation of reactive oxygen metabolites and this may cause the initiation of carcinogenesis [36]. The observed decrease in hepatic CAT activity of DMH treated animals in the present study indicates that the liver is susceptible to oxidative damage during colon carcinogenesis. ACME supplementation significantly (p ≤ 0.05) aided to maintain the CAT activity near to normal level in hepatic tissue. It is evidently clear that one of the ways by which ACME exerts its chemopreventive effect is by modulating the activity of CAT in hepatic tissue.

Histopathological examination revealed the hepatic damage and appearance of pre - neoplastic lesions in nts treated alone with DMH (Fig. 2C). Liver sections of ACME supplemented rats showed the signs of recovery from DMH induced hepatotoxicity and carcinogenicity in a dose dependent manner.

CONCLUSION

The results of the present study evidently indicate that the administration of colon specific procarcinogen DMH brings about profound alterations in tissue lipid peroxidation and antioxidant status of liver. It is obvious that one of the mechanisms of chemoprevention of colon tumorgenesis by ACME may be the enhancement of antioxidant enzyme systems in the liver and thereby the metabolic disposal of carcinogenic DMH metabolites. The role of ACME as a free radical quencher and its role in conserving the pre-oxidant –antioxidant balance suggested ACME a promising agent for chemoprevention with a good candidate for chemoprevention. Preliminary phytochemical screening of ACME revealed the presence of phytochemical constituents such as alkaloids, tannins, glycosides, phenols, flavonoids, saponins and carbohydrates [37]. Published report also establishes the presence of phenolic acid, luteol, stigmastrol, β- sitosterol, glucose, galactose, rhamnose and xylose in its corn [38]. The chemopreventive and antioxidant effect of the extract might be attributed to the presence of the identified class of chemicals in single or in combination. Nevertheless, more efforts are still required for the isolation, characterization and biological evaluation of the active principle(s) of the methanolic extract of A. campanulatus tuber.

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