Effect of *Clerodendrum serratum* leaf extract on biochemical and oxidative stress parameters of testis in 7, 12-dimethylbenz[a]anthracene induced skin carcinogenesis in Swiss albino mice

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Abstract

The biochemical contents and antioxidant potential of *Clerodendrum serratum* (Verbenaceae) leaf extract (CSLE) on 7, 12-dimethylbenz[a]anthracene (DMBA) induced skin carcinogenicity in testis of mice was investigated. Group I received distilled water served as control. The skin lesions were induced by twice-weekly topical application of DMBA for 2 weeks on the shaved backs of group II, III, IV and V mice. CSLE was administered to group III, IV and V mice at the dose of 300, 600 and 900 mg/kg b.wt/day, for 4 week before DMBA application, and continued till 45 days. On 46th day the mice were sacrificed, testis were dissected out freed from adherent tissue and weighed to nearest milligram and evaluated the biochemical contents DNA, RNA, protein, glycogen, cholesterol, lactate dehydrogenase (LDH), Succinic dehydrogenase (SDH), acid phosphatase (ACP) and alkaline phosphatase (AKP) activities, oxidative stress parameters, levels of glutathione (GSH), thiobarbaturic acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT) and glutathione-s-transferase (GST). DMBA induced skin carcinogenesis decreased body and testis weight, DNA, RNA, protein, glycogen, GSH level, SDH, ACP, SOD, CAT and GST activities. But there was increase in cholesterol content, LDH, ACP activities and TBARS level. DMBA act via generating reactive oxygen species (ROS) as tumor initiator and free radicals inducing oxidative stress. The results revealed that there was a recovery in biochemical contents, dehydrogenases, phosphatases and oxidative stress parameters in testis. Thus, the present study inferred that CSLE administration significantly curtailed tumor development and counteracted all the biochemical effects. Many plant secondary metabolites exhibit potent anticancerigenic potential and known to exert their effects by quenching reactive oxygen, inhibiting lipid peroxidation.

Keywords: *Clerodendrum serratum*, DMBA, Biochemical parameters, Reactive oxygen species, Mice.

INTRODUCTION

Skin is a barrier or an outer layer of the body that protects humans from heat or cold, chemicals, bacteria, UV and other harmful radiations. Skin, a major environmental interface for the body, is accidentally or occupationally exposed to a number of chemical mutagens and carcinogens [1]. Skin cancer is the most common form of human cancer in which cancer cells are found in the outer layers of the skin and its incidence is increasing rapidly all over the world. Basal cell carcinoma accounts for 80% whereas squamous cell carcinoma and melanomas accounts for 16% and 4% respectively of all skin cancers [2]. Squamous cell carcinoma is the most serious form of cancer than other skin cancers since they can spread into vital organs inside the body [3]. In India, skin cancer accounts for approximately 1-2% of all diagnosed cancers and the annual incidence of skin cancer will increase significantly in future due to its immense population [4].

7, 12-dimethylbenz[a]anthracene (DMBA), the organ specific potent carcinogen, is commonly used to induce skin cancer in Swiss albino mice. DMBA could either be used as an initiator or promoter for inducing skin carcinogenesis. Twice per week for two week topical application of DMBA induced skin carcinogenesis in Swiss albino mice [5]. DMBA induced skin cancer is therefore used as an ideal tool to test the antioxidant potential of medicinal plants and its constituents. Enzymatic activation of Pol aromatic hydrocarbons leads to the generation of active oxygen species such as peroxides and superoxide anion radicals, which induce oxidative stress in the form of lipid peroxidation [6,7]. Consequences of the damage initiated by these metabolic by products affect reproductive organ testis by large range of biological reactions, like increases in mutation rate, alteration of cellular membrane composition, structural proteins, metabolic, detoxifying enzymes and cellular signaling proteins [8].

All over the world, studies on plant materials have revealed their health promoting action including cancer prevention. The *Clerodendrum serratum*, Linn (Family: Verbenaceae) commonly known as “Bhangi” in the ayurvedic medicine of Indian system. The genus *Clerodendrum* L. is very widely distributed in tropical and subtropical regions of the world. Plants remain a large chemical library to be explored for new agents. It has been reported that, plant-derived terpenoids have attracted reasonable attention for their unique antineoplastic activity [9]. The major chemical components reported from the genus *Clerodendrum* are phenolics, steroids, di-terpenes and triterpenes, flavonoids, volatile oils, etc.
Carcinogen

MATERIALS AND METHODS

Carcinogen

The carcinogen chemical 7, 12-dimethylbenz[a]anthracene (DMBA) was procured from Sigma Chemicals Co., St. Louis, USA. DMBA is 95% potent carcinogen, with molecular formula C_{20}H_{16} and molecular weight 256.3.

Plant extract preparation

The leaves of Clerodendrum serratum were collected from Botanical garden of Karnataka University Dharwad. The plant was authenticated in P. G. Department of Botany, Karnataka University Dharwad. Methanolic leaf extract of Clerodendrum serratum was extracted by the Soxhlet apparatus by continuous cycle collection of the extract. The leaves of the plant were washed and dried at room temperature and crushed by the mechanical grinder to fine powder. The powder (500 gm) was then extracted with 2.5 litre of 90% methanol in a Soxhlet apparatus at 65°C, until the powder became exhausted totally. The resulting extract was filtered, concentrated, and dried in vacuo (yield 8.75% w/w). The extract was stored in a desiccator for administration orally to mice in three increasing graded dose.

Animals

Laboratory bred adult male Swiss albino mice were used in the experiments. Mice aged 90 days old weighing between 25-30 g was used. The study was approved by the Ethical Committee, Dept. of Zoology, Karnataka University, Dharwad, India; CPCSEA (639/02/a/CPCSEA) guidelines were followed for maintenance and use of experimental animals. They were housed in separate polypropylene cages containing sterile paddy husk as bedding material. The mice were provided with standard mice pellet diet and water ad libitum. The mice were maintained under normal day/night schedule (12 L: 12 D) at room temperature 25 ± 2°C.

Treatment

The chemical carcinogen, 7, 12-dimethylbenz[a]anthracene (DMBA) induced skin tumorigenesis in male Swiss albino mice [11]. It was applied topically on the dorsal skin surface of the mice, at a dose of 25 µl DMBA in 25 µl acetone (1:1v/v) per mouse twice a week for two weeks to respective groups with a suitable art brush. Methanolic leaf extract of Clerodendrum serratum was dissolved in physiological saline and administered orally in the graded dose of 300, 600 and 900 mg/kg b.wt/day for four weeks before topical application of DMBA on skin to respective groups and continued for two weeks while inducing to respective groups. After 45 days mice were sacrificed and the skin and testis was dissected out and stored in saline. The experiment was designed to determine the preventive effect of methanolic leaf extract of Clerodendrum serratum on 7, 12 dimethylbenz[a]anthracene (DMBA) induced skin carcinogenesis on body and testis weight, biochemical contents and oxidative stress parameters of the testis in albino mice.

Biochemical Studies

The biochemical contents such as DNA and RNA carried out as per the method described by [12], protein by [13], glycogen by [14], cholesterol by [15], activities of enzymes such as LDH by [16], SDH by [17], ACP and AKP by [18].

Oxidative stress parameters

The oxidative stress parameters such as GSH level was measured following the method of [19], the product of the reaction between malondialdehyde (MDA) and thiobarbaturic acid reactive substances (TBARS) by [20] were measured by a modified method of Esterbauer and Cheesman, (1990), SOD activity by [21], CAT activity by [22] and GST activity by [23].

Extraction of plasma membrane surface proteins and SDS-PAGE Electrophoresis

The extraction of surface proteins from plasma membrane of mice skin epidermis was carried with 3M KCl according to the procedure of [24]. The skin epidermis was removed at the time of autopsy, minced and washed with 5M phosphate buffered saline (PBS), pH 7.2 by centrifugation at 10,000 rpm for 10 min twice in order to remove the soluble extracts and intracellular components. SDS-PAGE was performed according to [25], to identify the tumor associated proteins with a medium range marker. Silver staining was performed to distinguish the bands as it was not clear with the commassie brilliant blue stain.

Statistical analysis

Statistical significance between the control and experimental data were subjected to analysis of variance (ANOVA) together with Dunnett’s test (P<0.05).

RESULTS

Body and organ weight

Change in the body and testis weight revealed that there was a significant decrease in the body and testis weight of DMBA treated control mice. However, there was increase in the body and testis weight of mice treated with DMBA along with higher dose 900 mg/kg b.wt/day of plant extract showing its recovery (Table 1).

Biochemical studies

Biochemical contents and the enzyme activities revealed that there was a significant decrease in the level of DNA, RNA, protein, glycogen, SDH and ACP activities in the testis of DMBA treated control mice. However, there was increase in the level of the nucleic acids, protein, glycogen, SDH and ACP activities in the mice treated with DMBA along with higher dose 900 mg/kg b.wt/day of plant extract showing its recovery. But there was a increase in the level of the cholesterol, LDH and AKP activities in the mice treated with DMBA along with higher doses of plant extract showing its recovery (Table 2 and 3).
Oxidative stress parameters

There was a decrease in the level of GSH, CAT, SOD and GST activity in the testis of DMBA treated control mice. Further, there was an increase in the level of GSH, CAT, SOD and GST activity in the mice treated with DMBA along with higher doses of plant extract showing its recovery. However, there was an increase in the level of TBARS of DMBA treated control mice and there was a decrease in the level of TBARS in the mice treated with DMBA along with higher doses of plant extract showing its recovery (Table 4).

Tumor associated proteins

The results of SDS PAGE of surface proteins in skin epidermis of mice showed the expression of the bands having molecular weights of 27 and 54 kDa indicating the presence of tumor associated proteins in DMBA treated control mice. However, there was expression of tumor associated proteins in DMBA along with plant extract treated mice (Fig. 1).

Table 1. Effect of Clerodendrum serratum leaf extract (CSLE) on body and testis weight of mice on treatment with 7, 12-Dimethylbenz[a]anthracene (DMBA)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment (mg/kg/d)</th>
<th>Change in body weight (g)</th>
<th>Relative testis weight /100 g body weight (Mean ± S.E.) (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal Control</td>
<td>2.14±0.32</td>
<td>0.36±0.14</td>
</tr>
<tr>
<td>II</td>
<td>DMBA Control (25µl)</td>
<td>-2.43±0.42*</td>
<td>0.21±0.23*</td>
</tr>
<tr>
<td>III</td>
<td>DMBA+CSLE (300)</td>
<td>0.62±0.38</td>
<td>0.26±0.15</td>
</tr>
<tr>
<td>IV</td>
<td>DMBA+CSLE (600)</td>
<td>1.12±0.25*</td>
<td>0.32±0.26</td>
</tr>
<tr>
<td>V</td>
<td>DMBA+CSLE (900)</td>
<td>2.52±0.28*</td>
<td>0.40±0.18*</td>
</tr>
</tbody>
</table>

Values are mean± SEM of 10 animals * Significant P ≤ 0.05 vs Control
Table 2. Effect of *Clerodendrum serratum* leaf extract (CSLE) on biochemical contents in the testis of mice on treatment with 7, 12-Dimethylbenz[a]anthracene (DMBA)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment (mg/kg/d)</th>
<th>DNA (µg / mg wet weight of tissue)</th>
<th>RNA (µg / mg wet weight of tissue)</th>
<th>Protein (µg / mg wet weight of tissue)</th>
<th>Glycogen (µg / mg wet weight of tissue)</th>
<th>Cholesterol (µg / mg wet weight of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal Control</td>
<td>2.68±0.05</td>
<td>4.84±0.03</td>
<td>158.03±5.08</td>
<td>6.98±0.03</td>
<td>8.98±0.12</td>
</tr>
<tr>
<td>II</td>
<td>DMBA Control (25µl)</td>
<td>2.05±0.02*</td>
<td>3.34±0.40*</td>
<td>123.23±6.02*</td>
<td>4.53±0.07*</td>
<td>9.84±0.62*</td>
</tr>
<tr>
<td>III</td>
<td>DMBA+CSLE (300)</td>
<td>2.12±0.08</td>
<td>3.60±0.67</td>
<td>133.34±6.15</td>
<td>4.86±0.32</td>
<td>9.62±0.45</td>
</tr>
<tr>
<td>IV</td>
<td>DMBA+CSLE (600)</td>
<td>2.28±0.34</td>
<td>4.34±0.83</td>
<td>146.48±7.18*</td>
<td>5.82±0.23*</td>
<td>9.43±0.62*</td>
</tr>
<tr>
<td>V</td>
<td>DMBA+CSLE (900)</td>
<td>2.52±0.23*</td>
<td>4.95±0.79*</td>
<td>161.58±6.24*</td>
<td>6.61±0.26*</td>
<td>9.23±0.87*</td>
</tr>
</tbody>
</table>

Values are mean± SEM of 10 animals * Significant P ≤ 0.05 vs Control

Table 3. Effect of *Clerodendrum serratum* leaf extract (CSLE) on dehydrogenases and phosphatases enzyme activities in the testis of mice on treatment with 7, 12-Dimethylbenz[a]anthracene (DMBA)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment (mg/kg/d)</th>
<th>Enzyme activity (µµ µµ moles/ min/ g tissue)</th>
<th>LDHa</th>
<th>SDHb</th>
<th>ACPc</th>
<th>AKPd</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal Control</td>
<td></td>
<td>9.28±0.19</td>
<td>12.42±0.18</td>
<td>18.46±0.18</td>
<td>14.32±0.24</td>
</tr>
<tr>
<td>II</td>
<td>DMBA Control (25µl)</td>
<td></td>
<td>11.05±0.27*</td>
<td>11.15±0.05*</td>
<td>16.15±0.38*</td>
<td>16.10±0.84*</td>
</tr>
<tr>
<td>III</td>
<td>DMBA+CSLE (300)</td>
<td></td>
<td>10.60±0.45</td>
<td>11.35±0.48</td>
<td>16.52±0.17</td>
<td>15.72±0.45</td>
</tr>
<tr>
<td>IV</td>
<td>DMBA+CSLE (600)</td>
<td></td>
<td>10.04±0.82</td>
<td>11.92±0.63</td>
<td>17.32±0.27*</td>
<td>15.12±0.56</td>
</tr>
<tr>
<td>V</td>
<td>DMBA+CSLE (900)</td>
<td></td>
<td>9.45±0.64*</td>
<td>12.32±0.53*</td>
<td>18.02±0.36*</td>
<td>14.62±0.62*</td>
</tr>
</tbody>
</table>

a µmoles of pyruvate formed/min/ g tissue  b µmoles of formazon formed/min/ g tissue

c µmoles of inorganic phosphorus formed/min/ g tissue  d µmoles of p-nitrophenyl formed/min/ g tissue

Values are mean± SEM of 10 animals * Significant P ≤ 0.05 vs Control

Table 4. Effect of *Clerodendrum serratum* leaf extract (CSLE) on oxidative stress parameters in the testis of mice on treatment with 7, 12-Dimethylbenz[a]anthracene (DMBA)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment (mg/kg/d)</th>
<th>Antioxidant (µmole/mg protein)</th>
<th>Oxidative stress byproduct (nmoles thiobarbaturic acid/gm protein)</th>
<th>Catalase (µmole H₂O₂/min/mg protein)</th>
<th>SOD (unit/mg protein)</th>
<th>GST (µmole/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal Control</td>
<td>9.27±0.08</td>
<td>0.23±0.03</td>
<td>148.60±2.18</td>
<td>45.36±2.74</td>
<td>2.91±0.08</td>
</tr>
<tr>
<td>II</td>
<td>DMBA Control (25µl)</td>
<td>6.63±0.09*</td>
<td>0.47±0.01*</td>
<td>126.34±2.84*</td>
<td>32.81±2.68*</td>
<td>1.23±0.06*</td>
</tr>
<tr>
<td>III</td>
<td>DMBA+CSLE (300)</td>
<td>7.23±0.07</td>
<td>0.34±0.02*</td>
<td>132.47±2.68</td>
<td>36.32±2.88</td>
<td>1.83±0.07</td>
</tr>
<tr>
<td>IV</td>
<td>DMBA+CSLE (600)</td>
<td>8.10±0.08*</td>
<td>0.26±0.03*</td>
<td>140.53±2.46*</td>
<td>42.38±2.28*</td>
<td>2.20±0.08</td>
</tr>
<tr>
<td>V</td>
<td>DMBA+CSLE (900)</td>
<td>8.95±0.08*</td>
<td>0.22±0.02*</td>
<td>146.40±2.54*</td>
<td>48.26±2.36*</td>
<td>2.46±0.09*</td>
</tr>
</tbody>
</table>

a µmole of glutathione (GSH)/ mg protein  b nmoles thiobarbaturic acid (TBARS)/gm protein
c µmole of H₂O₂  d super oxide dismutase (SOD) unit/mg protein
e Glutathione-s-transferase (GST) µmole/min/mg protein

Values are mean± SEM of 10 animals * Significant P ≤ 0.05 vs Control

**DISCUSSION**

Free radicals are “any species capable of independent existence that contain one or more unpaired electrons” [26]. Because of their very high chemical reactivity, free radicals are able to induce cellular damage in a variety of ways [27]. The most deleterious effects of free radicals are damage to DNA [28], which is associated with the process of carcinogenesis. This deleterious effect of free radicals can also be reduced by the natural or synthetic antioxidants. Antioxidants can terminate the free radicals chain reaction by...
donating hydrogen or electrons to free radicals and converting them to more stable products. The present study shows that the Clerodendrum serratum leaf extract contains secondary metabolites which include antioxidants which are implicated in lowering the lipid peroxidation and the risk of cancer.

The decrease in the body weight on treatment with DMBA may be due to suppression towards food and water intake. Similar results are reported on the body weight in the conditions of DMBA induced squamous cell carcinoma of skin [29]. There has been considerable scientific evidence, epidemiologic and experimental, accumulated in the past two decades indicating that modifications in lifestyle, including diet, can have a major effect on the risk for numerous cancers [30]. Further, the increase in the body weight in the DMBA treated along with Clerodendrum serratum leaf extract reveal that, cancer prevention involves pharmacological intervention with synthetic or naturally occurring chemicals or substances to prevent or inhibit or reverse the process of carcinogenesis or prevent the development of invasive cancer [31].

There is a decrease in the testis weight in DMBA induced mice and further the increase in weight shows the recovery in the plant extract treated group. Similar results are reported on the testis weight in the conditions of DMBA induced squamous cell carcinoma of skin [32]. As the testis is highly susceptible to damages caused by genetic disorders, environmental or occupational exposure to chemicals or by other means. Quality of sperm production has been adversely affected due to the exposure to UV rays and chemicals, particularly mutagens and carcinogens [33]. The decrease in the testis weight on treatment with DMBA may be due to the release of free radicals which may affect the production of sperms as there is DNA damage in the germ cells. Recovery in the weight is due to the antioxidants of the plant extract which scavenge the free radicals and lowers the lipid peroxidation.

The decrease in the biochemical contents of the testis in DMBA induced mice and further the increase in biochemical contents shows the recovery in the DMBA along with plant extract treated group. Similar results are reported on the testis weight in the conditions of DMBA induced squamous cell carcinoma of skin [32]. The decrease in the testis weight on treatment with DMBA may be due to the release of free radicals which may affect the production of sperms as there is DNA damage in the germ cells. Recovery in the weight is due to the antioxidants of the plant extract which scavenge the free radicals and lowers the lipid peroxidation.

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CONCLUSION

The present study conducted in a mouse skin carcinogenesis model clearly reveal that the methanolic leaf extract of Clerodendrum serratum major components flavonoids and phenolics can effectively reduce the incidence and multiplicity of skin papillomas, a precancer condition which precedes development of carcinomas. The recovery in the plant extract treated mice groups is due to their antilipidperoxidative and antioxidant functions during papilloma genesis [56,57]. Further this study demonstrates that the functional action of CSLE and its components is mediated by varied pathways, which includes activation of detoxification and prevention of cellular damage, inhibition of cell proliferation and induction of apoptosis. Similar observations are in accordance with a rat colon carcinogenesis model [58]. In view of this study it is suggested CSLE has a potential antioxidants for prevention of diseases involving oxidative damages, including cancer. The secondary metabolites isolation studies are therefore, required to accurately establish the cancer preventive metabolite of CSLE.

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