OBJECTIVE: The objective of this study was to evaluate the effects of ethyl acetate extract of Mucuna cochinchinensis seeds (EMC) on 7,12-Dimethylbenzanthracene induced mammary carcinoma in Sprague–Dawley rats and to understand the expression of B-cell lymphoma-2 (Bcl-2) an anti-apoptotic protein in experimental animals tissues.

METHODS: The antitumor activity was accessed by the effects of EMC on tumor weight, body weight, enzymic, and non-enzymic antioxidant parameters such as thiobarbituric acid reactive substances in plasma, superoxide dismutase, glutathione peroxidase, reduced glutathione (GSH), catalase, vitamin C and vitamin E in breast tissues and serum. The extract was administered orally at three different doses of low (200 mg/kg), high (400 mg/kg), and low dose with the standard. Western blot analysis was carried out for understanding Bcl-2 expressions. EMC was analyzed by high-performance liquid chromatography (HPLC)/electron spray ionization (ESI)-mass spectrometry (MS) to comprehend the active constituents.

RESULTS: Significant (p<0.01) reduction in tumor weight and gain in body weight were observed with EMC treatment. Enzymic and non-enzymic antioxidant levels in serum as well as in breast tissues were revert back significantly (p<0.01) on EMC treatment. Overexpression of Bcl-2 was observed in tumor control and intensities were reduced in the treatment group. HPLC/ESI-MS revealed the presence of genistein and daidzein.

CONCLUSION: The EMC seeds shown anticancer activity in tested animal model.

KEYWORDS: Mucuna cochinchinensis, Isoflavones, 7,12-Dimethyl benzanthracene, Mammary carcinoma, Liquid chromatography-mass spectrometry.
the experimental period. The animals received a balanced diet of commercially available pellet rat feed and water ad-libitum. EMC and tamoxifen dissolved in corn oil and administered intragastrically (Approval - IAEC/RMCP/2013/93).

Tumor induction

Mammary tumor was induced by a single dose of 20 mg of 7,12-Dimethyl benzanthracene (DMBA) dissolved in corn oil (1 ml) given through an oral gavage [16]. All the experimental animals were sacrificed after 90 days.

Experimental design

A total of 36 female Sprague–Dawley rats were divided into six groups of six rats each. Group I received normal saline (10 ml/kg body weight), Group II tumor control, Group III received standard tamoxifen (10 mg/kg body weight), Group IV received EMC (200 mg/kg body weight) and tamoxifen (10 mg/kg body weight), Group V and Group VI received EMC 200 and 400 mg/kg body weight, respectively. At the end of the experimental period, all the rats were alive and were anesthetized with diethyl ether and sacrificed by euthanasia. Animals were starved overnight before sacrifice. Blood was collected and the serum was separated by centrifugation. Breast was dissected out and washed with ice-cold 0.9% NaCl solution. The resultant solid tumor was considered to be prelate ellipsoid with one long axis and two short axes. The two short axes were measured with a vernier caliper. The tumor weight [17] was calculated using the following formula:

\[ \text{Weight (g)} = \frac{\text{Length (cm)} \times \text{Width (cm)}}{2} \]

Tissues (100 mg) were homogenized in 0.1 M Tris–HCl buffer (pH 7.4). The homogenate was used for the determination of various antioxidant biochemical parameters.

Biochemical analysis

The superoxide dismutase (SOD) activity was measured at absorbance 420 nm using a spectrophotometer as the degree of inhibition of autoxidation of pyrogallol in an alkaline pH according to the method of Marklund and Marklund [18]. The catalase (CAT) activity was assayed by the method of Sinha [19]. The activity of glutathione peroxidase (GPx) was assayed by the method of Rotruck et al. [20]. Reduced glutathione (GSH) was determined by the method of Moron et al. [21]. Vitamin E (α-tocopherol) levels were estimated by the method of Desai [22]. Vitamin C (ascorbic acid) was measured by the method of Omaye et al. [23]. Lipid peroxidation was estimated by the method of Högberg et al. [24].

Western blot analysis

Tumors were disaggregated by treatment with an enzymic mixture containing 2.0 g/l collagenase, 0.5 g/l proteases, and 2.0 g/l DNase for 90 minutes at 37°C. The resulting cell suspensions were filtered through a 30 μm nylon mesh. Centrifuged cells were washed in phosphate-buffered saline and boiled in Laemmli lysis buffer for 5 minutes. Breast tissue proteins (50 μg/lane) were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. The membrane was probed with B-cell lymphoma-2 (Bcl-2) specific antibody (1:2000 dilution) to determine their levels, and the intensities were measured using ImageJ/Fiji 1.46. β-actin was used as an internal control [25].

Statistical analysis

The treated groups were compared with the toxicant control groups. All results were expressed as a mean±standard error of the mean of six animals in each group. The results were analyzed statistically using one-way ANOVA followed by Newman-Keuls multiple comparison methods to compare the mean value of different groups using Graph pad 3.1 version, p<0.05 was considered significant.

RESULTS

EMC screened for phytoconstituent analysis revealed the presence of alkaloids, phenols, flavonoids, amino acids, quinones, steroids, and carbohydrate in various color reactions. The characterization of the compounds using UHPLC-ESI MS/MS analysis were done in negative ion mode. To confirm the nature of the flavonoid peaks observed in the EMC, MS/MS experiments were carried out to obtain the characteristic fragments of each peak. The data of typical MS/MS spectra of each type are shown in Fig. 1. Common features such as CO and CO₂ losses for the flavonoids were observed as a result of the contraction of ring C. Diagnostic fragments were observed for each type of the flavonoids. For example, significant Retro-Diels-Alder fragments such as Aion were obtained for isoflavones. For the isoflavones, Genistein and Daidzein, the ion loss of C₆O₂ was observed. This was attributed to the Diels-Alder fragmentation reaction between rings A and C [26]. Further the strong intense peak at 241, 225, 197, 181 m/z confirmed the structure of genistein, while 209, 181, 135 confirmed the presence of daidzein.

Table 1 presents the body weight, tumor weight and thiobarbituric acid-reactant substances of control and experimental animals. The body weight was found to be significantly decreased in Group II tumor-induced animals when compared with control animals (p<0.01). Conversely, the administration of EMC increased the body weight in Group III to VI when compared to Group II animals (p<0.01). The Groups III to IV animals exhibited significant (p<0.01) reduction of tumor weight when compared to Group II. The Groups V to VI animals exhibited significant (p<0.05) reduction of tumor weight when compared to Group II. Administration of EMC decreased the tumor weight significantly (p<0.05).

Activities of enzymic and non-enzymic antioxidants in breast homogenate and serum of control and experimental animals are presented in Tables 2 and 3, respectively. Group II cancer-bearing animals showed a significant reduction in both enzymic and non-enzymic antioxidant levels (p<0.01) when compared to control animals. Administration of tamoxifen and EMC in Groups III to VI animals significantly (p<0.01) increased the antioxidant levels when compared to Group II animals. Striking results were observed for body
Table 2 : Effect of EMC on enzyme and non-enzyme antioxidants in breast homogenate for control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/min/mg protein)</th>
<th>CAT (μM H₂O₂ consumed/ min/mg protein)</th>
<th>GPx (μmol/min/mg protein)</th>
<th>GSH (μg/mg protein)</th>
<th>Vitamin C (μg/mg protein)</th>
<th>Vitamin E (μg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>16.35±1.70</td>
<td>69.15±1.88</td>
<td>12.85±0.80</td>
<td>13.12±0.96</td>
<td>3.60±0.35</td>
<td>5.32±0.53</td>
</tr>
<tr>
<td>II</td>
<td>7.79±0.65</td>
<td>43.60±1.32</td>
<td>6.92±0.65</td>
<td>6.28±0.71</td>
<td>1.58±0.20</td>
<td>3.42±0.26</td>
</tr>
<tr>
<td>III</td>
<td>15.20±0.86</td>
<td>68.64±1.74</td>
<td>12.60±0.72</td>
<td>12.88±0.80</td>
<td>3.36±0.30</td>
<td>5.18±0.46</td>
</tr>
<tr>
<td>IV</td>
<td>16.66±0.92</td>
<td>68.75±1.76</td>
<td>12.71±0.76</td>
<td>12.96±0.86</td>
<td>3.44±0.32</td>
<td>5.24±0.48</td>
</tr>
<tr>
<td>V</td>
<td>15.15±0.80</td>
<td>67.95±1.48</td>
<td>11.95±0.68</td>
<td>12.12±0.66</td>
<td>3.15±0.24</td>
<td>4.96±0.36</td>
</tr>
<tr>
<td>VI</td>
<td>15.48±0.88</td>
<td>68.18±1.56</td>
<td>12.42±0.74</td>
<td>12.62±0.78</td>
<td>3.30±0.28</td>
<td>5.05±0.38</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. All values were found out by using one-way ANOVA followed by Newman-Kuels multiple range tests. *Values are significantly different from normal control at p < 0.01. Values are significantly different from breast cancer control at p < 0.01. EMC: Ethyl acetate extract of Mucuna cochinchinensis; SEM: Standard error of mean; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GSH: Reduced glutathione.

Table 3: Effect of EMC on enzyme and non-enzyme antioxidants in serum for control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/min/mg protein)</th>
<th>CAT (μM H₂O₂ consumed/ min/mg protein)</th>
<th>GPx (μmol/min/mg protein)</th>
<th>GSH (μg/mg protein)</th>
<th>Vitamin C (μg/mg protein)</th>
<th>Vitamin E (μg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>12.11±1.40</td>
<td>6.32±3.35</td>
<td>9.6±1.20</td>
<td>9.02±1.08</td>
<td>2.95±0.08</td>
<td>5.08±0.48</td>
</tr>
<tr>
<td>II</td>
<td>9.05±0.92</td>
<td>34.60±0.25</td>
<td>6.86±0.88</td>
<td>7.22±0.72</td>
<td>1.58±0.06</td>
<td>3.36±0.30</td>
</tr>
<tr>
<td>III</td>
<td>11.76±1.80</td>
<td>57.45±1.32</td>
<td>9.12±1.02</td>
<td>10.18±0.92</td>
<td>2.68±0.07</td>
<td>4.48±0.36</td>
</tr>
<tr>
<td>IV</td>
<td>11.89±1.18</td>
<td>59.20±2.30</td>
<td>9.18±1.08</td>
<td>10.44±0.98</td>
<td>2.79±0.08</td>
<td>5.44±0.40</td>
</tr>
<tr>
<td>V</td>
<td>11.26±0.15</td>
<td>51.66±2.96</td>
<td>8.44±0.96</td>
<td>9.30±0.82</td>
<td>2.26±0.06</td>
<td>4.16±0.31</td>
</tr>
<tr>
<td>VI</td>
<td>11.56±1.14</td>
<td>54.43±0.85</td>
<td>8.92±1.00</td>
<td>9.86±0.94</td>
<td>2.49±0.07</td>
<td>5.32±0.32</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. All values were found out by using one-way ANOVA followed by Newman-Kuels multiple range tests. *Values are significantly different from normal control at p < 0.01. Values are significantly different from breast cancer control at p < 0.01. EMC: Ethyl acetate extract of Mucuna cochinchinensis; SEM: Standard error of mean; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GSH: Reduced glutathione.

weight, enzymic and non-enzymic parameters for Group IV, by which the combined efficacy of standard and EMC at low dose is understood.

To assess the expression of Bcl-2 in breast tissues of experimental animals, SDS-PAGE and Western blot analysis were performed. Bcl-2 proteins were expressed in all the experimental animals and expressed in (Fig. 2). Intensities observed of treated and normal groups were significantly different from Group II. Group IV intensity demonstrated an excellent silencing of Bcl-2 expression in combined treatment pattern of standard and extract even with EMC treated alone. Genistein and Daidzein present in the extract are very much responsible for downregulating the bcl-2 gene.

DISCUSSION

Recent studies indicated that the use of HPLC and coupled techniques, such as ESI or diode array detector and mass spectra, is a powerful approach for the rapid identification of the constituents in medicinal plants and their preparations [27]. In this study for the qualitative analysis, acetonitrile, aqueous acetic acid was used as the suitable eluting solvent for the analysis of flavonoids in (EMC). The compounds were confirmed by small molecular databases and further fragmentation pattern was analyzed through previous studies.

The present study indicates that administration of plant extract resulted in substantial inhibition of breast tumor incidence or decrease in the initiation of tumor genesis and increase in body weight of animals treated with EMC at a dose of 200 mg/kg and 400 mg/kg body weight.

Toxic manifestation of DMBA is associated with its oxidative metabolism leading to the formation of reactive metabolites (epoxides and quinones) capable of generating free radicals. Metabolism of DMBA by the mixed function oxidases system often results in the formation of oxyradicals which bind covalently to nucleophillic sites on cellular macromolecules thereby eliciting cancerous responses [28]. The generation of reactive oxygen species (ROS) and the peroxidation of membrane lipids are well associated with the initiation of carcinogenesis affecting the normal biochemical process, which further leads to the reduction of body weight [29]. Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidant defense system that quench or scavenge them and thereby protect the body against pathogenesis [30]. It is evident from the results that increased level of lipid peroxides (LPO) was found in cancer-bearing animals when compared to control group. On the contrary, reduced level of LPO was observed in tamoxifen and EMC treated animals at both doses indicating that it is a good free radical scavenger. SOD and CAT act mutually supportive antioxidative enzymes, which provide protective defense against ROS [31]. The present study reveals that SOD levels are decreased in the cancer-bearing animal, which may be due to altered antioxidant status caused by carcinogenesis. The present study also shows that increased level of CAT observed in Group II cancer-bearing animals may be due to the utilization of antioxidant enzymes in the removal of H₂O₂ by DMBA.

GPx is an important defense enzyme against oxidative damage and this, in turn, requires GHS as a cofactor. GPx catalyses the oxidation of GSH using H₂O₂ [32]. Our findings agree well with this observation and also the activity of GPx significantly decreases in cancer-bearing animals.

The non-enzymic antioxidant systems are the second line of defense against free radical damage. GSH is an important non-protein cellular thiol that in conjunction with GPx plays a regulatory role in cell proliferation [33]. We observed decreased activity of GSH in cancer-bearing animals. The EMC increased the GSH levels, which clearly suggest their antioxidant property. Decreased levels of water soluble antioxidants found in cancer-bearing animal may be due to the utilization of antioxidants to scavenge the free radicals.

Vitamin E is a potent oxygen radical scavenger that protects cell membranes from oxidative damage initiated by carcinogens [34]. The present study found decreased levels of vitamin E in the cancer condition which may be due to excessive production of ROS by cancer cells. The free radical clearing capacity of vitamin E is due to the localization of an unpaired electron on its conjugated double bond. Thus, it is suggested that the occurrence of phytochemicals such as daidzein and genistein in EMC significantly attenuated the LPO activity and resulted in increased antioxidant levels.

The apoptotic signal transduction pathway commonly induced by anticancer agents is associated with the induction of Bax and cleaved PARP and the downregulation of Bcl-2 and pAkt. Bcl-2 expression is
Fig. 2: Expression of B-cell lymphoma-2 (Bcl-2) in experimental animal breast tissues. (a) Western blot analysis of Bcl-2 expression. (b) Relative intensities of expressed Bcl-2 in different groups

regulated by the ER-responsive element of the promoter region of the bcl-2 gene [35], such that overexpression of Bcl-2 might be expected to confer greater drug resistance on ER-positive breast cancer cells. Some reports suggest that Bcl-XL, but not Bcl-2, is capable of modulating apoptosis induced by tumor necrosis factor related apoptosis ligand. Usually, the downregulation of Bcl-2 expression by AS enhances drug sensitivity by modulating the apoptotic signal transduction pathway of Bcl-2 [36]. However, Bcl-2 inhibits Bid-induced apoptosis at the mitochondrial level by blocking cytochrome c release, whereas Bcl-XL does not affect the insertion of Bid into mitochondrial membranes [37]. So in this study, Bcl-2 is concentrated rather than any other member of the same family. Overexpression of Bcl-2 is observed more frequently than overexpression of Bcl-XL (70% vs. 40%) in breast cancer tissue, which suggests a more important role for Bcl-2 in conferring drug resistance. In our results greater suppression of Bcl-2 was achieved.

CONCLUSION

The biochemical alterations observed in cancer-bearing animals in the present study may be due to the induction of LPO and reduction of antioxidant level following carcinogen administration. However, administration of EMC significantly reversed the alteration to near normal level in cancer-bearing animals. From the results, it can be inferred that EMC positively modulated the antioxidant activity by quenching and detoxifying the free radicals induced by DMBA. The attenuation of DMBA induced oxidative stress by the plant extract could be attributed to the antioxidants activity of isoflavones (genistein and daidzein) present in the Mucuna, which is known to quench the free radicals by maintaining antioxidants levels and induce apoptosis also by downregulating antiapoptotic protein Bcl-2. Considering these properties of Mucuna, the extract can be supplemented with anticancer medicines.

REFERENCES


