Chemomodulatory potential of *Glycine max* against murine skin and cervical papillomagenesis

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In the present study, chemopreventive potential of *Glycine max* (*G. Max*) seeds was examined against DMBA-induced skin and MCA-induced cervical papillomagenesis in Swiss albino mice. Different doses (2.5, 5, and 7.5% w/w) of *G. max* were provided to animals in feed. Results exhibited a significant reduction in skin as well as cervical tumor incidence and tumor multiplicity (up to 75%) at all doses of test diet as compared to the control. Relatively, 7.5% test diet was most effective in protecting the animals against carcinogenesis. Further, detoxifying enzymes and antioxidative status was also evaluated in the liver of mice to understand the role of *G. max* in prevention of cancer. It was observed that the test diet containing *G. max* significantly elevated the specific activities of glutathione-S-transferase (GST), DT-diaphorase (DTD), superoxide dismutase (SOD), catalase (CAT), and glyoxalase I (Gly I). The test diet also elevated the content of reduced glutathione whereas it decreased the level of the peroxidative damage along with the specific activity of lactate dehydrogenase. It appeared that *G. max* seeds provided chemoprevention against skin and cervical papillomagenesis probably by modulating the detoxifying and antioxidative enzymes. It could be inferred that intake of *G. max* might help in reducing the risk of cancer.

**Keywords:** Biotransformation enzyme system, Chemoprevention, *Glycine max*, Papillomagenesis

Over the years, man has acquired extensive knowledge regarding the utilization of plants around him as food for maintenance of his health. *Glycine max* (soybean) is one of the most important crops for human and animal consumption, belonging to the family Fabaceae. It is a staple food in China and Japan. *G. max* is mainly cultivated for its seeds or grains, used commercially as human food, livestock feed and for extraction of edible oil. Soybean is a sweet, cooling and slightly bitter herb used in Chinese medicine for a variety of ailments. It has sedative, anti-spasmodic, diaphoretic (causes sweating) and anti-pyretic properties, with hormonal balancing effects and has great benefit to the liver and circulation. It contains phospholipids, soy protein, protease inhibitors, isoflavonoids and triterpene saponins. Soybean is a legume that contains no cholesterol and is low in saturated fat. It is also a good source of fiber, iron, calcium, zinc, and vitamin B. Commonly consumed Asian soy products include soymilk, tofu, miso, yuba, and tempeh. Extensive epidemiological studies have shown that consumption of soybean and soya products reduces the risk of osteoporosis, and cardiovascular diseases as well as several types of human cancer, including breast, prostate, and colon cancer.

Isoflavones, saponins, protease inhibitors and inositol are components of the soy seeds which have anticancer properties. Soybean is a rich and relatively important source of isoflavones. Soybean is known to contain the five isoflavone glycosides namely, genistein, daidzin, glycine 7-O-β-D-glucoside (glycitin), 6'-O-acetylgenistin, and 6'-O-acetyldaidzin and their corresponding aglycons such as daidzein, glycine, and genistein. Isoflavonoids possess numerous biological activities that may support chemoprevention through the promotion of apoptosis in diseased cells. Since soybean is widely consumed world-wide, it can be relevant to confirm its chemopreventive potential using various models of carcinogenesis. Available information suggests that chemopreventive action of soybean has not been tested against DMBA-induced skin and MCA-induced cervical papillomagenesis.

Therefore, in the present study, the chemopreventive efficacy of *G. max* has been evaluated against 7,12 dimethylbenz(a)anthracene (DMBA)-induced skin papillomagenesis and methylcholanthrene (MCA)-
induced cervical papillomagenesis in Swiss albino mice. Since, chemopreventive agents are expected to have antioxidant properties, an attempt has been made to study the modulatory effect of *G. max* on specific activities of glutathione-S-transferase (GST), DT-diaphorase (DTD), superoxide dismutase (SOD), catalase (CAT) and glyoxalase-I (Gly-I) as well as on the level of reduced glutathione (GSH). In addition, modulatory effect of *G. max* on peroxidative damage and on the specific activity of lactate dehydrogenase has also been studied.

**Materials and Methods**

Chemicals—7,12-Dimethylbenz(a)anthracene (DMBA), methylcholanthrene (MCA), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 2,6-dichlorophenolindophenol (DCPIP), ethylenediamine tetra-acetic acid (EDTA), reduced glutathione (GSH), pyrogallol, bovine serum albumin (BSA), methylglyoxal, Triton X-100, thiobarbituric acid (TBA), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The rest of the chemicals used were obtained from local firms (India) and were of highest purity grade.

Animals—Random bred female Swiss albino mice (6-8 weeks old) were used for the present study. The animals were maintained in the air-conditioned animal facility (Jawaharlal Nehru University, New Delhi, India) under 12 h light/dark cycle and provided (unless otherwise stated) with standard food pellets and drinking water *ad libitum*. Throughout the duration of experimentation, the animals were under strict observation, with respect to food and water consumption and for manifestation of any toxicity syndrome. The experimental studies were conducted according to the ethical guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, on the use of animals for scientific research.

Modulator—Seeds of *G. max* (soybean) were obtained from the market and were authenticated by a competent Botanist at the Institute. The seeds were dried in shade and grinded into a powder, with the help of a mixer grinder and mixed with the standard feed powder, according to the desired concentrations (2.5, 5 and 7.5%, w/w) and pellets were prepared. The pellets with different concentrations of the plant modulator were stored in clean bags in the feed store room of the animal house of the Jawaharlal Nehru University.

Preparation of chemicals and test diet—DMBA was dissolved in acetone at a concentration of 50 µg/50 µl acetone and was applied topically to the animals. Croton oil (2%) in acetone was prepared which was used as a promoter in skin papillomagenesis study. MCA was dissolved in beeswax. The food intake was monitored and found to be almost the same in each group.

**Experimental design**

In the present study, three separate experiments were performed to delineate specific objectives as mentioned earlier. Experiments I and II were aimed to evaluate the probable chemopreventive efficacy of *G. max* seeds against the skin and cervical papillomagenesis in murine model system. Further, experiment was also designed to study the chemomodulatory effect of *G. max* seeds on the enzymes involved in antioxidant function and toxicity in terms of peroxidative damage and activity of lactate dehydrogenase.

Experiment I—The modulation of DMBA-induced skin papillomagenesis by *G. max* seeds was carried out as described by Yasukawa et al. with some modifications. The hairs on the dorsal scapular region (2 cm diam) of the mice were clipped off three days before the application of the carcinogen and animals in the resting phase of hair growth cycle were selected for the experiment. The animals were randomly assorted into different groups. The body weights of the animals were monitored at weekly intervals. The numbers of papillomas appearing in the shaven area of the skin were noted down at weekly intervals. The papillomas of the size above 1 mm in diameter were included in data analysis. The animals were sacrificed 120 days after commencement of the treatments. In each group, the number of papillomas per mouse (tumor multiplicity) was counted at the termination of the experiment.

Experiment II—The experiment was undertaken to study the chemopreventive potential of *G. max* seeds on MCA-induced tumorgenesis in the uterine cervix of mice. Murphy’s string method as described by Manoharan and Rao was followed for the induction of tumors in the mouse uterine cervix. Briefly, sterile double cotton thread impregnated with beeswax containing approximately 600 µg of MCA was inserted into the canal of the uterine cervix by means of laparotomy under mild ether anesthesia.
Study of chemomodulatory effect of *G. max*

Preparation of homogenate, cytosol and microsome fractions—Animals were sacrificed and the entire liver was perfused immediately with ice cold NaCl (0.9%) and thereafter carefully removed, trimmed free of extraneous tissue and rinsed in chilled 0.15 M of Tris KCl buffer [(0.15 M, KCl + 10 mM, Tris HCl, (pH 7.4)]. The liver was then blotted dry, weighed quickly and homogenized in ice cold 0.15M, Tris KCl buffer (pH 7.4) to yield 10% (w/v) of homogenate. An aliquot of this homogenate (0.5 ml) was used for estimation of reduced glutathione content while the remainder was centrifuged at 10,500 × g for 45 min at 4 ºC using RC5C Sorvall centrifuge (SM 24 rotor). The resultant supernatant was centrifuged at 1,05,000 × g for 60 min at 4ºC in a Beckman ultracentrifuge (Model L 780M). The supernatant (cytosol fraction), after discarding any floating lipid layer and appropriate dilution, was used for the assay of glutathione-S-transferase (GST), DT-diaphorase (DTD), lactate dehydrogenase (LDH) and antioxidant enzymes. The pellet representing the microsomal fraction was resuspended in the homogenizing buffer and lipid peroxidation. Protein estimation was determined according to the method of Lowry et al. using bovine serum albumin (BSA) as standard.

Determination of glutathione-S-transferase and DT-diaphorase activity—The cytosolic GST activity was determined according to the method of Habig et al. The specific activity of GST was calculated using an extinction coefficient 9.6 mM⁻¹ cm⁻¹ at and expressed in terms of µmoles of GSH-CDNB conjugate formed/min/mg protein. The specific activity of DTD was determined by the method described by Ernster et al. The activity was calculated using an extinction coefficient of 21 mM⁻¹ cm⁻¹. One unit of enzyme activity is defined as that causing the oxidation of 1 µmole of NADH per min. Peroxidative damage in microsomes was estimated using the thiobarbituric acid reactive substances (TBARS) method as described by Varshney and Kale and is expressed in terms of malondialdehyde (MDA) formed per mg protein.

Statistical analysis—The results are represented as mean ± SD. The data was analysed using one way ANOVA followed by a Dunnett’s t test for the significant difference. A P value of < 0.05 was considered significant. In experiment II, the MCA-treated group was considered a positive control. Groups treated with MCA+ soya seeds (2.5, 5 and 7.5%) were considered as the experimental groups. DMBA. On the other hand, 92.3, 84.6 and 76.9% per cent in case of the control group treated with only DMBA. The tumor incidence was 100% respectively (Table 1). G. Max seeds found to be more

### Results

Inhibition of DMBA-induced skin papillomagenesis by *G. max* in mice—Effect of seeds of *G. max* in diet during the peri-initiational period on DMBA induced skin papillomagenesis in mice has been presented in Table 1. All the animals comprising the respective control and the experimental groups, maintained a healthy body weight. There was no manifestation of any kind of toxicity syndrome among the animals fed with *G. max* seeds diet. The tumor incidence was 100 per cent in case of the control group treated with only DMBA. On the other hand, 92.3, 84.6 and 76.9% tumor incidence was observed in the groups of animals which were given 2.5, 5 and 7.5% test diets, respectively (Table 1). *G. max* seeds found to be more
effective at 7.5% diet than 2.5 and 5% test diets for decreasing tumor multiplicity as well. In control group of mice the tumor multiplicity was 10.5 which was reduced to 6.8, 3.9 and 2.6 with three different doses of G. max seeds i.e. 2.5, 5 and 7.5% in test diet, respectively. This accounted for a significant decrease in tumor multiplicity by 35, 63 and 75%, respectively.

**Inhibition of MCA-induced cervical cancer by G. max in mice**—Effect of G. max on MCA-induced tumorigenesis in the uterine cervix of mice is given in Table 2. No adverse effect of G. max seeds in test diet was seen in terms of body weight gains or diet consumption. The animals that were inserted intracervically with MCA plus wax-impregnated threads were kept on a normal diet (group II). This group displayed 73.9% tumor incidence in their uterine cervixes. The animals of group III, inserted intracervically with MCA plus wax-impregnated threads and treated with 2.5% dose of G. max mixed diet, showed 71.4% tumor incidence in their uterine cervices. Group IV treated with 5.0% dose of G. max showed a reduction in tumor incidence to 40.9%. However, at high dose (7.5%) of G. max, the tumor incidence was reduced to 30.0%. Overall, these results suggest that soybean reduced the MCA-induced cervical tumor incidence significantly as well as dose-dependently in mice (Fig. 1A). Further, a dose-dependent increase in dysplasia was observed with the corresponding decrease in invasive carcinoma, suggesting that G. max inhibited the progression from dysplasia to invasive carcinoma (Fig. 1B and Table 2).

**Effect of G. max on antioxidant enzymes**—GST and DTD are known for their antioxidant function. These enzymes neutralize electrophilic metabolites into water soluble compounds so as to be readily excreted from the body. G. max seeds in diet significantly increased the specific activity of GST. As compared to the control group, the specific activity of GST was significantly enhanced by 1.21, 1.23 and 1.3 fold due to 2.5, 5 and 7.5% test diets of G. max seeds, respectively. The specific activity of DTD also showed an increase by 1.45, 1.65 and 1.56 fold relative to the control group, in case of the groups

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**Table 1**—Effect of different doses of Glycine max (Soybean) on DMBA-induced skin papillomagenesis in mice. [Values are mean ± SD of 13-15 animals]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Tumor incidence (%)</th>
<th>Tumor multiplicity*</th>
<th>% inhibition of tumor multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Final</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>26.92 ± 1.55</td>
<td>34.83 ± 1.80</td>
<td>100</td>
<td>10.5 ± 4.30</td>
</tr>
<tr>
<td>B</td>
<td>26.62 ± 2.26</td>
<td>35.08 ± 2.53</td>
<td>92.3</td>
<td>6.8 ± 2.8</td>
</tr>
<tr>
<td>C</td>
<td>28.46 ± 0.88</td>
<td>37.69 ± 2.81</td>
<td>84.6</td>
<td>3.9 ± 2.2**</td>
</tr>
<tr>
<td>D</td>
<td>28.80 ± 1.60</td>
<td>37.47 ± 2.56</td>
<td>76.9</td>
<td>2.6 ± 2.3**</td>
</tr>
</tbody>
</table>

(A)-DMBA; (B) - DMBA + Croton oil + 2.5% diet of Glycine max; (C)- DMBA + Croton oil + 5% diet of Glycine max; (D)- DMBA + Croton oil + 7.5% diet of Glycine max

*Number of tumors per mouse

** (P < 0.001), represents significant change against control
The specific activity of lactate dehydrogenase exhibited a significant decrease as compared to the control group. The content of GSH was also significantly enhanced by 1.23, 1.23 and 1.24 fold in case of the groups of animals treated with 2.5, 5 and 7.5% test diets of Glycine max seeds, respectively (Table 4). Since, Glycine max seeds enhanced the antioxidant status of animals, an attempt was also made to see its effect on peroxidative damage and the specific activity of lactate dehydrogenase.

Lactate dehydrogenase and peroxidative damage—The specific activity of lactate dehydrogenase exhibited a significant decrease as compared to the control group, by 0.78, 0.77 and 0.69 fold compared to the control group. The content of GSH was also significantly enhanced by 1.23, 1.23 and 1.24 fold in case of the groups of animals treated with 2.5, 5 and 7.5% test diets of Glycine max seeds, respectively (Table 4). Since, Glycine max seeds enhanced the antioxidant status of animals, an attempt was also made to see its effect on peroxidative damage and the specific activity of lactate dehydrogenase.

### Table 2—Chemopreventive action of Glycine max (Soybean) on MCA-induced cervical carcinogenesis in murine model system

<table>
<thead>
<tr>
<th>Group No</th>
<th>Treatment</th>
<th>Diet</th>
<th>Effective No. of mice</th>
<th>No. of mice normal or with cervical lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Nil</td>
<td>ND</td>
<td>10</td>
<td>6 3 1 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>II</td>
<td>MCA-wax</td>
<td>ND</td>
<td>23</td>
<td>0 1 1 0 1 0 3 6 11</td>
</tr>
<tr>
<td>III</td>
<td>MCA-wax</td>
<td>2.5%</td>
<td>21</td>
<td>1 0 0 1 0 1 3 5 10</td>
</tr>
<tr>
<td>IV</td>
<td>MCA-wax</td>
<td>5.0%</td>
<td>22</td>
<td>4 0 1 3 3 0 2 3 6</td>
</tr>
<tr>
<td>V</td>
<td>MCA-wax</td>
<td>7.5%</td>
<td>20</td>
<td>3 2 1 0 3 2 3 3 3</td>
</tr>
<tr>
<td>VI</td>
<td>Nil</td>
<td>7.5%</td>
<td>9</td>
<td>5 2 2 0 0 0 0 0 0</td>
</tr>
<tr>
<td>VII</td>
<td>wax</td>
<td>ND</td>
<td>10</td>
<td>7 0 2 0 1 0 0 0 0</td>
</tr>
</tbody>
</table>

H*: Mild Hyperplasia, H**: Moderate Hyperplasia, H***: Severe Hyperplasia
D*: Mild Dysplasia, D**: Moderate Dysplasia, D***: Severe Dysplasia
CIS- Carcinoma In Situ; InvC- Invasive Carcinoma; ND- Normal Diet

### Table 3—Modulatory influence of different doses of Glycine max (Soybean) on hepatic II drug metabolizing enzyme levels in Swiss albino mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>GST</th>
<th>DTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.94±0.26</td>
<td>0.0529±0.012</td>
</tr>
<tr>
<td>Glycine max (2.5% diet)</td>
<td>2.36±0.16</td>
<td>0.0766±0.012</td>
</tr>
<tr>
<td>Glycine max (5.0% diet)</td>
<td>2.41±0.29</td>
<td>0.0875±0.062**</td>
</tr>
<tr>
<td>Glycine max (7.5% diet)</td>
<td>2.61±0.51**</td>
<td>0.0832±0.013**</td>
</tr>
</tbody>
</table>

### Table 4—Modulatory influence of different doses of Glycine max (Soybean) on hepatic antioxidant related parameters in Swiss albino mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH</th>
<th>SOD</th>
<th>CAT</th>
<th>Gly 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.95±0.21</td>
<td>2.84±1.10</td>
<td>22.19±2.43</td>
<td>4.17±0.99</td>
</tr>
<tr>
<td>Glycine max (2.5% diet)</td>
<td>3.63±0.20**</td>
<td>5.81±0.84**</td>
<td>23.49±1.61</td>
<td>5.43±0.95*</td>
</tr>
<tr>
<td>Glycine max (5.0% diet)</td>
<td>3.64±0.15**</td>
<td>6.55±2.57**</td>
<td>29.35±2.54**</td>
<td>5.83±0.55**</td>
</tr>
<tr>
<td>Glycine max (7.5% diet)</td>
<td>3.67±0.14**</td>
<td>7.83±1.78**</td>
<td>28.44±5.29**</td>
<td>5.97±0.76**</td>
</tr>
</tbody>
</table>

### Notes
- Values in parentheses represent relative change in parameters assessed.
- * (P < 0.05) and ** (P < 0.01) represent significant changes against control.
- µmole CDNB-GSH conjugate formed/min/mg protein and µmole of DCPIP reduced/min/mg protein.
- Abbreviations- GST: glutathione S-transferase, DTD: DT-diaphorase.
- Treatment duration-15 days.
Discussion

Different parts of the plant including seeds are consumed by human beings throughout the world in their diet. Since, plants contain variety of chemical agents, the biological effect on consumption, could be a result of their coordinated and collective action. It becomes more important and relevant to examine the chemomodulatory effect by using whole part of the plant instead of single molecule. Keeping this in mind, in the present study, an attempt has been made to examine the chemopreventive potential of G. max seeds as well as its modulatory action on enzymes involved in antioxidant function in Swiss albino mice. Dietary administration of G. max seeds exerted a strong chemopreventive effect against skin and cervix papillomagenesis induced by DMBA and MCA, respectively in murine model system. A significant reduction in tumor incidence and tumor multiplicity in skin papillomagenesis was observed at all the three doses (2.5, 5.0 and 7.5%) of G. max seeds given in test diet with the highest dose (7.5%) showing more efficacy.

GST and DTD are known to be involved in antioxidant functions, GST catalyzes the conjugation of reactive chemicals with GSH and play a major role in protecting cells. DT-diaphorase is a flavoprotein that catalyzes the two electron reduction of quinones and nitrogen oxides. This reaction prevents one electron redox cycling of these groups, thereby preventing the formation of DNA damaging ROS. Reduction of quinones and nitrogen oxide might also make them available for conjugation with UDP-glucuronic acid, facilitating their excretion. Mounting evidence has indicated that the induction of phase II detoxification enzymes which detoxify carcinogens either by neutralizing their reactive centers or by conjugating them with endogenous ligands, and facilitating their excretion. Mounting evidence has indicated that the induction of phase II detoxification enzymes by numerous compounds and food phytochemicals result in protection against toxicity and chemical carcinogenesis, especially during the initiation phase.

Reactive oxygen species (ROS) which induce oxidative stress have been implicated in the etiology and progression of several diseases including cancer. Since, several enzymes metabolize reactive oxygen species or compounds which generate ROS are likely to decrease carcinogenic effect of toxic compounds. G. max seeds in diet treatment increased the activities of both of these enzymes. It may be noted that both GST and DTD are important members of phase II detoxifying enzymes which detoxify carcinogens either by neutralizing their reactive centers or by conjugating them with endogenous ligands, and facilitating their excretion.

Table 5—Modulatory influence of different doses of Glycine max (Soybean) on hepatic lipid peroxidation and LDH in Swiss albino mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>LDH⁶</th>
<th>LP⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.64±0.85</td>
<td>6.40±2.50</td>
</tr>
</tbody>
</table>
| Glycine max 2.5% diet | 3.64±0.32**| 3.80±1.60*\(|\(P<0.05)\) and ** (P<0.01) represent significant changes against control. \(\mu\)mole of S-D-Lactoylglutathione/min/mg proteins and \(\mu\)mole malondialdehyde formed/mg protein. Abbreviations- LDH: lactate dehydrogenase, LP: lipid peroxidation. Treatment duration-15 days

Values in parentheses represent relative change in parameters assessed.
radicals. The enhanced antioxidant status is expected to scavenge the free radicals generated during the metabolism of DMBA and MCA and in turn contribute the inhibition of skin and cervical papillomas.

Thus, findings of the present work suggested that *G. max* acted as a chemopreventive agent against skin and cervical papillomagenesis in the mouse models. It also inhibited the progression of cervical cancer in mice. Several earlier studies have shown the chemopreventive effect of *G. max* against different types of cancers. Studies addressing the chemopreventive effect of *G. max* in humans against skin and cervical cancer will further validate the findings from mouse models and will be useful in establishing soybean as a chemopreventive agent.

Acknowledgement

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