Antiatherogenic and antiperoxidative effects of garlic and soy proteins in alcohol fed rats

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Rats fed with alcohol (18%) at 3.76 g/day for 45 days showed significant reduction in body weight, glutathione (GSH) content and activities of superoxide dismutase (SOD) and catalase in liver. Lecithin cholesterol acyltransferase (LCAT) in plasma, levels of HDL cholesterol in serum, hepatic bile acid production and fecal excretion of neutral sterols also showed significant reduction. Simultaneous feeding of garlic protein (GP) or soy protein (SP) (500 mg/kg body weight/day for 45 days) to alcohol fed groups increased each of above parameters significantly towards normal values. Increase in GSH content and catalase activity in liver, was significantly higher for SP treated group than for GP treated group. However, increase in plasma LCAT was significantly higher for GP treated group than for SP treated group. Alcohol fed rats showed significant increase in liver weight, serum and tissue cholesterol, serum triacylglycerol (TAG), phospholipids (PL) and free fatty acid (FFA) levels and activity of HMGCoA reductase in liver and intestine. Lipid peroxidation, glucose-6 phosphate dehydrogenase (G6PD), glutathione peroxidase (GPx) and glutathione reductase (GR) in liver and incorporation of labeled acetate into liver cholesterol also showed significant increase. GP and SP treated rats showed decrease in these values towards normal. GP feeding showed a better effect than SP in lowering serum and heart total cholesterol, and in maintaining GPx at near normal level, while SP feeding showed a better effect in lowering serum FFA level and maintaining GR activity at near normal level. In suppressing incorporation of labeled acetate into serum cholesterol, GP feeding showed a better effect than SP. Antiatherogenic and antiperoxidative effects of these proteins may be due to lower lysine/arginine ratio.

Keywords: Alcohol, Cholesterol, Enzymes, FFA, Garlic protein, Lipid peroxidation, Soy protein, TAG

Vegetable proteins are hypolipidemic compared to animal proteins in human beings and nature of protein in diet has significant effect on lipid level both in human beings and animals. Several studies on soy protein showed hypolipidemic activity compared to casein. Feeding of soy protein to animals and humans compared to casein showed significant reduction in serum cholesterol.

Biju et al. reported hypolipidemic effects of garlic protein in high cholesterol diet fed rats. Rajasree et al. reported antiperoxidative and hypolipidemic effects of garlic protein in alcohol fed rats. Based on beneficial effects observed on soy and garlic protein treatments by different groups, a comparative study of soy and garlic protein feeding on selected serum and tissue parameters in rats subjected to alcohol feeding has been carried out.

Materials and Methods

Preparation of soy and garlic proteins — Soy flour purchased locally was defatted with petroleum ether and dissolved in NaCl (10%) solution. The solution was stirred for about 48 hr and centrifuged. Supernatant was dialyzed for 3-4 days against water at 5°C and pH was adjusted to 7, to precipitate the protein. The protein was centrifuged and collected. Precipitate was redissolved in NaCl solution, dialyzed and lyophilized to get soy protein (SP). Garlic protein (GP) was prepared as reported earlier. Amino acid composition of SP was determined by Sanger’s method using an amino acid analyzer as reported earlier for GP. Composition of both proteins is presented in Table 1. Data on SP are similar to previous report. Yield of SP was 40 g/100 g soy flour and GP 5.5 g/100 g garlic.

Animals and treatment — Male albino rats of Sprague – Dawley strain weighing 90-120 g were selected from University animal house. Mean body weight of each group was adjusted to 110 g/rat. Approval of departmental ethical committee (Reg. No. 218/CPCSEA.dt. 1/6/2000.) was obtained.
Rats were housed in conventional wire-mesh cages in a room temperature regulated at 21° ± 1°C, 45-50% RH and 12:12 hr L/D Cycle. Environmental conditions (humidity, heat, light, ventilation etc.,) were kept constant for 24 hr daily during the period of study. Ethanol was obtained from E. Merck. Rats were maintained on normal laboratory rat feed and water ad libitum and were divided into following 4 groups of 6 rats each

Group 1— Normal control given 2ml physiological saline daily
Group 2 — Rats were fed with alcohol (18%) at a dose of 3.76 g/kg body weight for 45 days
Group 3— Rats were fed with alcohol (18%) as above + garlic protein (GP) at a dose of 500 mg/kg body weight for 45 days
Group 4—Rat were fed with alcohol (18%) as above + soy protein (SP) at a dose of 500 mg/kg body weight for 45 days

Alcohol and saline suspension of SP and GP were given intragastrically. Diluted absolute alcohol (18%) was used to feed to Group 2, 3 and 4. All animals were pair fed and their body weights were recorded before and after experiment. Rats were deprived of food on 45th day overnight and on next day morning, 1 ml solution of 50 mM (5µ Ci) of 1,2 (14C ) labeled acetate was injected (ip) into each of the rats. After 3 hr rats were stunned by a blow at the back of the neck and were sacrificed by decapitation. Blood and tissues were collected into ice cold containers for estimations by standard procedures. Mean value ± S.D of 6 rats in each group was taken for all parameters.

Biochemical estimation — Activities of HMGCoA reductase, glutathione reductase, glutathione peroxidase (GPx), Superoxide dismutase (SOD), catalase and glucose 6 phosphate dehydrogenase, in liver and HMGCoA reductase in intestine and lecithin cholesterol acyltransferase (LCAT) in plasma were estimated. Concentrations of total cholesterol in serum, liver, heart and aorta, lipoprotein cholesterol, free fatty acids and triacylglycerol in serum and GSH and TBARS in liver were estimated. Fecal excretion of bile acids and neutral sterols and hepatic concentration of bile acids and incorporation of labeled acetate in serum free and total cholesterol were also assessed.

Fractionation of garlic protein and its electrophoresis — Garlic protein (GP, 150 mg) was dissolved in 3 ml Tris buffer (pH 8.6) and it was centrifuged for removing suspended impurities. Clear supernatant was used for chromatographic fractionation using weakly basic cellulose ion exchanger DEAE cellulose (Sigma Chemical Company, USA) as described earlier. DEAE cellulose was washed with HCl (0.1 N) and NaOH (0.1 N) in sequence. Ion-exchanger was further washed with distilled water, until the filtrate became neutral and was washed with 20 mM Tris buffer (pH 8.6). Slurry of cellulose in buffer was transferred into chromatographic column of size 20 x 20 cm and the column was filled with buffer for chromatography. Purified protein (150 mg/3 ml) solution was applied to column and was washed thoroughly with buffer. Column was connected to a gradient elution device with a linear gradient of NaCl (0.05 M) in 500 ml of Tris buffer. Eluted fractions (3 ml) were monitored by measuring absorbance at 280 nm. Three fractions (11, 15 and 18) gave protein peaks. Fraction No. 15 gave maximum O.D. representing major fraction in garlic protein preparation and was subjected to SDS-PAGE electrophoresis. Two blocks of SDS-PAGE were used for garlic protein fraction No. 15 and standard protein. Samples were subjected to electrophoresis in Hoeffer electrophoresis apparatus at 30 mA for 8 hr using SDS-PAGE (10%). Gels were stained with coomassie brilliant blue and destained. Molecular weight of garlic protein was estimated by PAGE.

Data were analysed statistically by ANOVA and t'-values for each group according to Bonferroni Multiple comparison procedure. Level of significance was fixed at P < 0.05.

Results and Discussion
Amino acid compositions of SP and GP were quite similar both qualitatively and quantitatively (Table 1) and effects of these proteins against alcohol induced damages in rats gave similar results. Changes in body and liver weights of various groups of rats were significant. Mean body weight decreased significantly in alcohol fed group. However, on treatment, body weight of GP and SP groups increased significantly (P<0.05) compared to alcohol group. Between the normal and treated groups or among the treated groups no significant difference was observed. Liver weight increased significantly for alcohol fed group compared to normal group, (P<0.05). Treatment with GP or SP significantly decreased liver weights to near normal.

Multiple comparison procedure
values. Between normal and treated groups or among treated groups no significant difference was observed.

Level of total concentration of cholesterol, lipoprotein fractions and atherogenic indices (total cholesterol/HDL cholesterol) in serum of various groups are given in Table 2. Total cholesterol in alcohol group showed significant increase and GP or SP treated groups showed significant decrease compared to control group. LDL + VLDL cholesterol level and atherogenic index (AI) showed significant increase in alcohol treated group but HDL cholesterol level decreased significantly. However, in GP or SP treated groups LDL + VLDL cholesterol and AI decreased significantly and HDL increased significantly towards normal level. Between treated groups GP treated group showed significant decrease for total serum cholesterol similar to the level in normal group.

Total cholesterol level in liver, heart and aorta increased significantly in alcohol fed group i.e. in liver from 485.8 ± 12.1 to 537.8 ± 13.9; in heart from 279 ± 15.8 to 346.9 ± 14.7 and in aorta from 157.6 ± 14.3 to 185.7 ± 12.5 mg/100 g wet weight, respectively. Treatment with GP and SP significantly decreased values as follows. In liver the level dropped to 493.4 ± 12.7 and 503.1 ± 12.1; in heart the values dropped to 227 ± 12.2 and 293.1 ± 13.2; in aorta the value dropped to 154.2 ± 16.64 and 144.4 ± 14.6. Between the treated groups there was a significant difference only for heart where GP treated group showed value far below the normal group.

In serum, triacylglycerol (TAG), phospholipids (PL) and free fatty acids (FFA) levels were altered by alcohol feeding and treatment with GP or SP. TAG in serum increased significantly from a normal level of 12.6 ± 0.71 to 15.9 ± 0.78 mg/dl in alcohol fed group. GP or SP feeding to alcohol fed groups significantly decreased the level to normal range of 12.6 ± 0.8 and 13.0 ± 0.65 mg/dl respectively. Similarly, PL level increased significantly from a normal value of 118.2 ± 7.1 to 185.3 ± 7.5 mg/dl on alcohol feeding. GP or SP feeding to alcohol fed groups showed significant decrease in PL level i.e 152.5 ± 8.0 and 142.0 ± 8.4 mg/dl respectively. Similarly serum FFA also showed an increase from a normal level of 78.9 ± 2 to 91 ± 1.8 mg/dl in alcohol fed group. Feeding GP or SP to alcohol fed groups the values decreased significantly to 77.3 ± 2.9 and 71.6 ± 2.1 respectively.

Ratio of mevalonate/HMGCoA, an index for HMGCoA reductase in liver, showed a significant increase from a normal value of 0.54 ± 0.022 to 0.64 ± 0.024 on alcohol feeding. Treatment of alcohol fed groups with GP or SP decreased the level significantly to near normal and normal level respectively. In alcohol fed group this level increased significantly in intestinal tissue from a normal level of 0.47 ± 0.02 to 0.58 ± 0.025. Treatment of alcohol fed groups with GP or SP showed significant decrease to near normal level. GSH content of liver decreased significantly from a normal level of 353 ± 9.4 mg/100 g wet tissue to 322 ± 7.4 mg on

### Table 1 — Amino acid composition of soy and garlic proteins

<table>
<thead>
<tr>
<th>Soy protein (%)</th>
<th>Garlic protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>3.61</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.32</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.80</td>
</tr>
<tr>
<td>Cysteine &amp; Cystine</td>
<td>1.51</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>16.01</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.62</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.51</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.90</td>
</tr>
<tr>
<td>Leucine</td>
<td>6.62</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.41</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.32</td>
</tr>
<tr>
<td>Phenyl alanine</td>
<td>4.41</td>
</tr>
<tr>
<td>Proline</td>
<td>4.60</td>
</tr>
<tr>
<td>Serine</td>
<td>4.52</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.20</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.21</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.30</td>
</tr>
<tr>
<td>Valine</td>
<td>4.21</td>
</tr>
<tr>
<td>Ammonia</td>
<td>14.70</td>
</tr>
</tbody>
</table>

### Table 2 — Serum concentration of total cholesterol, lipoprotein cholesterol and atherogenic index of normal, alcohol fed and GP or SP treated rats for 45 days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total cholesterol (mg/dl)</th>
<th>LDL + VLDL Cholesterol (mg/dl)</th>
<th>HDL Cholesterol (mg/dl)</th>
<th>Atherogenic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>69.9 ± 2.5a</td>
<td>33.5 ± 0.92a</td>
<td>35.7 ± 0.70a</td>
<td>1.94 ± 0.03a</td>
</tr>
<tr>
<td>Alcohol fed (AF)</td>
<td>92.2 ± 2.6b</td>
<td>60.4 ± 1.60b</td>
<td>32.3 ± 0.42b</td>
<td>2.88 ± 0.03b</td>
</tr>
<tr>
<td>AF + G.P</td>
<td>68.6 ± 2.2a</td>
<td>33.6 ± 2.9a</td>
<td>35.7 ± 0.58a</td>
<td>1.92 ± 0.07a</td>
</tr>
<tr>
<td>AF + S.P</td>
<td>74.2 ± 2.6a</td>
<td>38.5 ± 3.6a</td>
<td>35.6 ± 0.53a</td>
<td>2.06 ± 0.07a</td>
</tr>
</tbody>
</table>

ANOVA followed by ‘t’ test. Values with different superscripts show significant differences between groups. P values: <0.05
alcohol feeding. Treatment with GP or SP increased these values significantly to normal level of 351 ± 7.2 mg and 369 ± 7.1 mg respectively. Similar effects of amelioration in the GSH contents in heart and kidney were also observed on treatment with GP/SP. Activities of antioxidant enzymes in liver of various groups of rats are given in Table 3. Activities of SOD and catalase decreased significantly in the alcohol fed groups and GPx and glutathione reductase increased significantly in these groups compared to control group. These enzymes showed significant increase after the alcohol fed group was fed with GP or SP. SP treated group showed significant increase in the level of catalase compared to GP treated group.

GPx and glutathione reductase showed significant increase in alcohol fed rats. Treatment of alcohol fed rats with GP or SP restored the level of these enzymes to near normal level. Among the treated groups GP treatment showed significantly higher values of antioxidant enzymes over the SP group. However, only in SP treatment GR activity fell down to normal level.

Lipid peroxidation index TBARS, concentrations of hepatic bile acids, fecal bile acids and neutral sterols of various groups are given in Table 4. TBARS value increased significantly over the normal level in alcohol fed group. Feeding GP or SP to alcohol fed groups significantly decreased the level towards normal level.

In alcohol fed group, hepatic bile acid production decreased significantly from normal level and increased significantly after treatment with GP or SP. No significant difference was observed between treated groups. Fecal excretions of bile acids and neutral sterols showed significant decrease in alcohol fed group. GP or SP treatment significantly increased the level towards normal.

Lipogenic, G6PD activity in liver, plasma LCAT activity and in vivo synthesis of cholesterol from labeled acetate are given in Fig.1. G6PD activity increased significantly in alcohol fed group compared to control group and treatment with GP or SP significantly decreased the level, but far above normal value. Plasma LCAT level decreased significantly in alcohol fed group and treatment increased it slightly above the normal for GP group and just to normal level for SP group and a significant difference was observed between these values. Incorporation of 1,2\(^{14}\)C acetate into serum free and ester cholesterol increased significantly for alcohol fed group and treatment decreased the incorporation significantly to normal level by GP and slightly above normal by SP. Significant difference was observed between these two values.

Fractional separation of GP by Gel filtration gave two minor fractions Nos. 11 and 18 and a major middle fraction No. 15 with a double peak height of the other two. Middle fraction No. 15 protein was of molecular weight less than 14 kDa. (Fig. 2). Small impurities separated just above the band. Molecular weight of major protein fraction was about 11 kDa.

### Table 3 — Activities of antioxidant enzymes in liver tissue.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (units/mg protein)</th>
<th>Catalase (× 10(^{-3}) units/mg protein)</th>
<th>Glutathione Px (units/mg protein)</th>
<th>Glutathione reductase (× 10(^{-2}) units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10.2 ± 0.50(^a)</td>
<td>60.3 ± 3.6(^a)</td>
<td>0.25 ± 0.004(^a)</td>
<td>5.02 ± 0.01(^a)</td>
</tr>
<tr>
<td>Alcohol fed (AF)</td>
<td>6.81 ± 0.46(^b)</td>
<td>30.9 ± 2.1(^b)</td>
<td>0.45 ± 0.002(^b)</td>
<td>7.08 ± 0.06(^b)</td>
</tr>
<tr>
<td>AF + G.P</td>
<td>9.1 ± 0.21(^c)</td>
<td>43.3 ± 2.7(^c)</td>
<td>0.28 ± 0.005(^d)</td>
<td>5.93 ± 0.01(^c)</td>
</tr>
<tr>
<td>AF + S.P</td>
<td>9.25 ± 0.40(^d)</td>
<td>49.3 ± 2.4(^d)</td>
<td>0.20 ± 0.004(^c)</td>
<td>5.04 ± 0.02(^a)</td>
</tr>
</tbody>
</table>

ANOVA followed by ‘t’ test. P values: < 0.05. Differences in superscripts represent significantly different values.

### Table 4 — Level of lipid per oxidation product MDA in terms of TBARS in liver, hepatic bile acid levels and related fecal excretions

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS (mM/100g wet tissue)</th>
<th>Hepatic bile acids (mg/100 g wet tissue)</th>
<th>Fecal bile acid (mg/rat/day)</th>
<th>Fecal neutral sterol (mg/rat/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.759 ± 0.035(^a)</td>
<td>27.8 ± 1.18(^b)</td>
<td>10.9 ± 0.87(^a)</td>
<td>10.28 ± 0.63(^b)</td>
</tr>
<tr>
<td>Alcohol fed (AF)</td>
<td>0.850 ± 0.036(^b)</td>
<td>22.1 ± 1.7(^b)</td>
<td>9.1 ± 0.24(^b)</td>
<td>8.34 ± 0.66(^b)</td>
</tr>
<tr>
<td>AF + G.P</td>
<td>0.751 ± 0.05(^a)</td>
<td>28.34 ± 1.0(^b)</td>
<td>13.0 ± 0.36(^a)</td>
<td>11.36 ± 0.71(^a)</td>
</tr>
<tr>
<td>AF + S.P</td>
<td>0.730 ± 0.04(^d)</td>
<td>26.8 ± 1.02(^a)</td>
<td>12.7 ± 0.56(^a)</td>
<td>10.30 ± 0.94(^a)</td>
</tr>
</tbody>
</table>

ANOVA. Differences in superscripts represent significantly different values as in previous tables. P values: < 0.05.
Increases in tissue contents of GSH possibly helped to scavenge free radicals induced by alcohol feeding. Similarly, a 40% increase in liver GSH content and a significant increase in SOD and a decrease in GPx activities in tissues of chicks fed with 2% garlic diet was reported. Beneficial effects of GP and SP in alcohol fed rats may be due to their similar amino acid composition with a high level of essential amino acids. Higher hypolipidemic effect of GP may be due to higher level of sulfur containing amino acids. Lysine/arginine ratio of protein was suggested for determining the antiatherogenicity of each protein. Animal proteins have a lysine/arginine ratio greater than 1. Caseine with a ratio of 2, is atherogenic. Plant proteins like GP, with a ratio of 0.77, is more hypolipidemic than SP with a higher ratio of 0.84. Antioxidant activity has been ascribed to sulfur containing amino acids. Sulfur containing amino acids like methionine can give rise to sulfoxide form on metabolism and are hypolipidemic in action. Results from the present study showed efficacy of plant proteins with specific amino acid composition as functional foods to counteract damaging effects of alcohol in rats.

Substitution of soy protein (with low or high isoflavone content) for animal products reduced coronary artery disease risk due to moderate reductions in blood lipids, oxidized LDL, homocysteine and blood pressure in hyperlipidemic men and women. Clinical trials showed hypocholesterolemic effects of soy protein intake, however the constituents were not identified. Serum LDL cholesterol concentration in subjects consuming soy protein with high content of isoflavone decreased significantly compared with users of soy protein with low isoflavone. Isoflavone has LDL cholesterol lowering effect independent of soy protein. Isoflavone significantly decreased serum total cholesterol, LDL cholesterol and TAG and significantly increased serum HDL cholesterol.

Anticarcinogenic effects of garlic was attributed to selenium and sulfur containing compounds, viz; γ-glutamyl selenomethyl selenocysteine (GGMSC) and methyl selenocysteine (MSC) in it. GGMSC is present in garlic protein also. Rats challenged with a carcinogen and supplemented with GGMSC or MSC had only lower prevalence of premalignant lesions in mammary gland and fewer mammary carcinomas. Both these compounds have very similar mechanism of action. Garlic has plasma lipid lowering, anticoagulant and antioxidant properties and improved impaired endothelial functions. Chemopreventive doses of MSC altered circadian rhythm in rat mammary tissue.

Presence of two important proteins in garlic with molecular weights of 40 kDa (probably allinase) and 14 kDa has been reported. 40 kDa fraction is a minor fraction and is an enzyme in garlic. The fraction was not present in the protein isolated in the present study. 14 kDa protein may be a mixture of proteins obtained at fraction Nos. 11,15 and 18, of which the middle one was a major protein with a molecular weight of 11 kDa. Glycoprotein with molecular weight of about 14 kDa has been isolated from garlic and its activity was assessed. It may induce NK augmentation against K 562 tumor cell line. In vivo studies also confirmed that a fraction...
immunoboosting effects were reported\(^{33,54}\). Fraction 15 used in this study may be same as F\(_4\) as reported earlier\(^{53}\) with molecular weight 11 kDa.

Protein used in this study contained mainly protein fraction 15 with 11 kDa and fractions 11 and 18 with molecular weights in the range of 10-14 kDa. It is suggested that these three fractions together may be responsible for effects observed for the sample of GP. Further work is needed to elucidate effects of each fraction of garlic protein. However, both SP and GP may be used as functional foods to prevent damaging effects of alcohol.

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References


