Nutraceutical effects of garlic oil, its nonpolar fraction and a *Ficus* flavonoid as compared to vitamin E in CCl₄ induced liver damage in rats

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Received 2 July 2004; revised 18 January 2005

Carbon tetrachloride feeding (3.2g/kg/72hr) for one month increased significantly the serum and tissue lipid profile and deranged the enzyme levels viz; alkaline phosphatase, alanine transaminase, aspartate transaminase, glutathione reductase, HMGCoA reductase, catalase, gluc.6.PDH and malic enzyme in rats. Simultaneously the lipid peroxidation level in liver was also raised. On administration of garlic oil and its major nonpolar fraction (NPFGO) and a flavonoid isolated from the bark of *Ficus bengalensis* Linn, viz; leucopelargonin derivative respectively to different groups (50mg/kg/day) the deleterious effects of CCl₄ were significantly ameliorated. The liver damage by CCl₄ was satisfactorily prevented by these samples as effectively as Vit. E (50 mg/kg/day). The results prove that important nutraceuticals (phytonutrients) like bioflavonoids and theols i.e. allylic sulphide rich fractions give protection from toxins like CCl₄. The order of beneficial effects of the drugs are Leucopelargonin > NPFGO > Garlic oil and their effects are comparable to that of vitamin E used at a minimal dose.

Keywords: Carbon tetrachloride, Garlic oil, Lipids, Leucopelargonin, NPFGO, Nutraceuticals, Vitamin E.

The role of free radical has been implicated in several diseases. The damages produced by certain toxic chemicals or hepatotoxic agent like carbon tetrachloride (CCl₄) were found to be due to its metabolism in the liver. CCl₄ binds to cytochrome P₄₅₀ reductase. The enzyme substrate complex then loses a chloride ion and a free radical (CCl₃⁻) intermediate is generated.

\[
\text{CCl}_4 + \text{NADPH} \rightarrow \text{CCl}_3^- + \text{Cl}^- + \text{Cytochrome P}_{450} \text{ reductase}
\]

The above free radical then reacts with oxygen or takes a hydrogen from a suitable donor to yield a secondary radical or reacts with lipids or proteins.

\[
\text{CCl}_3^- + \text{O}_2 \rightarrow \text{CCl}_3^- \text{O}^- + \text{Cl}^- \quad \text{(methyl peroxy radical)}
\]

The methyl peroxy radical is much more reactive than CCl₃⁻ and is the main initiating species for lipid peroxidation that causes tissue injury in the hepatotoxicity of CCl₄.

Kupffer cells play a role in the mechanism of action of CCl₄, e.g. CCl₄ elevates Ca²⁺ and the release of toxic eicosanoids and cytokines by Kupffer cells is calcium dependent.

There are certain naturally occurring antioxidants that can give protection to liver from hepatotoxins. Schisandrin B an active ingredient of the fruit of *Schisandra chinensis* can enhance the hepatic glutathione antioxidant system in mice against CCl₄ toxicity². Similarly, oral administration of ellagic acid (a polyphenolic acid anhydride) to rats was found to reduce significantly the elevated levels of lipid peroxides and liver hydroxy proline after CCl₄ induced liver damage³. Treatment with ellagic acid rectified the liver pathophysiology and significantly reduced CCl₄ toxicity and subsequent fibrosis. Adequate liver protection by several defined constituents of garlic was shown with freshly prepared rat hepatocytes that had been treated with CCl₄, a strong toxicant. S-allyl cysteine sulfoxide (alliin) and related sulfur compounds in garlic were...
found to be effective in the neutralization of cytoxicity. Further, S-allyl mercaptocysteine and S-methyl mercaptocysteine (R-S-S-C3H5.CH(NH2)COOH or RS-Cysteine, where R=CH3 or C2H5) showed remarkable protection in vitro to hepatocyes against the toxic effects of CCl4 and galatosamine. Garlic oil (mixtures of RSS(O)R and RS2R) and possibly ajone (a polymer of allicin i.e. C3H7-S(O)CH2.CH=CH-S-S-C3H5) are also involved in the protective action of garlic principles. Another group of compounds viz; polyphenols and bioflavonoids have been found to exert their chemopreventive role through multiple pathways including free radical scavenging. Bhardwaj et al. found that fistein and resveratrol (a flavonoid and a polyphenol respectively) were as effective as vitamin E in their inhibitory action on lipid peroxidation. A Ficus flavonoid leucopelargonin was proved as a good hypoglycemic agent and an antioxidant. As many flavonoids were shown to inhibit CCl4 induced microsomal lipid peroxidation leucopelargonin was also selected for the present study. The flavonoids have a keto (=CO) function in the 4th place of the oxide ring and flavanoids have a methylene (-CH2) group in that place and both are polyphenols. In the present study garlic oil, its nonpolar fraction and leucopelargonin, a ficus flavanol derivative have been used. Flavanol derivatives are also termed flavonoids as a class. As the yield of the polar fraction of garlic oil was very poor (10% of the oil) its effects were not studied. These compounds with biological action in our food are newly termed as nutraceuticals. They are functional foods.

Materials and Methods

Preparation of garlic oil and its nonpolar fraction (NPFGO)—One kg of locally purchased fresh garlic (Allium sativum Linn) was cleaned and sliced. It was then ground into a pulp in a mortar. Enough methanol was added to keep it fully immersed and left in a conical flask overnight. The next day the mixture was filtered through an ordinary filter paper. The residue left behind was further extracted with methanol and the procedure was repeated to get the filtrate. Both the filtered extracts were combined. Methanol was distilled off and the oil was collected. This oil was extracted thrice with diethyl ether and the ether soluble fraction was collected. Ether was evaporated off on a water bath and the left over oil was collected and a part of this garlic oil (yield 4g/kg) was used for one experiment and the remaining was used for the preparation of the nonpolar fraction for another experiment.

Nonpolar fraction of garlic oil (NPFGO) was prepared from the above oil by extracting it with hexane thrice and the hexane soluble fraction was collected by filtration. Hexane was evaporated off on a water bath and the oil left behind was collected as nonpolar fraction (yield 3.5g/kg fresh garlic). Vitamin E (Sigma Chemicals Co St.Louis Mo) was used as a standard at a minimum dose.

Preparation of leucopelargonin derivative—A glycoside of leucopelargonidin (leucopelargonin derivative) was isolated from the bark of banyan tree (Ficus bengalensis Linn) according to the method of Prema and Misra. This compound was found to be a secretagogue of insulin and hence hypoglycemic in action. It is a flavanol derivative (yield 5 g/kg) under the general group flavonoids.

Experimental animals—Adult male albino rats (Sprague Dawley strain) weighing around 100 g were purchased from the animal colony of the Veterinary College at Thrissur. The rats were divided into 6 groups of 6 in each. They were maintained on Gold Mohur rat feed (Brooke Bond Lipton India Ltd, Bangalore). Each rat consumed about 10 g feed daily. Therefore equal amounts of rat feed (65 g) was supplied to each group so that they were pair fed. The oral dose of CCl4 (Sisco Laboratories, Bombay containing 1.6 g/ml) was 3.2 g/kg/72 hr according to the method of Recknagel and Gindele for developing cirrhosis in the test groups. Other treatment details are listed below for each group:

Group I—Normal rat feed was supplied with water ad libitum

Group II—Normal rat feed + CCl4 i.e. 0.2 ml of CCl4 was dissolved in 0.2 ml ground nut oil/100 g body weight and administered po, every 72 hr for one month.

Group III—Normal rat feed + CCl4 as above + garlic oil 100 mg/kg body weight and dissolved in 0.2 ml ground nut oil was administered ip, daily.

Group IV—Normal rat feed + CCl4 as above + NPFGO, 100 mg/kg body weight and dissolved in 0.2 ml ground nut oil was administered ip, daily.

Group V—Normal rat feed + CCl4 as above + leucopelargonin derivative, 100mg/kg body weight and suspended in 0.2 ml ground nut oil was administered ip, daily.
Group VI—Normal rat feed + CCl4 as above + Vitamin E, 50mg/kg body weight, dissolved in 0.2 ml ground nut oil was administered ip, daily.

Chronic administration of CCl4 for developing cirrhosis13 was carried out for groups II to VI along with treatment for one month as described.

The rats were sacrificed by decapitation after overnight fasting. Blood was collected in centrifuge tubes and was allowed to clot. Clear serum was separated by centrifugation and various estimations were carried out. Liver and heart were also collected in ice cold containers for various estimations. Lipids from tissues were extracted by Folch’s procedure14.

Total cholesterol15, LDL cholesterol16, HDL cholesterol17, TAG18 (triaclylglycerol) and FFA19 in serum and tissue lipid extracts as the case may be and protein and A/G ratio in serum20, TBARS21 (thiobarbituric acid reacting substances), conjugated dienes22 and activities of ALP23 (alkaline phosphatase), glutathione reductase24, glucose-6-phosphate dehydrogenase25, malic enzyme26 and HMGCoA reductase27 in liver homogenates and activities of ALT and AST28 (alanine and aspartate transaminases) and catalase29 in heart and liver homogenates were estimated by standard methods. In TAG18 determination a modification was that a florisil column was used to remove phospholipids. All the data were statistically analysed according to Student’s t test.

Liver tissues fixed in 10% formalin were processed for routine histopathological examination. Sections (5 μm thickness) were cut and stained with routine haematoxyline and eosin.

Results

The effects of drugs on lipid parameters in serum, liver and heart and on protein levels in serum are given in Table 1. CCl4 fed rats without treatment with drugs showed highly significant rise in total cholesterol, TAG, HDL chol., LDL chol., and FFA in serum and total cholesterol in liver and heart and TAG in liver. Those rats treated with garlic oil, its nonpolar fraction (NPFGO), leucopelargonin derivative and vitamin E in separate groups very significantly reduced the lipid components except HDL chol. in the serum, liver and heart as the case may be. HDL chol. increased significantly on treatment with each drug from the control value. The beneficial effects of each drug were comparable to that of vitamin E. The order of their effects on lipid
profile is—Leucopelargonin > NPFGO > Garlic oil > Vitamin E, as compared to the minimum standard dose of vitamin E. Serum protein level was significantly reduced in the \( \text{CCl}_4 \) fed group and both vitamin E and the drugs raised the A/G ratio significantly; the former by 1.5 fold and the latter by two fold.

Enzyme activities in serum and liver (ALP, ALT and AST) which were raised significantly by \( \text{CCl}_4 \) and ameliorated by drugs are presented in Table 2. Treatment with vitamin E and the above drugs significantly decreased the activities of the above enzymes. Here again the order of effects of each drug is more or less the same as mentioned above. \( \text{CCl}_4 \) feeding very significantly reduced the activities of the antioxidant enzymes viz, catalase and glutathione reductase and very significantly increased the activities of HMGCoA reductase and the lipogenic enzymes viz; glucose 6-phosphate dehydrogenase and malic enzyme as depicted in Table 3.

Treatment with each drug very significantly improved the activities of the antioxidant enzymes. However, vitamin E did the same to a highly significant level only in the case of catalase. Glutathione reductase activity was enhanced to a lesser level of significance. On the contrary, treatment with each drug and vitamin E very significantly lowered the activities of the remaining enzymes viz; HMGCoA reductase and the two lipogenic enzymes. Treatment with garlic oil decreased glucose 6-phosphate dehydrogenase activity even below normal. In this experiment near normal activities for most of the enzymes were obtained in the groups treated with the leucopelargonin derivative. The effects of each drug were comparable to that of vitamin E and the

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**Table 2**—Effects of nutraceuticals on certain enzymes as compared to vitamin E in \( \text{CCl}_4 \) induced liver damage in rats  
[Values are Mean ± S.D. of 6 rats]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Alkaline phosphatase (ALP)</th>
<th>Alanine transaminase (ALT)</th>
<th>Aspartate transaminase (AST)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Liver</td>
<td>Serum</td>
</tr>
<tr>
<td>I. Normal Group</td>
<td>23.03±3.38</td>
<td>1.49±0.59</td>
<td>22.68±1.2</td>
</tr>
<tr>
<td>II. ( \text{CCl}_4 ) group</td>
<td>45.87±1.99</td>
<td>4.25±1.12</td>
<td>46.5±1.7</td>
</tr>
<tr>
<td>III. ( \text{CCl}_4 ) + garlic oil</td>
<td>25.70±3.42</td>
<td>1.67±1.26</td>
<td>33.0±1.31</td>
</tr>
<tr>
<td>IV. ( \text{CCl}_4 ) + NPFGO</td>
<td>25.73±3.65</td>
<td>1.82±0.28</td>
<td>34.10±1.32</td>
</tr>
<tr>
<td>V. ( \text{CCl}_4 ) + Leucopelargonin</td>
<td>24.99±3.87</td>
<td>1.52±1.64</td>
<td>31.96±1.12</td>
</tr>
<tr>
<td>VI. ( \text{CCl}_4 ) + Vitamin E</td>
<td>28.54±2.67</td>
<td>1.96±0.87</td>
<td>39.37±1.5</td>
</tr>
</tbody>
</table>

Student's t—test

II<sup>th</sup> group Vs I<sup>th</sup> group and III<sup>th</sup> to VI<sup>th</sup> groups vs II<sup>th</sup> group are compared. *<sub>P<0.001</sub> and *<sub>P<0.01</sub> and *<sub>P<0.05</sub> as compared to each control.

Enzyme activities are expressed as IU/L serum and for the liver ALP as µmol/min/mg protein. ALT & AST as IU/100g wet tissue

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**Table 3**—Effects of nutraceuticals on antioxidant and lipogenic enzymes activities in liver as compared to vitamin E in \( \text{CCl}_4 \) induced liver damage in rats  
[Values are Mean ± SD of 6 rats]

| Units: ALP: IU/l serum (serum); µmole/min/mg protein (liver); ALT and AST IU/100g wet tissue |
|---|---|---|---|---|---|
| Groups                  | Glutathione reductase | Catalase | HMGCoA reductase | Glucose 6 phosphate dehydrogenase | Malic enzymes |
| I. Normal Group         | 5.51±0.22           | 65.25±0.78 | 0.30±0.06 | 65.53±1.79 | 8.39±0.51 |
| II. Carbotetraclorid group | 3.58±0.20          | 32.37±1.20 | 0.99±0.03* | 92.88±3.94* | 14.97±0.70* |
| III. \( \text{CCl}_4 \) + garlic oil | 4.27±0.16          | 91.14±1.12 | 0.35±0.03* | 47.7±2.48* | 10.76±0.36* |
| IV. \( \text{CCl}_4 \) + NPFGO | 4.45±0.13*         | 95.34±1.05* | 0.35±0.05* | 74.67±2.67* | 10.78±0.24* |
| V. \( \text{CCl}_4 \) + Leucopelargonin | 4.37±0.13*         | 75.76±0.99* | 0.33±0.03* | 70.94±2.18* | 10.75±0.28* |
| VI. \( \text{CCl}_4 \) + Vitamin E | 4.05±0.09b         | 69.16±1.51* | 0.44±0.02* | 82.89±1.79* | 12.22±0.45* |

Student's t test

II<sup>th</sup> group vs I<sup>th</sup> group and III<sup>th</sup> to VI<sup>th</sup> groups vs II<sup>th</sup> group are compared. Level of significance *<sub>P<0.001</sub> and *<sub>P<0.01</sub> as compared to each control.

Units: Glutathione reductase as values × 10<sup>-3</sup> µmol of NADPH oxidized/min.

Catalase: velocity constant /second; HMGCoA reductase: the ratio of Mevalonate / HMGCoA

Glucose 6 – phosphate DH and malic enzyme: change in O.D. of 0.01/units/min mg protein
Fig. 1—Values of TBARS (mg/100g wet tissue) and conjugated dienes (mM/100g wet tissue) levels in the liver of rats. (values are mean ± S.D. from 6 rats). Comparison was made between groups I and II and between group II Vs groups III to VI for each parameter. [Level of significance for TBARS P<0.05-0.02; Conjugated dienes P<0.01-0.001]

order of their effects were more or less the same as stated above.

CCL4 feeding increased, to a significant degree both TBARS and conjugated dienes in the liver tissue (Fig. 1). On treatment with each drug or vitamin E, lipid peroxidation was significantly decreased to various levels. Leucopelargonin derivative produced the maximum effect by bringing down the levels of TBARS and CD significantly and very significantly respectively in liver. This was followed by NPFGO and garlic oil. The corresponding effects of vitamin E were also significant with the least effect on TBARS in liver respectively. Histopathological changes of the liver in control and test groups revealed that the treatment prevented the damages by CCl4 (Fig. 2).

Discussion
The results of the present study clearly demonstrate that certain biochemical changes took place in the liver and serum of rats on chronic administration of CCl4 for one month. These included very significant increases in biochemical parameters like total cholesterol, HDL and LDL cholesterol, TAG, FFA, TBARS and conjugated dienes which duly developed liver cirrhosis and fibrosis (Fig. 2). Activities of two lipogenic enzymes (viz; glucose 6-phosphate dehydrogenase and malic enzyme) and HMGCoA reductase in the liver and enzymes like ALP, ALT and AST in serum and liver were also raised. The levels of antioxidant enzymes in liver were significantly lowered in the CCl4 fed group. CCl4 feeding also adversely affected the protein level and A/G ratio in serum. This observation very well correlates with the results of Lamb et al.30 who reported that CCl4 administration reduced the liver protein content by 18%. This is reflected in the level of serum proteins i.e. 27% fall in the present study. These deleterious effects of CCl4 were ameliorated to varying but significant degrees by each of the drugs used and by vitamin E.

On CCl4 administration total cholesterol in serum and liver of rats have been raised which may be due to its increased biosynthesis or decreased degradation31. The activity of HMGCoA reductase, the rate limiting enzyme in cholesterol biosynthesis was also raised in cirrhosis. In cirrhotic liver, activity of LCAT is significantly reduced which may account for the elevation of LDL cholesterol with an increase in the number of hepatocytes with fatty infiltration32. Kim and Labella33 reported that CCl4 caused significant increase in hepatic lipid peroxidation due to free radical injury in cirrhotic livers of rats. Sherlock and Walshe34 described an increase of ALP, ALT and AST activities and Stowell et al.35 that of ALP in the degenerating and neurotic liver cells and CCl4 induced liver cells respectively.

CCl4 induced membrane lipid peroxidation further adversely affected lipid metabolism and decreased protein biosynthesis in the injured hepatocytes36. Increased reactive oxygen species (ROS) can initiate lipid peroxidation, stimulate glycation of proteins, inactivation of enzymes and alteration in the structure and function of collagen, basement and other membranes and play a role in the long term complication of diseases like diabetes37. Supplementation with nontoxic antioxidants may have chemoprotective role in the prevention of diseases.

Liver sections of CCl4 fed rats showed significant alteration of normal lobular architecture with mild grades of fatty degeneration, focal cellular necrosis and peripheral fibrosis. These results have proceeded in an almost parallel manner to the corresponding histopathological reports by Sakr et al.38. The term “Fatty Changes” is used when there is morphological evidence of excess intracytoplasmic fat. The mechanism of this change is complex and may be produced by many factors e.g.; metabolic disorders, deficiency of essential lipotropic factors, excessive mobilization of fat from extrahepatic sources, etc.
Fig. 2—Light micrograph of liver. (a) normal: no pathological changes were observed; (b) carbon tetrachloride group. A striking alteration of the normal lobular architecture could be seen in liver sections from CCl₄ fed rats. Liver sections showed mild grades of fatty degeneration (fd), focal cellular necrosis (cn) and periportal fibrosis (pf); (c) garlic oil; (d) NPFGO; (e) flavonoid (leucopelargonin) and (f) vitamin E treated groups showing very mild grades of fd (white patches), pf (diffused shades), cn (fused dark spots) or any related pathological changes. Here garlic oil showed the best results in preventing liver damages followed by NPFGO, flavonoid and vitamin E.
Garlic oil, NPFGO, leucopelargonin and Vitamin E have very successfully inhibited fibrosis as well as fatty infiltration. Organic disulphides have inhibitory action on the thiol group enzymes, viz; fatty acid synthetase and HMGCoA reductase and thereby on atherosclerosis also which in turn lead to decreased fatty infiltration. It was also found that vitamin E inhibited lipid peroxidation, but not fat deposition. The naturally occurring antioxidants in garlic oil/NPFGO and related organic sulphides and the ficus flavonoid have certain biological actions other than that of vitamin E, viz; reactions with thiol group of enzymes, insulin sparing action, enhanced glucose uptake by peripheral tissues, insulin secretagogue action, and electron trapping capacity. The antioxidant and related reactions of organic disulphide compounds from various sources were described by Klanns – Dieter.

The thyl radical (RS') helps in the repair of free radicals and it does not react with critical molecules, hence it serves as a very good scavenger of free radicals. Flavonoids act as metal chelators and also as stimulants of microsomal activating enzymes. Pancreatic β cell stimulation by flavonoids and insulin sparing action by garlic sulphur compounds were known. Organic disulphides oxidize NADPH which is necessary for cholesterol and fatty acid synthesis.

\[
\text{RSSR} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{RSH} + \text{NADP}^+
\]

This reaction of disulphides explains the hypolipidemic effects of garlic allyl disulphides (C3H5- S-S-C3H5) and its polymers in NPFGO.

In a classical work by Hikino et al, they demonstrated that the volatile oil of garlic inhibited galactosamine induced liver injury and CCl4 induced free radical formation and lipid peroxidation in rats, more efficiently than that with vitamin E and it also indicated that the factor responsible for the inhibition of liver damage is the volatile oil of garlic which contains a mixture of both polar and nonpolar sulphur compounds. Cardioprotective action of garlic has also been reported by many workers, viz; garlic dialysate has a significant antiarhythmic effect in both ventricular and supraventricular arrhythmias.

The variation in the protective capacities of these nutraceuticals and vitamin E on liver/heart may be explained based on their various biological effects. Even though leucopelargonin is not lipid soluble, it is freely water soluble and endowed with antioxidant actions of flavonoids and such compounds were once termed by Szent Gyorgyi as vitamin P. Since the association of reduced risk for diseases like cancer, CHD, ageing, etc with a diet high in fruits and vegetables is strong, dietary constituents other than vitamin C and E or β carotene e.g. the above theols and flavonoid type compounds or polyphenols and terpenes may be making a greater contribution to this effect. Hence, we suggest that if garlic and ficus compounds are used as dietary components or herbal preparations they may give hepato and cardioprotective action. Further study is warranted on all nutraceuticals including the above.

Acknowledgement

The authors acknowledge with thanks the founder Director of the School of Medical Education, Dr. P.G.R. Pilla for his encouragement.

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