PROTECTIVE EFFECT OF AQUEOUS LEAF EXTRACTS OF AEGLE MARMELOS AND CAMELLIA SINENSIS IN OIL INDUCED DYSLIPIDEMIC RATS

Priyanga Suriyamoorthy¹, Margret Rosaland Fathima Mary², Hemmalakshmi Subrhamanian¹, Devaki Kanagasapabathy*¹

¹Department of Biochemistry, Karpagam University, Coimbatore - 641 021
²Department of Biochemistry, Shrimati Indira Gandhi College, Thiruchirappalli- 620 002

Short title: Protective effect of Aegle marmelos and Camellia sinensis.

ABSTRACT

The present study appraised the protective activity of aqueous leaf extracts of Aegle marmelos and Camellia sinensis and its possible mechanism of action. Male Wistar albino rats were randomly assigned to five groups: Groups I normal control; Group II hyperlipidemic with coconut oil and ground nut oil (8 ml/ rat/ day); Group III Hyperlipidemic treated with Aegle marmelos extract (250 mg / kg b. wt./ day) and Group IV Hyperlipidemic treated with Camellia sinensis extract (250 mg / kg b. wt./ day) orally. The whole study lasted for 28 days. The results clearly demonstrate the positive effects of Aegle marmelos and Camellia sinensis in oil administration. The amount of free and ester cholesterol were significantly reduced in the treatment group. Amount of TBARS was elevated in the induction group as an effect of high radical generation. This is a classical marker for the cell damage, as an increase of TBARS, the cell damage in the induction group is possible which was further evidenced by the elevated SGOT and ALP levels in induction group. Amount of SOD and CAT were decreased in the induction group. Upon treatment with the plant extracts there is significant raise in SOD and CAT levels and simultaneous decrease in the levels of TBARS, SGOT and ALP. The probable mechanism of action of the plants is assumed to be by their anti-oxidant property.

Keywords: Aegle marmelos, Camellia sinensis, TBARS, SOD, CAT.
INTRODUCTION
Dys-lipidemia is a major contributor towards many chronic non-infectious diseases like atherosclerosis, diabetes, Myocardial Infarction, angina, stroke etc. Dys-lipidemia are mainly related to some genetic variations in lipid metabolism or dietary food habits or both which are highly prevalent in Indian sub-continent\(^1\). Hyperlipidemia is classified into a primary and a secondary type, which indicates the complexities associated with disease. The primary disease may be treated using anti-lipidemic drugs but the secondary type originating from diabetes, renal lipid nephrosis or hypothyroidism demands the treatment of the original disease rather than hyperlipidemia\(^2\). Medicinal plants play a major role in hypolipidemic activity, literature suggests that the lipid lowering action is mediated through, inhibition of hepatic cholesterol biosynthesis and reduction of lipid absorption in the intestine\(^3\).

When the amount of cholesterol and lipid profile levels crossed the normal levels, it induces the radical generation by its abnormal metabolites such as oxidized LDL. This further damages the antioxidant defense system as a result SOD, CAT levels were significantly reduced and increase the lipid peroxidation levels\(^4\). Certain medications like statins, fibrates, and bile acid sequestrants are recognized to cause side effects in human as well as in experimental animals by impairing the defence ability.

The present study was undertaken to compare and analyse the antioxidant and anti-hyperlipidemic effect of Aegle marmelos and Camellia sinensis. Aegle marmelos Linn. also known as Bilwa in Hindi and Bel in Bengali. This plant has shown various activities including anti-diabetic, anti-inflammatory, anti-hyperlipidemic, anti-cancer and anti-viral properties\(^5\). Several chemical constituents have been isolated from various parts of the Bael tree. These include alkaloids, coumarins and steroids. The leaves contain skimianinc, sterol and aegelin. The active constituent of the fruit is marmorosin, which is identical to imperatorin. Odler coumarins contained in the fruits are Altoimperatorin and B Sitosterol. Roots of the tree have been found to contain psoralin, xanthotoxin, scopoletin and tembamide\(^6\). Aegle marmelos leaves contains sitosterol, aegelin, lupcol, rutin, marmesinin, eugenol, β-sitosterol, flavon, glycoside, montanine, o-isopentenyl halfordiol marmelin and phenylethyl cinnamamides\(^7\).

Tea (Camellia sinensis, Theaceace) is the second most popular beverage in the world next to water and has been extensively studied for its putative disease preventive effects\(^8\). The catechol group reacts readily with oxidants in the form of free radical reactive oxygen
species to form a stable radical, the semiquinone radical. The compounds with catechol or 1,4-dihydroquinone functionality are especially easy to oxidize because the resulting phenoxy radical can be stabilized on another oxygen molecule. An EGC and GA unit can also react readily with free radicals to form stable radicals\cite{9}.

**MATERIALS AND METHODS**

**Plants**

*Aegle marmelos* and *Camellia sinensis* were used as antihyperlipidemic drugs and were procured from natural herbal shop in Tiruchirapalli and Theni respectively.

**Hyperlipidemic induction**

Mixture of pure coconut oil and ground nut oil (1:1)\cite{10} was used as the Hyperlipidemic inducer in rats. The volume of oil was 8 ml/kg body weight/day for 28 days was used to induce hyperlipidemic condition.

**Animals**

Albino Wistar male rats, weighing 100-150g were obtained from the institutional animal house, Tamil University, Thanjavur for the present investigations. The animals were housed at a room temperature of 25±2°C, relative humidity of 75±5% and 12hrs dark-light cycle; animals were fed with standard laboratory diet and water ad libitum. The study was approved by IAEC and the experiments were conducted according to the ethical norms and Institutional Animal Ethics Committee Guidelines (IAEC).

**Procurement of diagnostic kits**

Diagnostic kits used for the estimation of alkaline phosphatase, SGOT were obtained from Agappe diagnostics Limited, Kerala. The reagents used for the estimation of, TBARS, SOD, CAT were purchased from Southern India chemicals Tiruchirappalli and were of analytical grade.

**Preparation of extract**

Fresh leaves of *Aegle marmelos* and *Camellia sinensis* were shade dried ground well until it becomes into a coarse powder. Air dried coarsely powdered plant material was extracted with water for 48 hours by maceration. The 100g dried leaf powder\cite{11,12} thus obtained from water extract were filtered and vacuum dried using vacuum flash evaporator to yield the solid residue of 8.8% respectively to the starting dry powder.
Dosage preparation

0.5mg of powdered *Aegle marmelos* and *Camellia sinensis* formulations was dispersed in saline and was administered with the dose of 250 mg/ kg body weight respectively for 28 days\cite{11,12}.

Experimental design

Animals were divided into four groups and each group contains four rats. Group I rats (100±10) orally treated with saline, group II rats orally received mixture of coconut oil and ground nut oil (8ml/100g b.w.,120±10), group III rats orally received mixture of coconut oil and ground nut oil (8ml/100g b.w.,) and treated with *Aegle marmelos* (250mg/kg b.w.,120±10) orally ,group IV rats orally received mixture of coconut oil and ground nut oil (8ml/100g b.w.,) and treated with *Camellia sinensis* (250mg/kg b.w.,150±10) orally for 28 days.

At the end of the experiment, all the rats were sacrificed by jugular vein cut. Blood was collected and centrifuged for serum separation. For plasma, blood was collected with anticoagulant and centrifuged (2000g for 20 min). The tissues were dissected out, weighed and washed using ice cold saline solution. Tissues were homogenized (10% w/v) in Tris-HCl buffer (0.1 M; pH 7.4) and centrifuged at 3000g for 20 minutes at 4°C. The resulting supernatant was used for various biochemical assays.

Preparation of liver homogenate

About 200mg of liver tissue was homogenized in 10 volume of 100mM potassium phosphate buffer containing 1mM EDTA, pH 7.4 and centrifuged at 12,000g for 30min at 4⁰C. The supernatant was collected and used for following experiments as described below.

Estimation of lipid peroxidation

Lipid peroxidation (LPO) was assayed using a colorimetric method using the thiobarbituric acid (TBA)\cite{13}. Briefly, the experimental samples containing 10% tissue homogenate (approximately 1mg protein) was diluted to 500µl using extraction buffer. The reaction mixture was incubated for 1hr at 37⁰C, treated with 1ml 20% TCA and 2ml 0.67% TBA and kept in boiling water bath for 30min. After cooling, samples from all the experimental groups were centrifuged at 3000rpm for 15min. Amount of TBARS formed were measured by taking the absorbance of the supernatant at 532nm. About 99% of TBARS was
malondialdehyde (MDA). The TBARS concentration of the samples was calculated using the extraction coefficient of MDA as $1.56 \times 10^5 \text{M}^{-1}\text{cm}^{-1}$.

**Assay of antioxidant enzymes SOD and CAT**

Liver homogenate containing about 5µg protein was mixed with 50mM sodium phosphate buffer, 6µM PMT and 10µM NBT. The reaction was started by the addition of NADH and the reaction mixture was incubated at 30°C for 90s. The reaction was stopped by the addition of 1ml of glacial acetic acid and the absorbance of the chromogen formed was measured at 560nm. One unit of SOD activity was defined as the enzyme concentration required to inhibit chromogen production by 50% in 1min under the assay condition[14,15].

CAT converts H$_2$O$_2$ formed via the action of SOD on superoxide radical into water. About 5µg protein contained in liver homogenate was mixed with 2.1ml of 7.5mM H$_2$O$_2$ and time scan was performed for 10mts at 240nm at 25°C. The disappearance of peroxide depending on the CAT activity was observed. One unit of CAT activity was defined as the amount of enzyme, which reduced 1µmol of H$_2$O$_2$ per min[16].

**Estimation of free and ester cholesterol**

To 0.2ml of homogenate in a test tube, 3ml of acetone ethanol mixture was added and kept on a water bath to raise its temperature just to boiling point. This was shaken for 15mts on a vortex mixer and the precipitated protein was separated by centrifugation. The protein precipitate was washed again with 3ml of acetone ethanol mixture and the supernatants were pooled. 1ml of glacial acetic acid was added followed by a drop of 10% glacial acetic acid and the contents were mixed well.

The tubes were securely closed and kept in a dark chamber for 16hrs. The precipitated cholesterol digitonide was removed by centrifugation at 1000g for 15mts. The precipitate was then dissolved in 10ml of glacial acetic acid by heating over in a water bath. To this 1ml of isopropanol was added followed by 2ml of 0.1% ferric chloride solution and mixed well. After 5mts, 2ml of concentrated H$_2$SO$_4$ was added dropwise with constant shaking. The colour developed was read at 540nm along with a standard solution of cholesterol[17]. The esterified cholesterol was calculated from the difference between total and free cholesterol levels. Free cholesterol and cholesterol ester content of the tissue was expressed as mg/g of wet tissue.
Serum hepatospecific markers

Activities of serum glutamate oxaloacetate transaminase (SGOT) and was estimated by the method of Retimen and Frankel\[18\]. 0.05 ml of serum with 0.25 ml of substrate (aspartate and α-ketoglutarate in phosphate buffer pH 7.4) was incubated for an hour and 30 min. After incubation 1 ml of 0.4N NaOH was added and absorbance was read at 505 nm in uv-vis spectrophotometer. Activities were expressed as IU/L.

Based on the method of King and Armstrong\[19\] alkaline phosphatase activity was assayed using disodium phenyl phosphate as substrate. 1000µl of working reagent and 20µl serum was added to the test tubes. The colour developed was read at 510 nm in uv-vis spectrophotometer after 10 min. Activities of ALP was expressed as IU/L.

Statistical analysis

The data obtained in present investigation was subjected to statistical analysis. The results are expressed as Mean ± S.D. The data was analyzed using SPSS software version (10.0).

RESULTS

When dyslipidemic rats were compared with normal (Table-1) we observed a significant increase in the TBARS level in oil induced group (16.18%) compared to normal. Treatment with herbal drugs like *Aegle marmelos* (-33.22%) and *Camellia sinensis* (-48.54%) showed a significant decrease in the TBARS level. In (Table-2) we identified a significant decrease in the SOD (12.53%) and CAT levels (106.9%) in oil induced group compared to normal. Treatment with herbal drugs like *Aegle marmelos* (-73.63%; -34.97%) and *Camellia sinensis* (-34.03%; -21.92%) showed a significant increase in the SOD and CAT levels. Likewise (Table-3) shows the changes in free cholesterol and ester cholesterol levels. There was a significant increase in the free cholesterol (66.5%) and ester cholesterol levels (535.24%) in oil induced group compared to normal. Treatment with herbal drugs like *Aegle marmelos* and *Camellia sinensis* showed a significant decrease in the free cholesterol (-17.65%; -27.21%) and ester cholesterol levels (-59.87%; -62.32%). In (Table-4) there was a significant increase in the SGOT (85.85%) and ALP levels (21.82%) in oil induced group compared to normal. Treatment with herbal drugs like *Aegle marmelos* and *Camellia sinensis* showed a significant decrease in the SGOT (-21.7%; -25.13%) and ALP (-23.57%; -34.15%) levels.
Table-1 Changes in the level of TBARS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS (^*)</td>
<td>22.8 ± 1.91</td>
<td>30 ± 2.74(^d) (16.18%)</td>
<td>17.69 ± 2.18(^{NS**}) (-33.22%)</td>
<td>13.63 ± 1.51(^c) (-48.54%)</td>
</tr>
</tbody>
</table>

\(^*\) mM/100g wet tissue
Group I- Normal rats; Group II- Oil induced rats
Group III – Induction of oil and *Aegle marmelos*; Group IV - Induction of oil and *Camellia sinensis*

c - Significant different from Group II \((p<0.001)\); d - Significant different from Group I
NS*- Non significant from Group I \((p<0.1)\); NS**- Non significant from Group II \((p<0.01)\)

Table-2 Changes in the level of SOD and CAT

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (^*)</td>
<td>51.86 ± 4.83</td>
<td>45.36 ± 4.90(^{NS}) (12.53%)</td>
<td>78.76 ± 6.27(^b) (-73.63%)</td>
<td>60.8 ± 6.86(^c) (-34.03%)</td>
</tr>
<tr>
<td>CAT(^**)</td>
<td>53.01 ± 1.26</td>
<td>25.62 ± 1.37(^a) (106.9%)</td>
<td>34.47 ± 0.75(^b) (-34.97%)</td>
<td>41.39 ± 1.90(^c) (-21.92%)</td>
</tr>
</tbody>
</table>

\(^*\) Units/mg protein
\(^**\) μM of H\(_2\)O\(_2\) consumed/min/mg protein

a - Significant different from Group I \((p<0.001)\); b - Significant different from Group II \((p<0.001)\)

c - Significant different from Group II \((p<0.001)\); NS- Non significant from Group I \((p<0.1)\)
Group I- Normal rats; Group II- Oil induced rats
Group III – Induction of oil and *Aegle marmelos*; Group IV - Induction of oil and *Camellia sinensis*

Table-3 Changes in the level of free cholesterol and ester cholesterol

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
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</thead>
<tbody>
<tr>
<td>Free cholesterol(^*)</td>
<td>20.42 ± 0.92</td>
<td>34 ± 5.29(^a) (66.5%)</td>
<td>28 ± 2.16(^{NS}) (-17.65%)</td>
<td>24.75 ± 2.21(^{NS**}) (-27.21%)</td>
</tr>
<tr>
<td>Ester cholesterol(^*)</td>
<td>1.22 ± 0.17</td>
<td>7.75 ± 0.36(^a) (535.24%)</td>
<td>3.11 ± 0.60(^b) (-59.87%)</td>
<td>2.92 ± 0.17(^c) (-62.32%)</td>
</tr>
</tbody>
</table>

\(^*\) mg/g of wet tissue

a - Significant different from Group I \((p<0.001)\); b - Significant different from Group II \((p<0.001)\)

c - Significant different from Group II \((p<0.001)\); NS*- Non significant from Group II \((p<0.1)\)
NS**- Non significant from Group II \((p<0.1)\)
Group I- Normal rats; Group II- Oil induced rats
Group III –Induction of oil and *Aegle marmelos*; Group IV - Induction of oil and *Camellia sinensis*
Table-4 Changes in the level of SGOT and ALP

<table>
<thead>
<tr>
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<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGOT*</td>
<td>106 ± 3.36</td>
<td>197 ± 12.52(^a) (85.85%)</td>
<td>154.25 ± 7.41(^b) (-21.7%)</td>
<td>147.5 ± 3.11(^c) (-25.13%)</td>
</tr>
<tr>
<td>ALP*</td>
<td>283 ± 1.63</td>
<td>344.75 ± 5.25(^a) (21.82%)</td>
<td>263.5 ± 8.34(^b) (-23.57%)</td>
<td>227 ± 26.26(^c) (-34.15%)</td>
</tr>
</tbody>
</table>

\(^a\)- IU/L

Group I- Normal rats; Group II- Oil induced rats

Group III –Induction of oil and Aegle marmelos; Group IV - Induction of oil and Camellia sinensis

a – Significant different from Group I (\(p<0.001\)); b- Significant different from Group II (\(p<0.001\))

c- Significant different from Group II (\(p<0.001\))

**DISCUSSION**

Hyperlipidemia (elevated levels of triglycerides or cholesterol) and reduced HDL-C occurs as a consequence of several interrelated factors that may be lifestyle, genetic, metabolic or other conditions that influence plasma lipoprotein metabolism\(^{20}\). Oxidative stress is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases\(^{21}\). A lot of oxygenated compounds, particularly aldehydes such as malondialdehyde (MDA) and conjugated dienes, are produced during the attack of free radicals to membrane lipoproteins and polyunsaturated fatty acids. Enzymic superoxide dismutase (SOD) and catalase (CAT) and nonenzymic antioxidants play an important role in alleviating tissue damage due to the formation of free radicals. A lot of studies have found that serum MDA are higher in subjects with hyperlipidemia and decrease following dietary supplementation with antioxidants. Similar observations have been reported in animal models of hyperlipidemia\(^{22,23}\). Elevated serum concentration of total cholesterol, LDL-C appears to increase the risk of an individual in developing Coronary Heart Disease (CHD). Lipid lowering therapy is indicated in the primary and secondary prevention of CVD in addition to the management of all other risk factors including smoking, diabetes and obesity\(^{24}\).

Level of TBARS was elevated (p<0.1) in the oil induced rats when compared to control. This variation was due to the formation of free radicals. Free radical generation which upregulate the level of TBARS. Upon treatment with Aegle marmelos and Camellia sinensis the level of TBARS was significantly reduced (p<0.001) in the treatment group. The possible mechanism
could be the phytochemicals terpenoids and coumarins present in *Aegle marmelos* and catechin in *Camellia sinensis* may scavenged the free radicals producing during the administration of high cholesterol. Being a lipid-soluble chain-breaking antioxidant, α-tocopherol reacts with superoxide and lipid peroxyradicals to inhibit lipid peroxidation. Similar results were observed with Rajadurai[25] on demonstration of their drug for the inhibition of free radical formation and decrease in the TBARS levels. Our drug may also use the same mechanism of reduction in the TBARS.

The level of SOD and CAT (p<0.1) was decreased in the oil induced rats when compared to the control group. The altered balance of antioxidant enzymes caused by decrease in SOD and CAT activities may responsible for the inadequacy of antioxidant defence. The decreased activity of CAT and SOD may response to increase production of H$_2$O$_2$ and O$_2$ by the auto oxidation of glucose and non-enzymatic glycation. On treatment with *Aegle marmelos* and *Camellia sinensis* the level of SOD and CAT was significantly increased (p<0.001) and brought back to near normal. Similar results were identified by Devendra Singh in his hepatoprotective study[26]. The polyphenols and epigallo catechin in *Camellia sinensis* and polyphenols, terpenoids in *Aegle marmelos* might be responsible for the free radical scavenging mechanism. It was already evidenced that the administration of plant drug treated rats showed decreased lipid peroxidation, which was associated with increased activity of SOD and CAT[27, 28].

Significant (P<0.001) increase in levels of both free and ester cholesterol were also observed in plasma of rats fed with high fat diet. This high cholesterol concentration in circulation may be damage the endothelial cells lining the large arteries and aorta and this may be an initial event in the etiology of atherosclerosis. Both plasma free and ester cholesterol reduced remarkably on treating the HFD rats with methanolic extract of *Ipomoea digitata*[29] and this supports the antioxidant activity of herbal drugs. In our experiment also we got similar results that the ester cholesterol and free cholesterol were significantly increased in the oil induced rats when compared to control. This elevated level of cholesterol (p<0.001) was due to the deposition of hepatic cholesterol as a result of administration of coconut and ground nut oil. Upon treatment with the drug *Aegle marmelos* and *Camellia sinensis*, the cholesterol level was brought back to near normal level (p<0.001). It was also evidenced that the treatment with the ethanolic extract of *Aegle marmelos* leaves inhibited the significant elevation of total cholesterol when compared to that of dyslipidemic animals[30].
There was a significant elevation of SGOT and ALP (p<0.001) in oil induced rats when compared to control. High cholesterol administration increases the radical generation in the hepatocytes by reducing SOD and CAT levels. This increase in radicals can directly damage the liver cells as a result SGOT and ALP were elevated in the blood of induction animals. On treatment with Aegle marmelos and Camellia sinensis there was a significant reduction (p<0.001) in the level of SGOT and ALP. The possible mechanism could be free radical scavenging ability of the plant drugs. Polyphenols in Camellia sinensis and alkaloids present in Aegle marmelos drug may scavenge the free radicals as a result the liver damage may also reduced in the treatment group.

CONCLUSION

The present study clearly demonstrates the positive effects of Aegle marmelos and Camellia sinensis in a high cholesterol administration. Therefore we suggest that active components of Aegle marmelos and Camellia sinensis have a significant antioxidant and anti-hyperlipidemic effect. Among the selected herbal drugs, Camellia sinensis proved better antioxidant activity than Aegle marmelos. Since the effect of drugs were highly beneficial without any side effects, the research about the Aegle marmelos and Camellia sinensis can be further extrapolated to the humans for the service of our society.

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