Appendices

Appendix-1

Institutional Animal Ethics Committee Approval

L.M.COLLEGE OF PHARMACY, AHMEDABAD
DEPARTMENT OF PHARMACOLOGY
INSTITUTIONAL ANIMAL ETHICS COMMITTEE
(Constituted as per CPCSEA, Ministry of Social Justice and Empowerment, Registration No. 228

CERTIFICATE

This is to certify that the Research Project No. LMCP/07/03 entitled

Investigations on therapeutic potential of Clitoria ternatea Linn. in hepatic dysfunction, inflammation and associated disorders using experimental animals.

submitted by Yogendra Sinh B. Slanki under the guidance of

Dr. S. M. Jain has been approved by the committee (IAEC).

It is expected that the animals are treated in human way and disposed off as per the norms and protocol given. Attention may be paid to save animals sanctioned.

(Dr. Ramesh K. Goyal)
Member Secretary

(Dr. Mukesh C. Gohel)
Chairman and Principal

(Dr. Rajesh Bhavsar)
CPCSEA, Nominee

Appendix-2

List of Publications


Presentation

Antihyperlipidemic activity of Clitoria ternatea and Vigna mungo in rats

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Abstract

Hyperlipidemia is one of the major risk factors for atherosclerosis and cardiovascular diseases. Some plants are effective in controlling hyperlipidemia.

Objective: To investigate the anti-hyperlipidemic effect of Clitoria ternatea L. and Vigna mungo L. (Fabaceae) on experimentally induced hyperlipidemia in rats.

Materials and methods: The poloxamer 407-induced acute hyperlipidemia and diet-induced hyperlipidemia models were used for this investigation.

Results: Oral administration of the hydroalcoholic extract of the seeds of C. ternatea and the hydroalcoholic extract of the seeds of V. mungo resulted in a significant (p<0.05) reduction of serum total cholesterol, triglycerides, very low-density lipoprotein cholesterol, and low-density lipoprotein cholesterol levels. The atherogenic index and the HDL/LDL ratio were also normalized after treatment in diet-induced hyperlipidemic rats. The effects were compared with atorvastatin (50 mg/kg, p.o.) and gemfibrozil (50 mg/kg, p.o.), reference standards.

Discussion: The cholesterol-lowering effect of C. ternatea might be attributed to increased biliary excretion and decreased absorption of dietary cholesterol. The cholesterol-lowering effects of V. mungo seeds might be because of decreased HMG CoA reductase activity, increased biliary excretion, and decreased absorption of dietary cholesterol. Additionally, they improved natural antioxidant defense mechanisms.

Conclusion: The findings of the investigation suggest that C. ternatea and V. mungo have significant antihyperlipidemic action against experimentally induced hyperlipidemia.

Keywords: Antihyperlipidemic activity; Clitoria ternatea; Vigna mungo; poloxamer 407

Introduction

Hyperlipidemia plays a significant role in the manifestation and development of atherosclerosis, leading to cardiovascular diseases (Ross, 1990). Atherosclerosis involves the interplay of several factors (Singh et al., 2002). There are three major factors, viz. hyperlipidemia, clotting factors, and oxidation of lipoproteins, which play a crucial role in atherosclerosis and collectively contribute to the development and rupture of atherosclerotic plaques. It is the oxidation of low-density lipoprotein (LDL) that plays a major role in atherosclerotic plaque development (Steinberg et al., 1989).

Various indigenous plants are used for antihyperlipidemic effects in the Ayurvedic system of medicine in India. Two such plants, Clitoria ternatea L. and Vigna mungo L., both belonging to the family Fabaceae, were reported to be used in a variety of disease conditions of the liver (Chopra et al., 1956; Kirtikar & Basu, 1976). Various parts of C. ternatea have been reported to have nootropic activity, anxiolytic activity, tranquillizing property, anti-inflammatory and analgesic activity, antipyretic activity, and antimicrobial activity (Mukherjee et al., 2008). C. ternatea has been reported to contain kaempferol and related glycosides, apurajitin, anthocyanins (Shrivastava & Pande, 1977), γ-sitosterol and related sterols (Sinha, 1960), hexacosanol, β-sitosterol, and anthoxanthins (Gupta & Lal, 1968). V. mungo is a rich source of protein, carbohydrates, oil, iron, potassium, and vitamin B (Swaminathan & Jain, 1975). It also contains phenolics (vitetin and isovitexin) (Peng et al., 2008), polyphenolics (phytic acid and tannic acid), trypsin inhibitors, and...
aromatic constituents such as hexanol, benzyl alcohol, \(\gamma\)-butyrolactone, methyl-2-propenol, and pentanol (Lee & Shibamoto, 2000). High-protein diets have been reported to reduce the risk of atherosclerosis (Meeker & Kesten, 1941; Wolfe & Giovannetti, 1992; Wolfe, 1995). Legumes are a rich source of dietary proteins. Hence, we investigated two leguminous plants, C. ternatea and V. mungo, for their possible antihyperlipidemic activity using poloxamer 407-induced acute hyperlipidemia and diet-induced hyperlipidemia models.

**Materials and methods**

**Plant collection and identification**

The plant C. ternatea is available in two varieties - blue and white. Since the blue variety is medicinally important, it was used for the present investigation. The plant was collected during April–May, 2007, from the fields and roadside of the Charotar region of Gujarat state, India. The seeds of V. mungo were purchased from the local market of the same region. Both plants were botanically identified by Dr. G. C. Jadeja, Professor and Head of Agricultural Botany Department, B. A. College of Agriculture, Anand, India. Specimens of each were stored in the museum of the department (specimen nos. 0701 and 0702). The quality of the plants was ascertained per the Ayurvedic formulary of India by determining foreign matter, total ash, acid-insoluble ash, alcohol-soluble extractive, and water-soluble extractive values (Anonymous, 2003).

**Extraction**

The dried, powdered (mesh no. 40) seeds of both plants were defatted with petroleum ether and then extracted with 50% v/v alcohol by maceration. The solvents were evaporated at 60°C to a pasty mass referred to as C. ternatea and V. mungo seed extracts, respectively. The roots of C. ternatea were directly extracted with 50% v/v alcohol by maceration and the solvents were evaporated at 60°C to yield the hydroalcoholic extract of the root, referred to as C. ternatea root extract.

**Chemicals and reagents**

All the chemicals used were of analytical grade. Poloxamer 407 (P-407) was obtained from Sun Pharmaceutical Ltd., Vadodara, Gujarat. Cholesterol and cholic acid were obtained from S. D. Fine Chemicals Ltd., Mumbai. Atorvastatin and gemfibrozil were received as gift samples from Zydus Research Center, Ahmedabad, Gujarat. The solvents and reagents were also from S. D. Fine Chemicals Ltd.

**Pharmacological evaluation**

**Animals**

Albino rats (SD strain) weighing 150–200 g of either sex were divided into different groups, each consisting of six animals. Animals were maintained on a commercial pellet diet (Pranav Agro Industries Ltd., Sangli, India) and water ad libitum throughout the study period. This study was approved by the institutional animal ethics committee in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, 2003).

**Drugs and extracts**

All the extracts were suspended in distilled water using 1% w/v gum acacia. The reference drugs atorvastatin and gemfibrozil were suspended in distilled water using 1% carboxymethylcellulose (CMC). The control group received 1% w/v gum acacia in distilled water and 1% CMC solution as vehicles.

**Acute toxicity studies**

Animals were treated with different doses up to 1500 mg/kg, p.o. of each extract. After single-dose administration, animals were observed for mortality or any other deformities up to 72 h.

**Poloxamer 407-induced hyperlipidemia in rats**

Acute hyperlipidemia was induced in rats using poloxamer 407 (Johnston & Palmer, 1993). Animals were divided into different groups (Table 1). All the extracts were administered orally about 1 h before the i.p. injection of 1 mL of 30% w/v solution of P-407. Animals were

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**Table 1. Poloxamer 407-induced acute hyperlipidemia model: summary of animal groups and treatments.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Vehicles (1 mL of 1% gum acacia and 1% CMC)</td>
</tr>
<tr>
<td>Hyperlipidemic control</td>
<td>Poloxamer 407 (1 mL of 30% w/v solution, i.p.)</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>Poloxamer 407 (1 mL of 30% w/v solution, i.p.) + atorvastatin (50 mg/kg, p.o.)</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>Poloxamer 407 (1 mL of 30% w/v solution, i.p.) + gemfibrozil (50 mg/kg, p.o.)</td>
</tr>
<tr>
<td>CT-seed extract</td>
<td>Poloxamer 407 (1 mL of 30% w/v solution, i.p.) + C. ternatea seed extract (500 mg/kg, p.o.)</td>
</tr>
<tr>
<td>CT-root extract</td>
<td>Poloxamer 407 (1 mL of 30% w/v solution, i.p.) + C. ternatea root extract (500 mg/kg, p.o.)</td>
</tr>
<tr>
<td>VM-seed extract</td>
<td>Poloxamer 407 (1 mL of 30% w/v solution, i.p.) + V. mungo seed extract (500 mg/kg, p.o.)</td>
</tr>
</tbody>
</table>

**Note.** CT, C. ternatea; VM, V. mungo; CMC, carboxymethylcellulose.
fed a normal chow diet throughout the study. Blood samples were collected at 15 and 24 h after P-407 injection and investigated for lipid profiles.

**Diet-induced hyperlipidemia in rats**

The method of Blank et al. (1963), with modification, was used to produce diet-induced hyperlipidemia. Animals were divided into different groups (Table 2). Briefly, the normal group received a standard chow diet and all other groups received a high-cholesterol diet consisting of normal chow diet 92%, cholesterol 2%, cholic acid 1%, and coconut oil 5% for 7 days. The reference drugs and extracts were administered once daily between 9:00 a.m. and 5:00 p.m. for 7 days. The daily food intakes were determined before treatments.

On the last day, animals were deprived of food but not water. Blood samples were collected by retroorbital puncture technique under light anesthesia. The animals were sacrificed and liver tissues were collected and preserved at -40°C for further analysis. The fecal matters of the last 24 h before fasting were collected, immediately dried in an oven at 24 h before fasting were collected, immediately dried in an oven at 24°C for 1 h, and stored at -40°C for further analysis.

**Estimation of biochemical parameters**

**Lipid profile**

The serum lipid profile was determined at 15 and 24 h after P-407 injection and on day 8 in the case of diet-induced hyperlipidemia. The total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) levels were estimated using commercially available kits (Erba; Transasia Bio-Medicals Ltd., Daman, India). Very low-density lipoprotein cholesterol (VLDL-C) was calculated as TG/5. LDL-cholesterol (LDL-C) levels were calculated using Friedewald's formula (Friedewald et al., 1972). The atherogenic index was calculated using the formula: atherogenic index (AI) = (VLDL-C + LDL-C)/HDL-C.

**HMG-CoA reductase activity**

The activity of the enzyme HMG-CoA (3-hydroxy-3-methyl-glutaryl-coenzyme-A) reductase was determined by an indirect method (Rao & Ramakrishnan, 1975). The method estimates the HMG-CoA/mevalonate ratio as an index of the activity of HMG-CoA reductase. The liver tissue was removed as quickly as possible and a 10% homogenate was prepared in saline arsenate solution. The homogenate was deproteinized using an equal volume of dilute perchloric acid and allowed to stand for 5 min, before centrifugation. To 1 mL of the filtrate, 0.5 mL of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG-CoA) was added. This was mixed, and 1.5 mL of ferric chloride reagent was added after 5 min. The absorbance was read after 10 min at 540 nm against a similarly treated saline arsenate blank. The ratio of HMG-CoA/mevalonate was calculated.

**Fecal cholesterol and bile acid excretion**

Fecal matter was collected during the last 24 h before fasting in the diet-induced hyperlipidemia model. The dried and powdered fecal matter was extracted with alkaline methanol. The resultant extract was then analyzed for cholesterol content in a manner similar to that of the serum. The cholesterol excreted in the fecal matter was calculated and expressed as mg/g of fecal matter. The method of Evrard and Janssen (1968) modified by Manes and Schneider (1971) was used for fecal bile acid extraction, and bile acid levels were estimated by the colorimetric method (Snell & Snell, 1954) and expressed as cholic acid equivalent per g of fecal matter.

**Phenobarbitone-induced sleeping time**

To investigate the hepatic HMG-CoA reductase enzyme inhibition, potentiation of the phenobarbitone-induced sleeping time was measured in rats (Walker & Parry, 1949). Animals were divided into different groups, each consisting of six. The control group received a single dose of phenobarbitone (80 mg/kg, i.p.). The treatment groups received 500 mg/kg, p.o. of each test extract 1 h before the phenobarbitone injection. The animals were observed for righting reflex. If the animals failed to maintain normal posture when placed on one side within 30 s, it was considered as a loss of righting reflex.

**Lipid peroxidation and antioxidant parameters in the liver**

In the diet-induced hyperlipidemia model, animals were dissected at the end of the study and the liver was collected, washed thoroughly in normal saline, bloated,

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**Table 2. Diet-induced hyperlipidemia model: summary of animal groups and treatments.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Vehicles (1 mL of 1% gum acacia and 1% CMC)</td>
</tr>
<tr>
<td>Hyperlipidemic control</td>
<td>High cholesterol diet</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>High cholesterol diet + atorvastatin (50 mg/kg, p.o.)</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>High cholesterol diet + gemfibrozil (50 mg/kg, p.o.)</td>
</tr>
<tr>
<td>CT-seed extract</td>
<td>High cholesterol diet + C. ternatea seed extract (500 mg/kg, p.o.)</td>
</tr>
<tr>
<td>CT-root extract</td>
<td>High cholesterol diet + C. ternatea root extract (500 mg/kg, p.o.)</td>
</tr>
<tr>
<td>VM-seed extract</td>
<td>High cholesterol diet + V. mungo seed extract (500 mg/kg, p.o.)</td>
</tr>
<tr>
<td>VM-seed powder</td>
<td>High cholesterol diet + V. mungo seed powder (10% of diet)</td>
</tr>
</tbody>
</table>

*Note:* CT, *C. ternatea*; VM, *V. mungo*; CMC, carboxymethylcellulose.

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and preserved at −40°C for further analysis. The liver homogenates were prepared in a Tris-hydrochloride buffer (0.1M, pH 7). They were subjected to protein (Lowry et al., 1951), malondialdehyde (Okhawa et al., 1979), superoxide dismutase (SOD) (Misra & Fridovich, 1973), catalase (Aebi, 1974), reduced glutathione (GSH) (Beutler et al., 1963), NO scavenging (Sreejayan & Rao, 1997; Nakagawa & Yokozawa, 2002), and myeloperoxidase activity (Zhang et al., 2001) estimation.

**Serum ascorbic acid levels**

Serum total ascorbic acid (TAA), l-ascorbic acid (LAA), and dehydroascorbic acid (DAA) levels were estimated according to the method of Schaffert and Kingsley (1955).

**Statistical analysis**

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's test. The level \( p < 0.05 \) was considered statistically significant.

**Results**

**Acute toxicity study**

No death or deformities were observed in any of the treatment groups up to 72 h. All of the extracts were found to be safe up to the dose level of 1 g/kg body weight.

**Food intake**

No significant difference in food intake among the different groups was observed (Table 3).

**Poloxamer 407-induced acute hyperlipidemia**

The serum TC, TG, LDL-C, and VLDL-C levels were significantly increased in the hyperlipidemic control group at 15 h (Table 4) and 24 h (Table 5), when compared with the normal group. Both *C. ternatea* root extract and *V. mungo* seed extract significantly reduced serum TC and TG levels at 15 and 24 h. These effects were comparable to those of the reference standard, atorvastatin. The *V. mungo* seed powder significantly increased serum HDL-C levels, and serum VLDL-C levels were significantly decreased in all the treatment groups at 24 h. The serum LDL-C levels were significantly decreased by gemfibrozil only at 15 h. However, the HDL-C/LDL-C ratio was not altered by any of the treatment groups (Table 6).

**Diet-induced hyperlipidemia**

There was a significant increase in the serum levels of TC, TG, LDL-C, VLDL-C, and AI in the hyperlipidemic control group as compared with the normal control group. All the treatment groups produced a significant decrease in serum TC, TG, HDL-C, and VLDL-C levels (Table 7) and AI (Table 6). In addition to the above, the serum LDL-C levels were significantly decreased by the *C. ternatea* root extract and the *V. mungo* seed extract.

**Fecal bile acid and cholesterol excretion**

The *C. ternatea* seed and root extracts and *V. mungo* seed extract significantly increased fecal cholesterol excretion (Table 8). The fecal bile acid excretion was significantly increased by all the treatment groups except atorvastatin.

**Liver HMG-CoA reductase activity**

The HMG-CoA/mevalonate ratio was significantly increased by the *V. mungo* seed extract as compared to the hyperlipidemic control group (Table 9).

**Lipid peroxidation**

Lipid peroxidation and antioxidant status were studied in the liver tissues of diet-induced hyperlipidemic rats. The liver MDA level, SOD level, NO scavenging activity, and myeloperoxidase activity were studied in the liver tissues of diet-induced hyperlipidemic rats. The liver MDA level, SOD level, NO scavenging activity, and myeloperoxidase activity were studied in the liver tissues of diet-induced hyperlipidemic rats.
Table 4. Effect of *C. ternatea* and *V. mungo* on serum lipid profile 15 h after poloxamer 407-induced acute hyperlipidemia in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>TC (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>LDL (mg/dL)</th>
<th>VLDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>244.30 ± 5.89*</td>
<td>396.01 ± 4.82*</td>
<td>82.60 ± 1.44*</td>
<td>82.09 ± 8.02*</td>
<td>79.60 ± 0.96*</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>198.14 ± 7.47**</td>
<td>315.99 ± 6.02**</td>
<td>70.14 ± 2.40</td>
<td>64.80 ± 9.42</td>
<td>63.20 ± 1.20</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>183.53 ± 6.67**</td>
<td>352.83 ± 2.53*</td>
<td>77.61 ± 1.79</td>
<td>35.35 ± 2.37**</td>
<td>70.57 ± 0.51</td>
</tr>
<tr>
<td>CT-seed extract</td>
<td>230.36 ± 11.44</td>
<td>327.12 ± 7.53**</td>
<td>80.84 ± 2.52</td>
<td>84.10 ± 10.43</td>
<td>65.42 ± 0.51</td>
</tr>
<tr>
<td>CT-root extract</td>
<td>203.29 ± 4.91**</td>
<td>332.95 ± 9.03**</td>
<td>61.19 ± 3.10**</td>
<td>75.52 ± 5.41</td>
<td>66.59 ± 1.81</td>
</tr>
<tr>
<td>VM-seed extract</td>
<td>194.80 ± 5.14**</td>
<td>300.30 ± 14.74**</td>
<td>85.31 ± 5.99</td>
<td>49.43 ± 11.91</td>
<td>60.06 ± 2.95</td>
</tr>
</tbody>
</table>

*Note. All values represent mean ± SEM from six animals. *Compared with normal group (p<0.05). **compared with hyperlipidemic control group (p<0.05).* TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol; CT, *C. ternatea*; VM, *V. mungo.*

Table 5. Effect of *C. ternatea* and *V. mungo* on serum lipid profile 24 h after poloxamer 407-induced acute hyperlipidemia in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>TC (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>LDL (mg/dL)</th>
<th>VLDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>66.15 ± 2.35</td>
<td>54.56 ± 1.72</td>
<td>23.72 ± 0.92</td>
<td>31.52 ± 2.82</td>
<td>10.91 ± 0.34</td>
</tr>
<tr>
<td>Control</td>
<td>35.07 ± 2.71</td>
<td>35.35 ± 9.03**</td>
<td>61.19 ± 3.10**</td>
<td>75.52 ± 5.41</td>
<td>66.59 ± 1.81</td>
</tr>
</tbody>
</table>

*Note. All values represent mean ± SEM from six animals. *Compared with normal group (p<0.05). **compared with hyperlipidemic control group (p<0.05).* TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol; CT, *C. ternatea*; VM, *V. mungo.*

Table 6. Effect of *C. ternatea* and *V. mungo* on atherogenic index and HDL/LDL ratio.

<table>
<thead>
<tr>
<th>Group</th>
<th>TC (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
<th>LDL-C (mg/dL)</th>
<th>Diet-induced hyperlipidemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>133.64 ± 2.97</td>
<td>100.46 ± 4.16</td>
<td>61.12 ± 1.32</td>
<td>52.43 ± 2.45</td>
<td>20.09 ± 0.83</td>
</tr>
<tr>
<td>Hyperlipidemic control</td>
<td>328.23 ± 6.95*</td>
<td>303.80 ± 12.59*</td>
<td>104.56 ± 3.54*</td>
<td>162.91 ± 9.58</td>
<td>60.76 ± 2.52*</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>162.09 ± 6.97**</td>
<td>162.99 ± 1.95**</td>
<td>66.87 ± 2.47*</td>
<td>63.25 ± 6.48**</td>
<td>32.60 ± 0.39**</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>196.41 ± 6.32**</td>
<td>128.46 ± 4.23*</td>
<td>71.67 ± 1.04*</td>
<td>99.64 ± 7.09*</td>
<td>25.68 ± 0.85*</td>
</tr>
<tr>
<td>CT-seed extract</td>
<td>242.26 ± 3.45**</td>
<td>108.42 ± 12.12**</td>
<td>66.50 ± 2.41*</td>
<td>154.08 ± 3.61</td>
<td>21.68 ± 0.42*</td>
</tr>
<tr>
<td>CT-root extract</td>
<td>207.56 ± 8.91**</td>
<td>130.51 ± 5.43**</td>
<td>84.92 ± 2.31*</td>
<td>116.62 ± 7.72**</td>
<td>26.18 ± 1.09*</td>
</tr>
<tr>
<td>VM-seed extract</td>
<td>238.56 ± 7.98**</td>
<td>127.67 ± 7.10**</td>
<td>73.37 ± 2.50*</td>
<td>139.65 ± 7.14**</td>
<td>25.53 ± 1.42*</td>
</tr>
<tr>
<td>VM-seed powder</td>
<td>238.72 ± 2.48**</td>
<td>115.42 ± 9.37**</td>
<td>71.88 ± 1.98*</td>
<td>143.75 ± 4.06</td>
<td>23.09 ± 1.87*</td>
</tr>
</tbody>
</table>

*Note. All values represent mean ± SEM from six animals. *Compared with normal group (p<0.05). **compared with hyperlipidemic control group (p<0.05).* TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol; CT, *C. ternatea*; VM, *V. mungo.*
myeloperoxidase activity were significantly increased in the hyperlipidemic control group (Table 10). The GSH level and catalase activities were significantly decreased in the hyperlipidemic control group, suggesting the presence of significant oxidative stress. The liver MDA, SOD, myeloperoxidase, and NO scavenging activities were significantly reduced by pretreatment with all the extracts. The liver GSH and catalase activities were increased in all the treatment groups when compared with the hyperlipidemic control group. The V. mungo seed extract showed a significant decrease in myeloperoxidase activity compared with all other treatments, but the V. mungo seed powder did not produce an increase in serum GSH levels.

Serum TAA, LAA, and DAA levels

The serum total ascorbic acid levels were significantly increased by all the treatment groups. Considering L- and D-ascorbic acids, L-ascorbic acid is biologically more active. The serum L-ascorbic acid levels were significantly increased by all the extracts except C. ternatea root extract. The dehydroascorbic acid levels were significantly increased by V. mungo seed powder (Table 11).

Potentiation of phenobarbitone-induced sleeping time

To investigate hepatic enzyme inhibition as a possible mechanism, we studied the effects of all the extracts on the phenobarbitone-induced sleeping time. The V. mungo seed extract significantly prolonged the phenobarbitone-induced sleeping time (Table 12). The C. ternatea seed extract significantly decreased the phenobarbitone-induced sleeping time. The C. ternatea root extract did not alter the phenobarbitone-induced sleeping time.

Discussion

In the present study, we have investigated the effect of C. ternatea (seed and root extracts) and V. mungo (seed extract and powdered seeds) against experimentally induced hyperlipidemia in rats. All the extracts at the dose of 500 mg/kg, p.o. significantly reduced serum TC and TG levels. The C. ternatea seed extract mainly affected serum TG levels and the root extract affected both TC and TG levels in the P-407-induced acute hyperlipidemia model. The serum TC and TG levels were reduced by both extracts of C. ternatea in the diet-induced hyperlipidemia model. V. mungo seed extract as well as seed powder reduced both serum TC and TG levels in both hyperlipidemia models. Additionally, this decrease in TC levels corresponded significantly to a reduction in LDL-C levels. These findings were supported by a decrease in atherogenic index and an increase in the HDL-C/LDL-C ratio. Since P-407-induced hyperlipidemia is mainly due to inhibition of the extractable (heparin releasable) pool of lipoprotein lipase (Johnston & Palmer, 1993), the serum TG-lowering effects can be attributed to the activation of lipoprotein lipase.

It is reported that cholesterol homeostasis is maintained by the control of two processes, viz. cholesterol biosynthesis, in which HMG-CoA reductase catalyzes the rate-limiting process, and cholesterol absorption of both dietary cholesterol and cholesterol cleared from the liver through biliary secretion. The HMG-CoA/mevalonate ratio has an inverse relationship to the activity of HMG-CoA reductase (Rao & Ramakrishnan, 1975). The results of the study indicated that the activity of the enzyme was significantly depressed by the V. mungo seed extract as was evident by the increase in the ratio. Furthermore, there was also an increase in the cholesterol content of the fecal matter, indicating that all the extract either promoted the excretion of cholesterol or prevented the absorption of cholesterol. Since the fecal bile acid levels were significantly increased, they might have promoted the cholesterol excretion.
Table 10. Effects of C. ternatea and V. mungo on lipid peroxidation and antioxidant parameters in liver tissues of diet-induced hyperlipidemic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>Hyperlipid. control</th>
<th>Atorvastatin</th>
<th>Gemfibrozil</th>
<th>CT-seed extract</th>
<th>CT-root extract</th>
<th>4-seed extract</th>
<th>VM-seed powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>2.39 ± 0.22</td>
<td>8.82 ± 0.68*</td>
<td>4.17 ± 0.35**</td>
<td>4.37 ± 0.29**</td>
<td>4.19 ± 0.31**</td>
<td>3.10 ± 0.33**</td>
<td>1.77 ± 0.38**</td>
<td>2.33 ± 0.35**</td>
</tr>
<tr>
<td>SOD (U/min/mg protein)</td>
<td>2.00 ± 0.20</td>
<td>31.01 ± 2.96*</td>
<td>11.96 ± 2.18**</td>
<td>12.67 ± 1.67**</td>
<td>9.54 ± 1.06**</td>
<td>3.94 ± 0.45**</td>
<td>1.39 ± 0.34**</td>
<td>4.48 ± 0.77**</td>
</tr>
<tr>
<td>Catalase (U/min/mg protein)</td>
<td>0.36 ± 0.03</td>
<td>0.26 ± 0.02</td>
<td>0.30 ± 0.002</td>
<td>0.31 ± 0.02</td>
<td>0.39 ± 0.02**</td>
<td>0.34 ± 0.01**</td>
<td>1.48 ± 0.02**</td>
<td>0.35 ± 0.02**</td>
</tr>
<tr>
<td>GSH (µg/mg protein) × 10⁴</td>
<td>0.0419 ± 0.0046</td>
<td>0.0119 ± 0.0002*</td>
<td>0.0125 ± 0.0002</td>
<td>0.0151 ± 0.0008</td>
<td>0.0240 ± 0.0012**</td>
<td>0.0248 ± 0.0016**</td>
<td>3.35 ± 0.0049**</td>
<td>0.0201 ± 0.0011**</td>
</tr>
<tr>
<td>NO scav. activity (nmol/mg protein)</td>
<td>134.45 ± 5.73</td>
<td>1545.35 ± 160.72*</td>
<td>546.02 ± 34.66**</td>
<td>372.93 ± 36.15**</td>
<td>351.19 ± 32.90**</td>
<td>380.33 ± 27.89**</td>
<td>1.33 ± 0.35**</td>
<td>415.63 ± 20.30**</td>
</tr>
<tr>
<td>Myeloperoxidase activity × 10⁴ (U/mg protein)</td>
<td>2.18 ± 0.22</td>
<td>5.67 ± 0.64*</td>
<td>3.38 ± 0.31**</td>
<td>4.41 ± 0.24**</td>
<td>1.423 ± 0.10**</td>
<td>3.07 ± 0.29**</td>
<td>1.82 ± 0.04**</td>
<td>3.79 ± 0.47**</td>
</tr>
</tbody>
</table>

Note. All values represent mean ± SEM from six animals. *Compared with normal group (p < 0.05). **compared with control group (p < 0.05). †compar th atorvastatin (p < 0.05).
Table 11. Effect of C. ternatea and V. mungo on serum total ascorbic acid (TAA), L-ascorbic acid (LAA), and dehydroascorbic acid (DAA) levels in diet-induced hyperlipidemic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>TAA (mg/dl)</th>
<th>LAA (mg/dl)</th>
<th>DAA (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.69 ± 0.07</td>
<td>2.00 ± 0.05</td>
<td>1.69 ± 0.11</td>
</tr>
<tr>
<td>Hyperlipidemic</td>
<td>3.26 ± 0.13</td>
<td>2.58 ± 0.14</td>
<td>0.68 ± 0.13*</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>3.41 ± 0.07</td>
<td>2.66 ± 0.14</td>
<td>0.78 ± 0.12</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>3.55 ± 0.17</td>
<td>2.50 ± 0.08</td>
<td>1.05 ± 0.12</td>
</tr>
<tr>
<td>CT-seed extract</td>
<td>4.51 ± 0.26**</td>
<td>4.05 ± 0.19**</td>
<td>0.62 ± 0.08</td>
</tr>
<tr>
<td>CT-root extract</td>
<td>3.05 ± 0.10</td>
<td>2.62 ± 0.08</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>VM-seed extract</td>
<td>5.15 ± 0.43**</td>
<td>4.33 ± 0.33**</td>
<td>0.81 ± 0.11</td>
</tr>
<tr>
<td>VM-seed powder</td>
<td>5.86 ± 0.27**</td>
<td>4.22 ± 0.21**</td>
<td>1.63 ± 0.16**</td>
</tr>
</tbody>
</table>

Note. All values represent mean ± SEM of six animals. *Compared with normal (p < 0.05). **Compared with hyperlipidemic control (p < 0.05). Values are in mg/g of liver tissue. CT, C. ternatea; VM, V. mungo.

Table 12. Effects on phenobarbitone-induced sleeping time.

<table>
<thead>
<tr>
<th>Group (n = 10)</th>
<th>Treatments</th>
<th>Induction time (min)</th>
<th>Sleeping time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle + phenobarbitone</td>
<td>50.6 ± 1.69</td>
<td>78.6 ± 2.71</td>
</tr>
<tr>
<td>CT-seed extract</td>
<td>C. ternatea seed extract (500 mg/kg, p.o.) + phenobarbitone (80 mg/kg, p.o.)</td>
<td>51.2 ± 2.21</td>
<td>45.3 ± 1.78*</td>
</tr>
<tr>
<td>CT-root extract</td>
<td>C. ternatea root extract (500 mg/kg, p.o.) + phenobarbitone (80 mg/kg, p.o.)</td>
<td>54.5 ± 1.99</td>
<td>79.7 ± 4.08</td>
</tr>
<tr>
<td>VM-seed extract</td>
<td>V. mungo seed extract (500 mg/kg, p.o.) + phenobarbitone (80 mg/kg, p.o.)</td>
<td>25.3 ± 3.91**</td>
<td>307.0 ± 4.18**</td>
</tr>
</tbody>
</table>

Note. All values represent mean ± SEM of 10 rats. p < 0.05 was considered statistically significant (one-way ANOVA followed by Tukey’s test). **Significant increase when compared with control group. *Significant decrease when compared with control group. CT, C. ternatea; VM, V. mungo.

Lipid peroxidation is the key factor leading to atherosclerotic plaque formation. It is the oxidized LDL that is responsible for chemotaxis of macrophages and the subsequent cascade of events to form plaques (Schaffert & Kingsley, 1955). The results of the present study indicated that lipid peroxidation was significantly reduced by all the extracts. Additionally, they improved the liver antioxidant status by improving the activities of the various enzymes.

The L-ascorbic acid is the only biologically active form, playing a vital role as a natural antioxidant against a variety of stress conditions including lipid peroxidation (Frei et al., 1990). It is easily converted into dehydroascorbic acid, thereby regenerating vitamin E. It also maintains high intracellular levels of glutathione (Meister, 1994).

The findings of this study showed significantly higher levels of the serum total and L-ascorbic acid levels in all the extract-pretreated groups, suggesting a marked reduction in oxidative stress. This can partly be supported by increased glutathione levels in the liver tissues.

In conclusion, C. ternatea seed and root extracts and V. mungo seed extract possess significant lipid-lowering activities against experimentally induced hyperlipidemia. The TG-lowering effects might be attributed to an increase in lipoprotein lipase activities. The cholesterol-lowering effects of V. mungo seeds can be related partly to the decreased activity of hepatic HMG-CoA reductase enzyme and partly to increased fecal excretion by promoting biliary excretion and preventing absorption of dietary cholesterol. The cholesterol-lowering effect of C. ternatea root extract might be because of increased fecal excretion by promoting biliary excretion and preventing absorption of dietary cholesterol. Further, the extracts improved the natural antioxidant status in the liver tissues.

Acknowledgements

This work is financially supported by a grant from the Gujarat Council of Science and Technology (GUJCOST), Gandhinagar, Gujarat, India.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


C. ternatea and V. mungo in hyperlipidemia


Immunomodulatory activity of Ayurvedic plant Aparajita (Clitoria ternatea L.) in Male Albino Rats

Y.B. Solanki, Sunita M. Jain

Abstract - The present study was undertaken to investigate immunomodulatory activity of Clitoria ternatea seed and root extracts. Effects on humoral immune response were investigated in SRBCs-sensitized rats. Effects on cell mediated immunity were studied by measuring delayed type hypersensitivity (DTH) response in SRBC-sensitized rats. Neutrophil recruiting and phagocytosis were measured by studying neutrophil adhesion and carbon clearance method respectively. Further the effects on hematological parameters were also studied. C. ternatea seed and root extracts showed significant immunosuppressive effects as evident from significant decrease in primary and secondary antibody titers in specific immune response could be attributed to decreased immune cell sensitization, immune cell presentation and phagocytosis. The anti-inflammatory and antioxidant properties of plant might be playing major role in immunomodulatory activity. The present study provided evidence for the traditional uses of the plants in Indian system of medicine.

Keywords - DTH response, Immunosuppressive, Neutrophil, Phagocytosis, Primary antibody titer.

I. INTRODUCTION

Clitoria ternatea L. belonging to family ‘Fabaceae’, is popularly known as a “Butterfly pea” in western countries and as “Aparajita” in the traditional Ayurvedic system of medicine. Clitoria ternatea (CT) is one of the important plants of Ayurvedic system of medicine and is official in the Ayurvedic Pharmacopoeia of India (Anonymous, 2003). It is reported to have brain tonic activity, and is popularly known as 'shankhapushpi' (Upadhye & Kumbhojkar, 1993) in southern India. In traditional system of medicine, it is employed against different disease conditions such as cathartic, purgative, demulcent, emetic and anti-inflammatory in swollen joints (Kirtikar & Basu, 1976; Chopra et al. 1956). Ayurvedic system prescribed various part of the plant in inflammation, hepatic disorders and as a brain tonic (Anonymous, 2003). Various parts of CT have been reported to have nootropic activity, anxiolytic activity, tranquillizing property, anti-inflammatory and analgesic activity, antipyretic, and antimicrobial activity (Mukherjee et al., 2008).

It is also reported to have immunomodulatory activities in alloxan-induced diabetic rats (Daisy et al. 2004). The plant is found to possess antibacterial activity (Malabadi et al. 2005). The flavonol glycoside present in roots is reported to have antibacterial activity (Yadava & Verma, 2003). CT has been reported to contain kaempferol and related glycosides, aparajitin, anthocyanins (Shrivastava & Pande, 1977) and anthoxanthins (Gupta & Lal, 1968). However, no study had been reported on immunomodulatory activities especially of seeds and roots in animal models. Hence, we conducted the present study to evaluate an immunomodulatory activity of seeds and roots of CT in male albino rats.

II. MATERIALS AND METHODS

Plant collection and Identification

The plant is available in two varieties – blue flowered and white flowered. It is a climbing vine found on road side and field sides throughout India. Since, the blue variety is medicinally more important, we used only blue variety for the present investigation. The plant was collected in the month of March (2007) from the fields and road side of the Charotar region of the Gujarat state, India. The pods were allowed to dry sufficiently under shade, and finally seeds were collected manually. The plant was botanically identified by Dr. G. C. Jadeja, Professor and Head of Agricultural Botany Department, B. A. College of Agriculture, Anand Agricultural University, Anand, India. The specimens of the sample were stored in the museum of the department (specimen no. 0701). The quality of plant was ascertained as per Ayurvedic Pharmacopoeia of India by determining foreign matters, total ash, acid insoluble ash, alcohol soluble extractive, and water soluble extractive values (Anonymous, 1999).

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**Preparation of extracts**

The dry powdered (40/4) seeds (1kg) were extracted with petroleum ether by percolation until the percolate was free of green color. The residues were extracted with 50% v/v alcohol by heating on boiling water bath under reflex for 3 h. The solvents were evaporated to have pasty mass, referred as CT seed extract. The dry powdered (40/4) roots were directly extracted with 50% v/v alcohol by heating on the boiling water bath under reflex for 4 h. The solvents were evaporated at room temperature to have pasty mass, referred as CT root extract.

**Preliminary phytochemical screening**

CT seed and root extracts were screened for phytochemicals using the method specified by Kokate, (2003).

**Chemicals and reagents**

All the chemicals used in the present study, were of analytical grade and purchased from S. D. Fine chemicals Limited, Mumbai. Dexamethasone (DMS) was obtained from Cadila Zydus Research Centre, Ahmedabad. In the studies herein unless otherwise indicated, all chemicals were of analytical grade as purchased from S.D. Fine Chemicals Limited, Mumbai. Dexamethasone (DMS) was obtained from Cadila Zydus Research Centre, Ahmedabad.

**Pharmacological evaluation**

**Animals**

Male albino rats (Wistar strain) weighing 150-200 g were divided into different groups, each consisting of six animals. Animals were maintained on a commercial chew diet (Pranav Agro Industries Ltd., Sangli, Maharashtra, India) and water at libitum throughout the study period. This study was approved by the institutional animal ethics committee in accordance with the guidelines of Committee for the Purpose of Supervision and Control of Experiments on Animals (CPCSEA), (CPCSEA, 2003). For each experiment outlined below, rats were randomized into various groups that received CT seed extract, CT root extract, vehicle, or dexamethasone (DMS) as a reference immunosuppressive drug.

**Treatment regimens**

CT seed and root extracts were suspended in distilled water using 1% w/v gum acacia. DMS was suspended at a concentration of 0.8 μg/mL in distilled water using 1% w/v CMC. In the studies herein unless otherwise indicated, treatment rats received CT seed and root extracts at 500 mg/kg body weight (BW) in 1 mL doses daily by gavage. The control group rats received vehicle, i.e., a single 2 mL bolus bearing 1 mL each of the 1% w/v gum acacia and 1% w/v CMC solutions, in parallel daily. The rats in the reference drug group received DMS at the dose of 0.25 mg/kg BW in 1 mL volume, daily by gavage.

**Antigen preparation**

Fresh blood was collected from sheep sacrificed in the local slaughter house, and placed in Alsever’s solution. During the experiment, adequate amount of stock solution of sheep red blood cells (SRBC) stored in Alsever’s solution, was taken and allowed to stand at room temperature. It was washed three times with normal saline. The settled SRBC were then suspended in normal saline. The SRBC of this suspension were adjusted to a concentration of 5x10⁹ SRBC/mL for immunization and challenge (Bafna and Mishra, 2005).

**Acute toxicity study**

Animals were treated with different doses 250, 500, 750 and 1000 mg/kg, p.o. of each extract. After single dose administration, animals were observed for death or any other deformities up to 72 h.

**SRBC-induced humoral antibody (HA) titer**

The method described by Atal et al. (1986) was utilized to examine the rats provided CT seed and root extracts once daily by gavage, starting 7 days prior to sensitization and continuing up to the second time of challenge (i.e., Day -7 up to and through Day +14; for a total of 21 d). Control and DMS-treated rats received vehicle or the drug, respectively, in parallel each day.

To specifically assess effects on antibody formation, groups of six rats per treatment were immunized with 20 μL of SRBC suspension (5x10⁹ SRBC/mL) injected subcutaneously into right hind foot pad. The day of immunization was referred to as Day 0. Seven days later (Day +7), the rats were challenged by injecting 20 μL of SRBC suspension (5x10⁹ SRBC/mL) intradermally into the left hind foot pad. Blood samples were collected from all the animals separately by retro-orbital puncture under light ether anesthesia on Day +7 (after challenge) for assessment of primary antibody titer and on Day +14 (after challenge) for measures of secondary antibody titer. Antibody levels were determined by the method described by Shinde et al. (1999). After allowing the collected blood to clot, serum was isolated and 25 μL was placed into one well of a 96-well microtiter plate. Serial two-fold dilutions of the serum were made using 25 μL of normal saline each time of transfer across the plate. To the 25 μL of diluted serum in each well was then added 25 μL of 1% w/v SRBC suspension in normal saline. The microtiter plate was maintained at room temperature for 1 h and then well contents examined for haemagglutination i.e., until control wells showed unequivocally negative patterns. The value of the highest serum dilution showing haemagglutination was defined as the antibody titer for the given rat.

**SRBC-induced delayed-type hypersensitivity (DTH) Response**

The method of Lagrange et al. (1974) was used to analyze effects on DTH responses in the treated rats. Daily treatment with CT seed and root extracts (500 mg/kg, by gavage) began 14 days prior to the challenge i.e., starting
on the same day as immunization with SRBC. Control and DMS-treated rats received vehicle or the drug, respectively, in parallel each day.

On Day 0, all rats were immunized with 20 μL SRBC solution (5 x 10^6 SRBC/mL) injected subcutaneously into their right hind footpad. After 14 days of gavage treatment, the thickness of each rat’s left footpad was measured just before the challenge; using a Schnelltaster caliper (H.C. Kroplin Hessen, Schluchtern, Germany) that could measure to a minimum unit of 0.01 mm. The rats were then challenged by injecting 20 μL SRBC solution (5 x 10^6 SRBC/mL) intradermally into their left hind footpad (deemed time 0). Foot thickness was the re-measured after 24 h. The difference between the thicknesses of left foot just before and after challenge (in mm) was taken as a measure of DTH (Doherty, 1981).

**Neutrophil adhesion test**

The method described by Wilkinson (1978), was used for evaluating the effect of CT seed and root extracts on neutrophil adhesion. After 14 days of gavage treatment, blood samples were collected from rats in each group by retro-orbital puncture under light ether anesthesia in heparinized vials and subjected to total as well as differential leukocyte count. After performance of the initial counts, the incubated samples were again analyzed for total and differential leukocyte count. The product of total leukocyte count and the percentage (%) neutrophil (known as neutrophil index) was determined for each rat of the respective groups (Fulzele et al., 2002). The % neutrophil adhesion for each of the test rat was then calculated as 

\[
\% \text{ Neutrophil Adhesion} = \frac{N_{tu} - N_{Li}}{N_{Li}} \times 100
\]

Where ‘Ntu’ is the neutrophil index of the blood samples before nylon fiber treatment and ‘Nli’ the index after nylon fiber treatment.

**Carbon clearance test**

The method of Böszö et al. (1953) was used to analyze phagocytic activity among the white blood cells in the rats. For each treatment regimen, a total of 6 rats were utilized. Daily treatment with CT seed extract (500 mg/kg, by gavage) occurred for 5 day prior to the assessment of in situ phagocytic activity. Control and dexamethasone – treated rats received vehicle or the drug, respectively, in parallel each day. A colloidal carbon ink suspension was injected via the tail vein into each rat 48 h after the final treatment. From each rat, blood samples (25 μL) were then withdrawn from the retro-orbital pouch under mild ether anesthesia, immediately after the injection and then 5, 10, and 15 min thereafter. Each blood sample was lysed with 2 mL of 0.1% acetic acid and the absorbance of the resulting solution evaluated at 675 nm (Damre et al., 2003). A graph of absorbance vs. time post-injection was prepared for each animal and the in situ phagocytic index calculated using the following formula,  

\[
\text{Phagocytic Index (PI)} = \frac{K_{sample}}{K_{standard}} \times 100
\]

wherein \( K_{sample} \) represents the slope of the absorbance vs. time curve of blood samples from rats in the extract – treated or Dexamethasone – treated group and \( K_{standard} \) represents the slope of the absorbance vs. time curve of blood samples for the rats in the control group.

**Hematological profile**

After 8 days of the repeated gavage treatment, blood was collected from each rat via their retro-orbital plexus under light ether anesthesia. Various parameters such as total white blood cell (WBC), differential WBC, red blood cell (RBC), platelet counts, as well as hemoglobin (Hb) levels were then evaluated using a Sysmex XN-800i automated hematology analyzer (TOA Medical Electronic Co., Tokyo, Japan).

**Statistical analysis**

Statistical analysis was carried out using one way ANOVA followed by Tukey's test, using the SigmaStat™ 2.03 software and computer with Intel Pentium® dual core® processor. A value of \( p < 0.05 \) was considered a statistically significant difference between analyzed groups.

**III. RESULTS**

In the present study, the immunomodulatory effect of CT seed and root extracts were investigated using various experimental models. The effect on humoral immunity was estimated by measuring primary and secondary antibody titers in SRBC sensitized rats. Effect on innate or cell mediated immunity was studied against delayed type of hypersensitivity (DTH) response. Further, neutrophil recruiting and phagocytic activity of the reticuloendothelial system were measured by neutrophil adhesion and removal of carbon particles from the blood circulation.

Acute toxicity study showed that CT seed and root extracts were safe up to the dose of 1000 mg/kg, p.o. The preliminary phytochemical screening showed presence of glycosides, tannins, saponins, phenolics, flavonoids, proteins, and carbohydrates.

**SRBC–induced antibody (HA) titer**

In SRBC-sensitized rats, the primary titer was significantly decreased by CT seed (0.05 ± 0.01) and root (0.03 ± 0.01) extracts on day 14 and secondary titer was significantly decreased by both the extract (0.06 ± 0.01 and 0.03 ± 0.01) respectively on day 21 when compared with the control group (3.52 ± 0.76) on day 14 and (3.00 ± 0.76) on day 21. Reference immunosuppressive drug dexamethasone showed significant decrease in primary titer (0.15 ± 0.02) and secondary titer (0.19 ± 0.05) (table 1).
GJSFR. 10(3), xxx-xxx, 2010 (In press)

Table 1: Effects on antibody formation by SRBC-sensitized rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Primary titer</th>
<th>Secondary titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.52 ± 0.76</td>
<td>5.00 ± 0.76</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.15 ± 0.02*</td>
<td>0.19 ± 0.05*</td>
</tr>
<tr>
<td>CT seed extract</td>
<td>0.05 ± 0.01*</td>
<td>0.06 ± 0.01*</td>
</tr>
<tr>
<td>CT root extract</td>
<td>0.03 ± 0.01*</td>
<td>0.03 ± 0.01*</td>
</tr>
</tbody>
</table>

All values represent mean ± SEM; n = 6 per treatment group. Statistical analysis was carried out using One Way ANOVA followed by Tukey’s multiple range test. *: Value significantly different (p < 0.05) compared with the control group. C. ternatea, treatments began in period starting 7 d prior to sensitization and continuing up to time of challenge (i.e., Day -7 up to and through Day +7).

SRBC-induced DTH response

The cell-mediated immune responses of CT seed and root extracts were assessed by DTH reaction, i.e. foot pad reaction. The DTH response was measured as difference in thickness of hind paw before and after the challenge with SRBC solution. CT seed (0.31 ± 0.01) and root (0.40 ± 0.02) extracts produced significant (p < 0.001) decrease in the DTH response when compared with the control group (0.85 ± 0.02). These effects were comparable with that of reference immunosuppressant drug - dexamethasone (0.36 ± 0.01) (table 2).

Neutrophil adhesion test

The % neutrophil adhesion was significantly (p < 0.01) decreased by CT seed (14.32 ± 1.09) and root (8.71 ± 0.81) extracts at the dose of 500 mg/kg, p.o., when compared with the control group (23.33 ± 1.02). These effects were comparable with the reference drug - dexamethasone (14.00 ± 3.07). The finding suggested possible immunosuppressive or immunoinhibitory action of both the extracts (table 3).

Table 2: Effects on DTH response and the phagocytic index.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DTH Response</th>
<th>Phagocytic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.85 ± 0.02</td>
<td>1.00</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.36 ± 0.01*</td>
<td>0.24 ± 0.005*</td>
</tr>
<tr>
<td>CT seed extract</td>
<td>0.31 ± 0.01*</td>
<td>0.31 ± 0.005*</td>
</tr>
<tr>
<td>CT root extract</td>
<td>0.40 ± 0.02*</td>
<td>0.40 ± 0.004*</td>
</tr>
</tbody>
</table>

All values represent mean ± SEM; n = 6 per treatment group. Statistical analysis was carried out using One Way ANOVA followed by Tukey’s multiple range test. *: Value significantly different (p < 0.05) compared with the control group. C. ternatea. * Value in parentheses indicate decrease or increase in DTH response relative to control rat value. Control value set to 1.00 for comparative purposes.

Carbon clearance test

The in vivo phagocytic activities were measured by carbon clearance method. CT seed (0.31 ± 0.01) and root (0.40 ± 0.01) extracts produced significant decrease in phagocytic index. The phagocytic index of the control group was considered as unite. The dexamethasone produced significant decrease in the phagocytic index (0.21 ± 0.01) (table 2).

Effects on hematological profile

The CT seed and root extracts significantly decreased blood lymphocyte, and RBC counts, as well as Hb content when compared with the control group. The reference drug dexamethasone significantly decreased blood total WBC, neutrophil, RBC counts, and Hb content (table 4).

Table 3: Effect of CT seed and root extracts on neutrophil index and neutrophil adhesion.

<table>
<thead>
<tr>
<th>Group</th>
<th>TLC (10³/mm³) (X)</th>
<th>% Neutrophil (Y)</th>
<th>Neutrophil Index (X x Y) x10³</th>
<th>% Neutrophil Adhesion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UnB FTB</td>
<td>UnB FTB</td>
<td>UnB FTB</td>
<td>UnB FTB</td>
</tr>
<tr>
<td>Control</td>
<td>6.38 ± 0.57</td>
<td>6.10 ± 0.60</td>
<td>14.34 ± 2.55</td>
<td>11.41 ± 1.85</td>
</tr>
<tr>
<td>DMS</td>
<td>1.42 ± 0.34*</td>
<td>1.43 ± 0.31*</td>
<td>3.51 ± 0.09*</td>
<td>3.10 ± 0.08*</td>
</tr>
<tr>
<td>CT seed extract</td>
<td>6.83 ± 0.66</td>
<td>6.52 ± 0.64</td>
<td>32.83 ± 6.66</td>
<td>30.60 ± 6.55</td>
</tr>
<tr>
<td>CT root extract</td>
<td>4.82 ± 0.43</td>
<td>5.26 ± 0.57</td>
<td>17.60 ± 0.63</td>
<td>14.93 ± 0.91</td>
</tr>
</tbody>
</table>

All values represent mean ± SEM; n = 6 per treatment group. Statistical analysis was carried out using One Way ANOVA followed by Tukey’s multiple range test. P < 0.05 was considered statistically significant. *: significant when compared with the control group. CT: C. ternatea, DMS: dexamethasone. TLC: total leukocytes count; UnB: untreated blood; FTB: nylon fiber-treated blood.
GJSFR. 10(3), xxx-xxx, 2010 (In press)

<table>
<thead>
<tr>
<th>Table: Effects of CT seed and root extracts on hematological parameters.</th>
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<tbody>
<tr>
<td>Group</td>
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<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>DMS</td>
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<tr>
<td>CT seed extract</td>
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<td>CT root extract</td>
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All values represent mean ± SEM; n = 6 per treatment group. Statistical analysis was carried out using One Way ANOVA followed by Tukey’s multiple range test. *: P < 0.05 was considered statistically significant. #: significant when compared with the control group. CT: C. termatea, DMS: dexamethasone. WBC: White blood cells, RBC: Red blood cells, Hb: Hemoglobin.

IV. DISCUSSION

In the present study, the immunomodulatory activity of CT seed and root were investigated using experimental models. The humoral response was measured as primary and secondary antibody titers in sheep red blood cells (SRBC) sensitized rats; the cell mediated immune response was measured as delayed type of hypersensitivity (DTH) response in SRBC sensitized rats. The neutrophil recruiting and Phagocytic activity of Reticuloendothelial system was measured as neutrophil adhesion and carbon clearance method. Further, the effects of CT seed and root extracts on hematological parameters were also investigated.

When animal hosts are non-intravenously sensitized with sheep red blood cells (SRBC), this 'antigen' initially becomes diffused within the extra vascular space and ultimately, via the lymphatic system, enters regional lymph nodes. Macrophages in the lymphoid tissues or lining the sinuses are then able to phagocytize the antigen, process it for presentation (in the context of surface major histocompatibility Class II molecules), and become antigen-presenting cells (APC) to many cells, including lymphocytes. Another APC is the B-lymphocyte; like macro-phages, B-lymphocytes are not very effective at presenting this or other antigen to naïve T-lymphocytes. They are, however, effective in presenting antigen to memory lymphocytes, especially when antigen level is low. Once the antigen has been fragmented and processed, helper Tg2 T-lymphocytes can then interact to assist/stimulate the B-lymphocytes to produce antibody against the SRBC. In general, during a first (primary) response to exposure to the SRBC/antigen, IgM is secreted initially, followed by a switch to IgG (Goldby et al., 2003). On re-exposure to the antigen, a secondary response is elicited that is characterized by a rapid onset and highly amplified level of antibody production. Thus, Antibody molecules, a product of B-lymphocytes and plasma cells, are central to humoral immune responses, IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins, etc. (Miller, 1991). At neutral pH, red blood cells possess negative ions cloud that makes the cells repel from one another, this repulsive force is referred to as zeta potential. Because of its size and pentameric nature, IgM can overcome the electric barrier and get cross-link red blood cells, leading to subsequent agglutination. The smaller size and bivalency of IgG, however, makes them less capable to overcome the electric barrier. This characteristic may accounted for, IgM being more effective than IgG in agglutinating red blood cells (Kuby, 1994).

In the present study, anti-SRBC antibody titers - during both primary and secondary responses - were found significantly decreased in the hydroalcoholic extracts-treated rats. The inhibition of the humoral response by CT seed and root extracts that were noted here could indicate that there was decreased responsiveness of macrophages/B-lymphocytes subsets in these hosts.

Phagocytosis represents an important innate defense mechanism against ingested particulates including whole pathogenic microorganisms. The specialized cells that are capable of phagocytosis include blood monocytes, neutrophils and tissue macrophages. Once particulate material is ingested into phagosomes, the phagosomes fuse with lysosomes and the ingested material is then digested. Thus, it is not only ingesting and removing microorganisms but also malignant cells, inorganic particles and tissue debris (Miller 1991). In general, the rate of in situ carbon particle clearance is frequently used as a measure of reticuloendothelial system (RES) competency. Specifically, a faster removal of particles is correlated with an enhanced phagocytic activity of RES cellular components (Abbas and Litchman, 2001). In the study here, prophylactic treatment with CT seed and root extracts inhibited the rate of carbon clearance seen among control group rats.

The neutrophil, an end cell unable to divide and with limited capacity for protein synthesis is, nevertheless, capable of a wide range of responses, in particular chemotaxis, phagocytosis, exocytosis and both intracellular and extracellular killing (Dale and Foreman, 1984). Normally, a more rapid clearance of exogenous particulates
from the blood by macrophages would arise from opsonization of the material with antibodies/complement C3b. The decrease in neutrophil function (i.e., adhesion activity) strongly suggests that the function in the treated rats’ phagocytes was inhibited (i.e., immunoinhibited).

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). CMI responses are critical to defense against infectious organisms, infection of foreign grafts, tumor immunity and delayed-type hypersensitivity reactions (Miller, 1991). Delayed type hypersensitivity reaction is characterized by large influxes of non-specific cytokines (lymphokines).

These cells generally appear to be a TH 1 subpopulation of chemokines and effective killing. Several lines of evidence suggest that DTH is explained partly by its inhibitory effects on humoral mechanisms carried out by T lymphocytes and their inflammatory protein 1h.

In addition to the above-noted outcomes, the DTH response - the magnitude of which can be directly correlated, with the competence of a host’s cell-mediated immune function that was decreased in rats that received CT seed and root extracts. Apart from the key role of memory (sensitized) T-lymphocytes in this reaction, the role of local macrophages (initially) and then recruited monocytes/other phagocytes are critical as well. From the data here, no specific conclusions about the functionality of memory T-lymphocytes can be predicted; however, decreases in anti-SRBC titers in CT seed and root extracts treated rats were suggestive of decreased activation of T-lymphocytes. The decreased phagocytic activities of local/recruited phagocytes would also be a major factor for the substantive decrease observed in DTH among extracts-treated rats.

The majority of the cells involved in the immune system are produced from common hematopoietic stem cells found in the bone marrow. This site also provides a microenvironment for antigen-dependent differentiation of B-lymphocytes (Raphael & Kuttan, 2003). Since CT seed and root extract treatments were seen here to give rise to decreased circulating antibody titers (specifically against the SRBC), it would be expected then that there should have also been decreases induced in levels of one or more of the cell types involved in the humoral response to this antigen.

In the present study, the evaluations of peripheral blood of extracts-treated rats confirmed the suppression of total WBC counts. These outcomes suggested strongly that the potential effect of CT seed and root extracts was an impact on hematopoietic processes and on the bone marrow in particular. Intensity of inflammatory immune responses is controlled by recruitment of inflammatory cells into inflammatory lesions. This process is tightly governed by expression of certain inflammatory chemokines, such as monocyte chemotactic protein 1 (MCP-1), Macrophage inflammatory protein 1a (MIP-1a), Macrophage inflammatory protein 1h (MIP-1h), and CC-Chemokine ligand 5 (CCL5) (Baggiolini and Dahinden, 1994; Kallinch, et al., 2005); and adhesion molecules, such as lymphocyte function-associated antigen 1 (LFA-1), and cluster of differentiation 44 (CD44), by the inflammatory cells, and inter-cellular adhesion molecules (ICAM-1), and vascular cell adhesion molecule (VCAM-1) by the endothelial cells (Cartier et al., 2005). Given the central role of chemokines and adhesion molecules in orchestrating the immune response, interference with the expression of these mediators substantially alters the quality of the immune response, leading to either enhancement or inhibition of the ongoing immune response.

Thus, one potential mechanism that might mediate the inhibitory effect of CT on inflammatory immune responses is an alteration of trafficking of the inflammatory cells via modulating expression of chemokines and/or adhesion molecules. Thus, the immuno inhibitory effect of CT can be explained partly by its inhibitory effects on humoral antibody formation, phagocytosis, delayed type hypersensitivity response, and immune cell activities. The anti-inflammatory activity of CT seed and root extracts against carrageenan-induced hind paw edema, pleurisy and cotton pellet granuloma model, suggesting inhibition of inflammatory components of immune response by CT.

V. CONCLUSION

CT seed and root extracts showed profound immunosuppressive activity in male albino rat model. The antioxidant and anti-inflammatory activities of plant may be playing major role in immunoinhibition. The immunomodulatory activity might be attributed to the presence of flavonoid and phenolic compounds. The present study demonstrated and provided evidence for the traditional uses of Clitoria ternatea. Further studies might be required to determine detailed mechanisms and active phytochemicals responsible for immunomodulatory activity.

VI. ACKNOWLEDGEMENT

The authors want to thanks Gujarat Council of Science and Technology (GUJCOST), Gandhinagar for their financial support. We also thank Dr. G.C. Jadeja, Professor and Head, Department of Agricultural Botany, Anand agricultural University, Anand, India; for his help in the identification of the plant.
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