Mechanism of action of antiatherogenic and related effects of *Ficus bengalensis* Linn. flavonoids in experimental animals

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One month treatment of alloxan diabetic dogs with a glycoside, viz. leucopelargonin derivative (100 mg/kg/day) isolated from the bark of *F. bengalensis* decreased fasting blood sugar and glycosylated haemoglobin by 34% and 28% respectively. Body weight was maintained in both the treated groups while the same was decreased significantly by 10% in the control group. In cholesterol diet fed rats, as the atherogenic index and the hepatic bile acid level and the faecal excretion of bile acids and neutral sterols increased, the HMGCoA reductase and lipogenic enzyme activities in liver and lipoprotein lipase activity in heart and adipose tissue and plasma LCAT activity and the incorporation of labelled acetate into free and ester cholesterol in liver decreased significantly. On treatment with the two ficus flavonoids, viz. leucopelargonina and leuco cyanin derivatives and another flavonoid quercetin (100 mg/kg/day) the above said effects except on bile acids and sterols and lipogenic enzymes were significantly reversed in the cholesterol fed rats. However in the treated rats the hepatic level of bile acids and the faecal excretion of bile acids and neutral sterols still further increased and the action of lipogenic enzyme glucose 6 phosphate dehydrogenase was still further decreased. These effects of leucopelargonin and quercetin were better than that of the second. Toxicity studies are required to be carried out to find out if the ficus flavonoids could be used as health promoters as they are hypcholesterolemic and antioxidant in action.

According to the Ayurvedic system of medicine *Ficus bengalensis* Linn (Banyan tree) is well known to be useful in diabetes. This attracted the attention of many earlier workers who studied the hypoglycemic effect of extracts from the bark of *F. bengalensis* and tried to isolate active compounds. We also studied the hypoglycemic and related effects of the flavonoids isolated from the above extracts particularly using diabetic dogs and rats. Similar studies by Shukla et al. confirmed the antidiabetic action of *F. bengalensis* extracts. Our studies showed that the mechanism of action of the ficus flavonoids (Compounds I&II) is through stimulation of insulin secretion. Babu et al. and Shukla et al. have reported not only the hypoglycemic but also the hypcholesterolemic effects of some purified compound isolated from *F. bengalensis*. In yet another study Basakar and Nath reported that quercetin and related flavonoids showed significantly high hypolipidemic effects on feeding to rats maintained on a 1% cholesterol diet. In a similar study we also demonstrated the hypolipidemic effects of ficus flavonoids in 2% cholesterol rich diet fed rats. According to the study of Shukla et al. the aqueous extract of the bark of *F. bengalensis* lowered the total cholesterol, LDL cholesterol and triacyl glycerol on one hand and increased HDL cholesterol on the other hand in the serum of rabbits fed cholesterol for more than a month. These workers further stated in a recent review that a water soluble compound having hypoglycemic activity at a very low dose of 10 mg/kg and a water insoluble derivative also with the same effect (dose not mentioned) were purified and their mechanism of action and lack of toxic effects were studied and applied for patent.

In the present study we carried out some more detailed investigations like the effect of the above mentioned ficus flavonoids at a dose of 100 mg/kg/day on glycosylated haemoglobin and body weights of alloxan diabetic dogs and also on serum total cholesterol/HDL cholesterol ratio labelled acetate incorporation into liver cholesterol, and certain enzymes not yet studied by others in tissues and blood of rats fed cholesterol rich diet, after treatment as compared to tolbutamide and quercetin as the case may be.
Materials and Methods

In our experiments leucopelargonin derivative (Compound I), leucocyanin derivative (Compound II) a known flavonoid quercetin and a known oral hypoglycemic agent tolbutamide were used as drugs. Hypoglycemic drug tolbutamide was supplied by Hoesch Pharmaceutical Ltd, Mumbai. The flavonoid derivatives compounds I and II, were isolated from the bark of banyan tree according to the method of Prema and Misra. Standard flavonoid quercetin was obtained from Sigma, U.S.A.

Experiment with alloxan diabetic dogs

The diet composition, induction of diabetes and maintenance of the dogs were the same as described in a previous work. When the blood sugar of the diabetic dogs were stabilized after a period of a fortnight they were divided into groups of six and used for the experiments. As drugs only compound I and tolbutamide were used here.

- Group I — Control dogs were orally administered with one ml of normal saline daily through a gastric tube.
- Group II — Dogs were orally administered with leucopelargonin derivative (Compound I) in one ml normal saline as above (daily dose 100 mg/kg body wt).
- Group III — Dogs were orally administered with tolbutamide (dose same as above) as a suspension in normal saline as above.

After 30 days treatment, their body weights were determined and fasting blood glucose and glycosylated Hb were estimated. The values were statistically assessed by ANOVA and the significant results at 5% level were noted.

Experiment with cholesterol diet fed rats

Male albino rats Sprague —Dawley Strain, (weight 100-120 g) were divided into five groups of 12 rats in each. Here the compounds I and II and quercetin were used as drugs. The grouping and treatment were as follows.

- Group I — Normal control. Normal rats fed with control diet.
- Group II — Cholesterol control. Normal rats fed with 2% cholesterol diet.
- Group III — Test group for leucopelargonin derivative. Normal rats fed with 2% cholesterol diet + compd I (100 mg/kg/day).
- Group IV — Test group for leucocyanin derivative. Normal rats fed with 2% cholesterol diet + compd II (100 mg/kg/day).
- Group V — Test group for quercetin. Normal rats fed with 2% cholesterol diet + quercetin (100 mg/kg/day).

Control diet — Corn starch, 71 g; Casein, 16 g; ground nut oil, 8 g; salt mixture, 4 g and vitamin mixture, 1 g.

Cholesterol diet — Corn starch, 62 g. coconut oil, 15 g; cholesterol, 2 g; and other constituents as in the control diet.

Fig. I — Structures of flavonoids used in the study
The rats were housed individually in polypropylene cages in rooms maintained at 25°C ± 1°C. The diet consumption was adjusted to be the same by supplying measured quantities to all groups. The flavonoid derivatives were administered as suspensions in normal saline through a gastric tube at a dose of 100mg/kg body weight/day. Water was given ad libitum. Duration of the experiment was 90 days. At the end of this period their body weights were determined and feces were collected for bile acid estimation from one half of each group. The rats of these subgroups i.e., six rats from each group were deprived of food overnight and the next day they were sacrificed by decapitation. Blood and tissues were removed to ice-cold containers. Their gain in body weights and liver weights were also assessed.

Serum lipoproteins HDL and LDL + VLDL were separated by a procedure described by Warnick and Albers and total cholesterol and lipoprotein related cholesterol were determined by the method of Abell et al. LDL cholesterol was separately assessed by subtraction of VLDL cholesterol equivalent to 1/5 TAG. Hepatic bile acids were determined by the method of Rao and Ramakrishnan and that of lipoprotein lipase by the method of Palmer. Activities of two hepatic lipogenic enzymes, viz. glucose 6-phosphate dehydrogenase and malic enzyme were determined by standard methods. Activity of HMGoA reductase in liver was determined by the method of Krauss and Windmuller and that of plasma LCAT by the method of Sperry and Webb.

The rats left behind in the other half of each group i.e., six rats from each group were deprived of food overnight for 16 hr and they were injected (ip) with 0.5ml solution of 1,2-14Cacatate into hepatic cholesterol as described below. The rats were deprived of food overnight for 16 hr and they were injected (ip) with 0.5ml solution of 1,2-14C-sodium acetate (10 μCi/100 g) at 9hr. After 3 hr, the rats were sacrificed by decapitation. The liver was quickly removed to ice-cold containers and gently blotted and weighed. The liver was extracted with chloroform: methanol (10:1) and the incorporation of 1,2-14C into hepatic cholesterol was determined by the procedure of Folch et al. Free cholesterol and ester cholesterol in the extract were separated by TLC over silica gel G using n-hexane:ether: acetic acid in the ratio of 30:6:0.5 (v/v/v) as solvent system. The activity was counted in liquid scintillation counter. The scintillant fluid was 6 g 2,5-diphenyl oxazole (PPO) and 0.2 g 1,4-biz (2-(5-phenyl oxazolyl) (POPOP)/L toluene.

In order to compare the beneficial effects of the three flavonoids, statistical significance was determined by analysis of variance (ANOVA) and the results, significant at 1-5% level are considered in this study.

Results

There is a significant reduction in the fasting blood glucose level of the diabetic dogs treated for a month with the leucopelargonin derivative (Compound I) or tolbutamide (100 mg/kg/day for each drug) as compared to their initial values i.e. 33% and 44% respectively. The body weights of the animals in both the test groups are more or less maintained at the initial level. However in the control group the body weight decreased by 10% which is significant (P<0.05) and the change in FBS is minimal. The percentage of glycosylated haemoglobin (GHb) in the whole blood showed a significant decrease (P<0.05) of 28% in both the treated groups and no such change was found in the control group.

In cholesterol diet fed group the body and liver weights increased by 22% and 12% respectively and that too significantly (P<0.05) over the normal values. Treatment with the three flavonoid compounds for 90 days significantly decreased the same (7-9%) in groups 3-5 over the cholesterol control (P<0.05).

The concentrations of serum total cholesterol and that of HDL and LDL cholesterol and the atherogenic index are given in Table 1. Total serum cholesterol and LDL cholesterol increased significantly in the cholesterol diet fed rats and these values decreased significantly on treatment with the three flavonoids for a period of 90 days. On the contrary serum HDL cholesterol decreased only slightly in the cholesterol diet fed rats, but the same improved significantly in the three other groups treated with the three flavonoids. The atherogenic index (total cholesterol/HDL cholesterol) increased significantly by 106% in the cholesterol fed control group but the same decreased significantly in the three drug treated groups, viz. 45.5%, 40% and 46.8% respectively.

The results on the levels of hepatic bile acids and that of faecal excretion of bile acids and neutral sterols are given in Table 2. There was a significant increase in the concentration of total bile acids in the liver of rats fed cholesterol rich diet (27%) along with a concomitant increase in the fecal excretion of bile acids (38%) and sterols (111%) as compared to those fed normal diet. A further significant increase in the concentrations of hepatic bile acids (30-38%) fecal excretion of bile acids (22-46%) and neutral sterols (54-70%) respectively was observed in the treated groups as compared to the control cholesterol diet fed
Table 1 — Concentration of cholesterol in serum and lipoproteins
[Values are mean ± SE of six rats in each group]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total cholesterol (TC) (mg/100ml serum)</th>
<th>Conc. of serum lipoproteins (mg/100ml serum)</th>
<th>Atherogenic index TC/HDLc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normal diet (Normal control)</td>
<td>70.2 ± 1.23</td>
<td>47.3 ± 0.53</td>
<td>1.48 ± 0.09</td>
</tr>
<tr>
<td>2. 2% Cholesterol diet (Cholesterol control)</td>
<td>143.2 ± 1.90b</td>
<td>61.2 ± 0.59</td>
<td>3.05 ± 0.032a</td>
</tr>
<tr>
<td>3. 2% Cholesterol diet +Compound I (100 mg/kg body wt)</td>
<td>101.8 ± 2.07b</td>
<td>39.5 ± 0.50</td>
<td>1.66 ± 0.016b</td>
</tr>
<tr>
<td>4. 2% Cholesterol diet +Compound II (100 mg/kg body wt)</td>
<td>110.2 ± 1.82b</td>
<td>47.9 ± 0.61</td>
<td>1.83 ± 0.016b</td>
</tr>
<tr>
<td>5. 2% Cholesterol diet +Quercetin (100 mg/kg body wt)</td>
<td>101.7 ± 1.76b</td>
<td>38.1 ± 0.55</td>
<td>1.62 ± 0.114b</td>
</tr>
</tbody>
</table>

Second group values a except HDL-C are significantly higher than the normal (P<0.01) and 3-5 groups values b are significantly lower than that of the 2nd. 3-5 HDL-C values a are significantly higher than the 2nd (P<0.01).

Table 2 — Concentration of hepatic bile acids and faecal excretion of bile acids and neutral sterols
[Values are mean ± SE of six rats in each group]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hepatic bile acids (mg/100 g wet tissue)</th>
<th>Faecal excretion (mg/rat/day)</th>
<th>Bile acids</th>
<th>Neutral sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normal diet (Normal control)</td>
<td>30 ±0.15</td>
<td>19.7±0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 2% Cholesterol diet (Cholesterol control)</td>
<td>38.9±0.20a</td>
<td>41.6±0.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 2% Cholesterol diet + Compound I (100 mg/kg body wt)</td>
<td>53.2±0.54b</td>
<td>69.8±0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 2% Cholesterol diet + Compound II (100 mg/kg body wt)</td>
<td>50.4±0.45b</td>
<td>64.2±0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. 2% Cholesterol diet + Quercetin (100 mg/kg body wt)</td>
<td>53.8±0.41b</td>
<td>71.3±0.68</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2nd values a are significantly higher than the normal (P<0.01) and 3-5 groups’ values b are significantly higher than that of group 2 (P<0.01).

group. The leucopelargonin derivative and quercetin showed a slightly better and significant effect than the leucocyanin derivative. The activities of the lipogenic enzymes, viz. malic enzyme and glucose 6 phosphate dehydrogenase in liver are given in Table 3. Significantly decreases in the activities of these enzymes were observed in rats fed a cholesterol rich diet as compared to the normal group. In flavonoid treated groups glucose 6-phosphate dehydrogenase activity decreased further, but there was no such change observed with malic enzyme (Table 3).

Results on HMGCoA reductase are also given in Table 3. Feeding of high cholesterol diet significantly decreased the activity of HMGCoA reductase in liver. The ratio of HMGCoA/Mevalonate indicates the activity of HMGCoA reductase in a reverse order i.e., with lower ratio higher activity and with higher ratio lower activity. The decrease in enzyme activity was restored to near normal levels on treatment with the three flavonoids. The leucopelargonin derivative and quercetin showed slightly better effects than the leucocyanin derivative.

Results on the in vivo incorporation of labelled acetate into hepatic cholesterol are given in Table 4. Incorporation of labelled acetate into free and ester cholesterol in the liver of rats fed cholesterol rich diet was significantly lower by 35% and 49% respectively as compared to the normal group. However on treatment with the flavonoids the incorporations of labeled acetate into hepatic free and ester cholesterol increased significantly and were nearer to normal value. The leucopelargonin derivative and quercetin showed slightly better effects than the leucocyanin derivative.

Results on heart and adipose tissue lipoprotein lipase (LPL) and plasma LCAT are given in Table 5. There was a significant reduction in the LPL enzyme activity in the heart (34%) and adipose tissue (24%) of rats fed cholesterol rich diet as compared to the normal group. Treatment with the flavonoids significantly increased the enzyme activity towards normal.
Table 3 — Activity of hepatic lipogenic enzymes (Units/g protein) and the rate of cholesterol synthesis

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ratio of HMGCoA/Mevalonate*</th>
<th>Malic enzyme</th>
<th>Glucose-6-phosphate dehydrogenase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normal diet (Normal control)</td>
<td>2.31 ± 0.028</td>
<td>1053.9 ± 13.97</td>
<td>112.2 ± 1.69</td>
</tr>
<tr>
<td>2. 2% Cholesterol diet (Cholesterol control)</td>
<td>3.15 ± 0.036*</td>
<td>857.4 ± 11.92</td>
<td>76.9 ± 0.66*</td>
</tr>
<tr>
<td>3. 2% Cholesterol diet + Compound I (100 mg/kg body wt)</td>
<td>2.42 ± 0.027*</td>
<td>853.9 ± 11.58</td>
<td>51.6 ± 0.41*</td>
</tr>
<tr>
<td>4. 2% Cholesterol diet + Compound II (100 mg/kg body wt)</td>
<td>2.59 ± 0.023*</td>
<td>851.7 ± 12.25</td>
<td>61.9 ± 0.42*</td>
</tr>
<tr>
<td>5. 2% Cholesterol diet + Quercetin (100 mg/kg body wt)</td>
<td>2.40 ± 0.028*</td>
<td>859.3 ± 11.74</td>
<td>51.4 ± 0.38*</td>
</tr>
</tbody>
</table>

*One unit is defined as the amount of enzyme which causes an increase of 0.01 in O.D/min/g protein.
+One unit is defined as the amount of enzyme which causes an increase of 1.0 in O.D/min/g protein.
Ratio of HMGCoA/Mevalonate is inversely proportional to HMGCoA reductase activity. 2nd values show significantly lower activities of HMGCoA reductase and the other two enzymes than the normal (P<0.01). Except malic enzyme the other enzyme activities are significantly increased by each drug therapy (P<0.01).

Quercetin showed a slightly better effect (34%) than the two Ficus flavonoids (30% and 20%). A similar pattern of results are observed with plasma LCAT, i.e., the enzyme activity decreased significantly (37%) in the rats fed cholesterol rich diet and that the treatment with the flavonoids ameliorated the enzyme activity towards normal. Here leucocyanin derivative showed a slightly better effect (40%) than the leucopelargonin derivative (27%) and quercetin (20%). Here we can observe that one or more of the flavonoids increased the enzyme activities which were decreased by cholesterol feeding, more or less in the same ranges.

Discussion

The three bioflavonoids of Ficus bengalensis Linn. isolated by our team and proved to be hypoglycemic in action are methoxy derivatives of leucopelargonin, leucocyanin and leucodelphinin. Water soluble leucopelargonin derivative was found to be more effective as a hypoglycemic agent. The hypoglycemic action of the former and its similarity in action to tolbutamide in controlling GHb in diabetic dogs and its significant hypocholesterolemic action in cholesterol rich diet fed rats warrant detailed studies. In this connection it is interesting to mention that Shukla et al. isolated a highly potent water soluble compound from F. bengalensis which is active at a very small dose of 10 mg/kg, body wt. The same compound also possesses hypocholesterolemic and hypolipidemic effect by lowering total and LDL cholesterol, TAG and increasing HDL chol. in blood. But its structure has not been mentioned. Therefore it is not known whether it is a flavonoid or not. They have recently reported that this compound when given orally at even 15 times the effective dose for 3 months, it is nontoxic to rats. Plant foods containing flavonoids may protect the diabetics while they are on the standard drugs as usual.

In cholesterol fed rats the antiatherogenic effects of the two flavonoid compounds isolated from F. bengalensis are compared with quercetin at a dose of 100 mg/kg/day for 90 days. The effects of the three drugs were more or less the same in lowering body
Table 5 — Activity of lipoprotein lipase and plasma lecithin: cholesterol acyl transferase

[Values are mean ± SE of six rats in each group]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lipoprotein lipase (µ mole of glycerol/hr/g protein)</th>
<th>Plasma LCAT*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
<td>Adipose tissue</td>
</tr>
<tr>
<td>1. Normal diet (Normal control)</td>
<td>29.4 ± 0.20</td>
<td>127.6 ± 0.79</td>
</tr>
<tr>
<td>2. 2% Cholesterol diet (Cholesterol control)</td>
<td>18.7 ± 0.19*</td>
<td>96.3 ± 0.69*</td>
</tr>
<tr>
<td>3. 2% Cholesterol diet + Compound I (100 mg/kg body wt)</td>
<td>24.3 ± 0.16b</td>
<td>113.1 ± 0.79b</td>
</tr>
<tr>
<td>4. 2% Cholesterol diet + Compound II (100 mg/kg body wt)</td>
<td>22.5 ± 0.17b</td>
<td>105.8 ± 0.97b</td>
</tr>
<tr>
<td>5. 2% Cholesterol diet + Quercetin (100 mg/kg body wt)</td>
<td>25.1 ± 0.14b</td>
<td>117.9 ± 0.93b</td>
</tr>
</tbody>
</table>

*Activity of LCAT expressed as % increase in the ratio of ester cholesterol to free cholesterol during incubation. Higher ratio indicates higher enzyme activity.

2nd group values a are significantly lower than the normal (P<0.01) and 3-5 group values b are significantly higher than that of the 2nd (P<0.01).

weights and liver weights. Leucopelargonin derivative showed a better effect than the other two drugs in lowering the liver weight. The hypoglycemic, hypolipidemic and antioxidant effects of different compounds from F. bengalensis were reported earlier and such effects may be responsible for the beneficial action of these drugs on body weight and liver weights.

The significant decrease observed in serum cholesterol in rats treated with the flavonoids is accompanied with a decrease in LDL cholesterol and an increase in HDL cholesterol as compared to the control group. These results are in agreement with those obtained by Rimi Shukla et al. in cholesterol fed rats treated with an aqueous extract of F. bengalensis or various flavonoids related phenols by others. Increase in HDL-cholesterol was also reported in earlier occasions on feeding various hypolipidemic agents of plant origin to cholesterol diet fed rats. According to the Framingham study, the risk for CHD decreased with the rise in the level of HDL-C in the serum of people because of the inverse relationship between them. High levels of HDL particles in the blood inhibits hydroperoxidation of LDL, lipids and oxidative modification of apo B. Several workers have reported a decrease in the activity of lipogenic enzymes in cholesterol fed rats and a still further decrease of glucose-6 phosphate dehydrogenase and significant increases of lipoprotein lipase and LCAT on feeding Ficus flavonoids and quercetin to such groups is quite interesting as found in the present study. The decreased lipogenic enzyme activity leads to decreased lipogenesis and this together with increased lipoprotein lipase may account for lower concentrations of triacylglycerols and phospholipids reported earlier for liver.

The decrease in the activity of hepatic HMGCoA reductase (i.e. an increased ratio) observed in cholesterol fed rats may be due to a feed back inhibition. However on treatment with the flavonoids the enzyme activity was increased significantly (i.e. a decreased ratio) which may be due to an enhanced degradation of cholesterol to bile acids and neutral sterols as observed in the results that in turn may remove the feed back inhibition on the enzyme. Further the reversal on the inhibition of incorporation of labelled acetate into hepatic free and esterified cholesterol in cholesterol fed rats treated with the flavonoids also support the above suggestions that hepatic cholesterolgenesis is improved possibly by these drugs through a mechanism that helps the degradation of cholesterol and its removal from liver. Flavonoids have the capacity to bind with bile acids and bile salts and that enhance their removal. The degradation of cholesterol may be more than its synthesis in the treated rats which may help them to maintain a lower serum cholesterol level but which may not be exactly so with liver cholesterol.

Increased activity of LPL correlates well with a higher level of HDL-cholesterol in plasma and the increased activity of plasma LCAT observed in treated rats clearly indicates an increased rate of esterification of cholesterol for its removal. Atherogenic effects of cholesterol are reversed by the ficus flavonoids and quercetin thus placing them among the list of hypolipidemic agents. A better action of the leucopelargonin derivative over the leucocyanin derivative may be due to several factors. The former contains only a monosaccharide unit linked through
only a monosaccharide unit linked through an α-glycosidic bond and the latter contains a trisaccharide unit linked by a β-glycosidic bond. Therefore digestion and absorption of the two may differ to a great extent. In addition to that the positions of the methylated groups are also different in these two glycosides (Fig. 1). All these points may account for a better action for the former as compared to the latter. Here it is pertinent to state that the hypolipidemic action of the former flavonoid is as potent as quercetin with out the disadvantages of the latter keto flavonoid. Herbal preparation with antioxidant effects are reported as good health promoters 35. Further studies are warranted to know whether the ficus flavonoids can be safely included in the diet in minimal quantities as hypolipidemic and antioxidant agents.

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