

Hypolipidaemic effect of leucodelphinidin derivative from *Ficus bengalensis* Linn on cholesterol fed rats

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Abstract

Administration of leucodelphinidin derivative isolated from the bark of *Ficus bengalensis* and another flavonoid quercetin (100mg/kg/day) in hypercholesterolemic rats provoked significant reduction in serum total cholesterol, LDL-cholesterol and an increase in the HDL-cholesterol levels. Significant decrease in atherogenic index was noted in these rats treated with the different flavonoids. There was an increased concentration of total bile acids in the liver and also increase in the fecal excretion of bile acids and neutral sterols in the rats fed cholesterol containing diet as compared to those fed normal diet. Feeding the flavonoids further significantly increased the concentrations of hepatic bile acids and the fecal excretion of bile acids and neutral sterols as compared to the control cholesterol diet fed group. The activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase was not inhibited by the flavonoids, instead there was an increased cholesterologenesis as was evident by an increased incorporation of labeled acetate into both free and esterified cholesterol on treatment with leucodelphinidin and quercetin. These results demonstrated that the flavonoids exerted their hypocholesterolemic effects by increasing fecal bile acids and cholesterol excretion. Acute toxicity studies with the leucodelphinidin derivative showed no toxic reactions up to a dose of 4g/kg dose level.

Keywords: *Ficus bengalensis*, leucodelphinidin, quercetin, hypocholesterolemic effect.

Introduction

Phytochemicals have drawn a considerable amount of attention as they play an important role in healthcare. Drug discovery using natural products is pivotal in current herbal medical research¹. Among the different phytochemicals, flavonoids, which are polyphenolic compounds, have attracted considerable attention for their wide variety of biological activities². *Ficus bengalensis* Linn (Banyan tree) is a large evergreen tree with aerial roots. Medicinal properties of this plant have been described in the literature of traditional systems of medicine like ayurveda, siddha, unani and homeopathy. Bioactive compounds isolated from different parts of the plant are effective in the treatment of various ailments including dysentery, diarrhea, diabetes, piles, rheumatism, leucorrhoea and menorrhagia³. The infusion of the bark of this tree is used for the treatment of diabetes mellitus⁴. We have studied the hypoglycemic effects of flavonoids isolated from extracts of the bark in diabetic rats, rabbits and dogs^{4,5,6}. Two flavonoids namely 5,7-dimethyl ether of leucopelargonidin-3-O- α -L rhamnoside

and 5,3'-dimethyl ether of leucocyanidin 3-O- α -D galactosyl cellobioside were reported to possess insulinogenic action from our laboratory^{7,8}. The antioxidant and related properties of these flavonoids were also demonstrated by us^{9,10,11}. The present study aimed to evaluate the hypolipidaemic effects of a flavonoid namely 5,7,3'-trimethylether of leucodelphinidin 3-O- α -L rhamnoside which was isolated from the bark of *F. Bengalensis*. Studies were carried out in 2% cholesterol fed rats and the results compared with those of a structurally similar synthetic flavonoid quercetin with known hypolipidaemic action. The structures of both flavonoids used in this study are shown in figure 1A and 1B.

Material and Methods

Chemicals: Quercetin dehydrate was purchased from Sigma (St Louis, MO USA). [¹⁴C] acetate (specific activity 1.5466 GBq/mmol) was purchased from BRIT (Mumbai, India). All other chemicals used were of high purity and analytical grade and were purchased from Sisco Laboratories Kochi, Kerala.

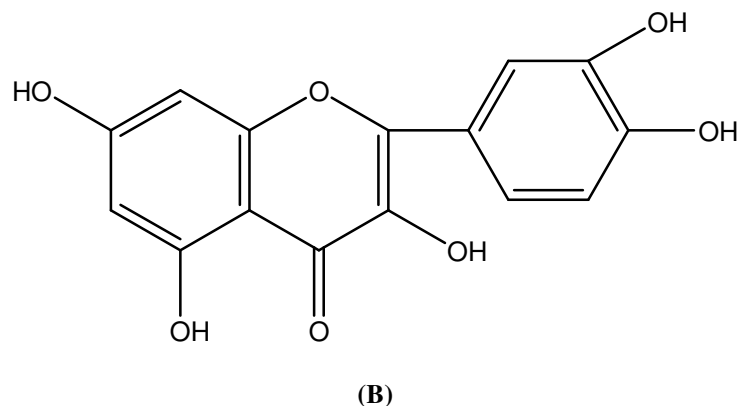
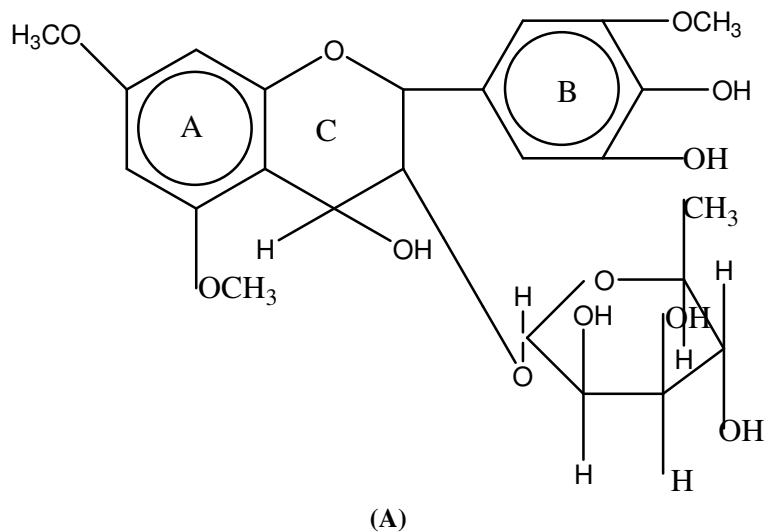


Figure-1

Structure of flavonoids used in this study

A: Leucodelphinidin derivative (5, 7, 3' trimethylether of leucodelphinidin 3-0- α -L rhamnoside, B: Quercetin

Plant material and extraction: 5, 7, 3' trimethylether of leucodelphinidin 3-0- α -L rhamnoside was isolated from the alcoholic extract of the defatted bark of *F. bengalensis* Linn according to the method of Subramaniam and Mishra with slight modifications¹². Fresh bark of banyan tree (after authentication) was collected locally and the middle saffron colored part of it was separated and dried under the sun. It was then cut into small pieces, powdered and defatted by extraction with petroleum ether (B.P.40-60°C) and solvent ether exhaustively (24 h each) in a soxhlet apparatus. These extracts were discarded. The bark powder was taken out of the soxhlet and dried again to remove the solvents. It was put into the soxhlet and extracted exhaustively with double distilled alcohol (8h). The alcoholic extract was collected and the solvent removed under reduced pressure. The tarry residue left behind was stirred with enough water to dissolve the entire water soluble fraction. The mixture was allowed to

stand overnight when a red precipitate and a red solution were separated. The precipitate was filtered out and allowed to dry in the funnel. This water insoluble residue was dissolved in methanol:chloroform (30:2.5V/V) mixture and chromatographed over silica gel-G of 60-120 mesh.

Elution of column with methanol-chloroform (1:11 V/V) gave the red coloured compound 5, 7, 3' trimethylether of leucodelphinidin 3-0- α -L rhamnoside whose structure is shown in figure1A. Yield obtained was 200 mg/100 gm bark powder. The compound with a melting point of 171°C is soluble in ethyl alcohol, methyl alcohol and ethyl acetate. With alcoholic hydrochloric acid, it developed a purple colour, which deepened on warming. With FeCl₃ it gave a blue colour which is characteristic of flavonoids.

Animals and diet: Male albino rats Sprague-Dawley Strain, (weight 100-120 g) were randomly divided into four groups of 12 rats each. The grouping and treatment were as follows.

- Group 1- Rats fed control diet (normal control).
Group 2- Rats fed 2% cholesterol diet (cholesterol control).
Group 3- Rats fed 2% cholesterol diet + leucodelphinidin derivative (100 mg/kg body wt/day).
Group 4- Rats fed 2% cholesterol diet + quercetin (100 mg/kg body wt/day).

The rats were housed in plastic cages individually in a controlled environment conditions (22-28°C, 60-70% relative humidity-and 12-h and 12-h dark/light cycle), fed *ad libitum* on standard laboratory feed from Hindustan Lever Ltd, Bangalore, India (approximate composition protein, 221 g/kg; fat, 40 g/kg; fibre, 36g/kg; minerals, 50 g/kg; energy, 15.16kJ/g) and water. The flavonoid derivatives were administered as suspensions in normal saline through a gastric tube at a dose of 100mg/kg body weight/day. Duration of the experiment was 90 days. The experiments were carried out with the approval of the Institutional Animal Ethics Committee.

Sampling procedures: At the end of the experimental period their body weights were determined and fecal samples were collected from one half of each group and stored at -20°C for bile acid estimation. The rats of these subgroups i.e., six rats from each group were deprived of food for 16 h and then anesthetized with ether inhalation and killed by decapitation. Blood samples were collected into tubes without an anticoagulant, kept at room temperature for 1h, and serum separated by centrifugation at 4°C for 20 min at 1500 g. Serum was stored at -20°C until further analyzed. Their gain in body and liver weights were also assessed. Livers were removed to ice-cold containers, sliced and portions equivalent to about 500 mg were taken for analysis.

Analytical procedures: Serum total cholesterol was determined by the cholesterol oxidase method¹³. Triacylglycerols in the serum were estimated by the glycerol phosphate oxidase method¹⁴. Serum VLDL+LDL was precipitated using heparin-MnCl₂ solution and the lipids in the VLDL+LDL fraction were extracted by and cholesterol estimated^{13,15}. LDL cholesterol was separately assessed by subtraction of VLDL cholesterol equivalent to 1/5 TAG. Fecal samples from the rats of all groups were homogenized with an equal volume of water and lyophilized to a fine powder. From this powder fecal neutral sterols and bile acids were extracted and estimated¹⁶. Activity of 3-hydroxy-3-methylglutaryl- coenzyme A (HMG-CoA) reductase in liver was determined by estimating the ratio of HMG-CoA/Mevalonate¹⁷.

The rats left behind in the other half of each group i.e., six rats each in all groups were used for studying the incorporation of 1,2-¹⁴C-acetate into hepatic cholesterol as described below. The rats were deprived of food overnight for 16hr and they were injected (ip) with 0.5 ml solution of 1,2-¹⁴C sodium acetate (10 μ Ci/100gm) 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 and individual lipids were separated by TLC on silica gel G plates with a solvent mixture of hexane-diethyl ether- acetic acid (80:20:1, by vol). Authentic lipid standards were run concurrently and the TLC plates were developed using iodine vapor. Individual lipids were located as spots corresponding to standards. Silica gel corresponding to the lipids was scraped and extracted with chloroform and the radioactivity associated with each lipid was measured separately by liquid scintillation counter.

Acute toxicity study: Acute toxicity of the leucodelphinidin derivative was carried out in rats according to standard protocol¹⁸. Different doses of the derivative dissolved in distilled water was administered orally at doses up to 4000 mg/kg body weight and animals were observed closely for a period of 72 hr for behavioral changes, toxic reactions and mortality.

Statistical analysis: All values are expressed as means and standard deviations. Data were analyzed by one-way ANOVA and the results significant at 1-5% level are considered in this study. The statistical analysis were done using SPSS Statistical Package for Windows version 10.0 (SPSS, Chicago, IL, USA).

Results and Discussion

Animal growth: The diet consumption was found to be more or less the same for all the groups (11.2 \pm 1.1 g/rat). Feeding of high cholesterol diet significantly increased the body and liver weights over the normal control by 21% and 13% (p<0.05). Treatment with the flavonoids for 90 days significantly decreased the same (7-9%) in groups 3 and 4 over the cholesterol control (p<0.05).

Serum lipids: To compare the effect of the two flavonoids on serum lipids, serum collected from rats of all four groups were analyzed for total cholesterol as well as LDL-cholesterol and HDL-cholesterol. These results and the calculated atherogenic index (Total cholesterol/HDL-C) are given in table-1. The concentration of total cholesterol and LDL cholesterol in the serum of cholesterol fed rats were significantly higher as compared to that of the normal diet fed rats (p<0.01). There was significant reduction in total cholesterol in the serum of rats treated with the flavonoids (p<0.01). Serum HDL cholesterol was slightly lowered in the cholesterol fed rats, but treatment with both the flavonoids resulted in a significant increase in the levels of serum HDL-C as compared to the cholesterol diet fed rats (p<0.01).and. Similarly the atherogenic index (Total cholesterol/ HDL-C) in the treated group of rats was also significantly lowered as compared to that of the control (p<0.01). The effect of both the flavonoids on these parameters were more or less the same.

Hepatic bile acids and excretion of fecal bile acids and neutral sterols: The concentration of hepatic bile acids and the excretion of fecal bile acids and neutral sterols were estimated and the results are given in table-2. There was a significant increase in the concentration of total bile acids in the liver and also increase in the fecal excretion of bile acids and neutral sterols in the rats fed cholesterol containing diet as compared to those fed normal diet ($p < 0.01$). Feeding the flavonoids further significantly increased ($p < 0.01$) the concentrations of hepatic bile acids and the fecal excretion of bile acids and neutral sterols as compared to the control cholesterol diet fed group. The leucodelphinidin derivative showed a slightly better effect than quercetin.

Synthesis of hepatic cholesterol: The synthesis of hepatic cholesterol was estimated by assaying the activity of HMG-CoA reductase (Ratio of HMG CoA/Mevalonate) and the

concomitant in vivo incorporation of labeled acetate into hepatic cholesterol. These results are given in table-3. The ratio of HMG CoA/Mevalonate indicates the activity of HMG CoA reductase in a reverse order i.e., lower ratio indicating higher activity and higher ratio lower activity. Feeding of high cholesterol diet significantly decreased HMG CoA reductase activity in liver ($p < 0.01$). This was reflected in the significant decrease ($p < 0.01$) in the incorporation of labeled acetate into free and esterified cholesterol in the liver of rats fed cholesterol rich diet. The decrease in activity was restored to near normal levels on treatment with the flavonoids ($p < 0.01$) as was shown by a significant increase in the incorporation of labeled acetate into both free and esterified cholesterol in the flavonoid treated groups ($p < 0.01$). The effects of both the flavonoids on hepatic cholesterol synthesis were more or less the same.

Table-1

Concentration of cholesterol in serum and lipoproteins (Mean values \pm standard deviations for six rats per group) TC, total cholesterol. Second group values^a except HDL-C are significantly higher than the normal ($p < 0.01$) and 3 and 4 group values^b are significantly lower than that of the 2nd. 3 and 4 HDL-C values^c are significantly higher than 2nd ($p < 0.01$)

Groups	TC (mg/100ml serum)	Serum lipoproteins (mg/100ml serum)		Atherogenic index TC/HDLc
		HDLc	LDLc	
Normal diet (Normal control)	68.2 \pm 1.21	43.2 \pm 0.50	16.6 \pm 0.23	1.57 \pm 0.006
2% Cholesterol diet (Cholesterol control)	159.2 \pm 1.91 ^a	41.8 \pm 0.56	93.8 \pm 1.5 ^a	3.80 \pm 0.038 ^a
2% Cholesterol diet+ Leucodelphinidin (100mg/kg body wt)	108.2 \pm 1.81 ^b	63.9 \pm 0.53 ^c	36.6 \pm 0.63 ^b	1.69 \pm 0.008 ^b
2% Cholesterol diet+ Quercetin (100mg/kg body wt)	106.2 \pm 1.81 ^b	64.9 \pm 0.53 ^c	34.6 \pm 0.63 ^b	1.63 \pm 0.009 ^b

Table-2

Concentration of hepatic bile acids and fecal excretion of bile acids and neutral sterols. (Mean values \pm standard deviations for six rats per group). Second group values^a are significantly higher than the normal ($p < 0.01$) and 3 and 4 group values^b are significantly higher than that of 2nd ($p < 0.01$)

Groups	Hepatic bile acids (mg/100gm wet tissue)	Fecal excretion (mg/rat/day)	
		Bile acids	Neutral sterols
Normal diet (Normal control)	29.2 \pm 0.12	23.6 \pm 0.16	17.6 \pm 0.11
2% Cholesterol diet (Cholesterol control)	39.4 \pm 0.21 ^a	31.8 \pm 0.52 ^a	43.2 \pm 0.53 ^a
2% Cholesterol diet + Leucodelphinidin (100mg/kg body wt)	60.8 \pm 0.51 ^b	55.9 \pm 0.53 ^c	86.1 \pm 0.73 ^b
2% Cholesterol diet+Quercetin (100mg/kg body wt)	57.1.2 \pm 0.42 ^b	52.5 \pm 0.53 ^c	79.6 \pm 0.62 ^b

Table-3

Rate of cholesterol synthesis and in vivo incorporation of (1,2-¹⁴C) acetate into hepatic cholesterol.(Mean values ± standard deviations for six rats per group). *Ratio of HMGCoA/Mevalonate is inversely proportional to HMG CoA reductase activity. Second group values^a show significantly lower activities of HMG CoA reductase and also decreased incorporation of (1,2-¹⁴C) acetate into hepatic cholesterol. 3 and 4 group values^b show significantly higher activities of HMG CoA reductase and also increased incorporation of (1,2-¹⁴C) acetate into hepatic cholesterol. (p<0.01)

Groups	Ratio of HMG CoA/ Mevalonate*	Free cholesterol (count/min/g tissue)	Ester cholesterol
Normal diet (Normal control)	2.28±0.025	1480±17.12	390.1±3.42
2% Cholesterol diet (Cholesterol control)	3.42±0.038 ^a	943.5±6.52 ^a	216.2±2.50 ^a
2% Cholesterol diet + Leucodelphinidin (100mg/kg body wt)	2.33±0.029 ^b	1325.0±10.25 ^b	376.1±3.34 ^b
2% Cholesterol diet + Quercetin (100mg/kg body wt)	2.31±0.022 ^b	1309.5±10.39 ^b	382.6±3.69 ^b

The results obtained in this study clearly indicate that the flavonoids studied have beneficial effects to a significant level on metabolism of rats fed cholesterol, containing diet. It is noteworthy to observe that the hypolipidaemic effect of leucodelphinidin is more or less the same as quercetin. The significant decrease observed in serum cholesterol in the rats treated with leucodelphinidin derivative and quercetin further extended an influence to decrease serum LDL-C also. Furthermore, their HDL-C showed significant increase on treatment as compared to the cholesterol diet fed rats. Studies in our laboratory and elsewhere with other ficus bioflavonoids have shown to decrease both total and LDL-C in hyperlipaemic rats and rabbits^{10,19}. In Germany a study in overweight subjects with a high cardiovascular risk phenotype have provided strong evidence supporting the cardioprotective properties of quercetin. Treatment with quercetin at a dose of 150 mg/d for six weeks was found to reduce systolic pressure and plasma oxidized low-density lipoprotein concentrations²⁰. Significant decreases in serum total and LDL-C were attained in female chickens treated with quercetin²¹. The protective effect of flavonoids as observed in our study on HDL-C is in agreement with similar results obtained by other workers using flavonoids from green tea, and fenugreek seeds^{22,23}. Bruteridin and melitidin, flavonoids of bergamot juice are structural analogues of statins and were shown to inhibit HMG-CoA reductase the enzyme that catalyzes conversion of HMG-CoA to mevalonate, which is the rate limiting step in the cholesterol synthesis pathway²⁴. However, in the present study such an inhibitory effect on HMG-CoA reductase was not observed on treatment with leucodelphinidin or quercetin. Instead the increased incorporation of labeled acetate into both free and esterified cholesterol in the treated groups indicate increased hepatic cholesterogenesis. The fact that the concentration of total bile acids was significantly higher in rats treated with the ficus flavonoid and quercetin indicates an increased hepatic degradation of cholesterol to bile acids. Increased excretion of neutral sterols and bile acids was also observed indicating that more of the cholesterol is degraded in the liver

and removed via bile acids in hyperlipidaemic rats treated with the flavonoids. Gallic acid, catechin and epicatechin, the major polyphenolic compounds in grape seed show cholesterol lowering activity by inhibiting pancreatic cholesterolesterase, binding of bile acids and reducing solubility of cholesterol in micelles which may result in delayed cholesterol absorption²⁵. The hypocholesterolemic effects of Chinese green tea, silimarin and glycyrrhiza glabra root powder also have shown to be mediated via accelerated bile acid and neutral sterol elimination through feces with an increased hepatic bile acid production^{22,26,27}.

Atherosclerosis is a progressive inflammatory disease affecting large and medium-sized arteries that may manifest as coronary heart disease, cerebrovascular disease or peripheral vascular disease. Coronary artery obstruction and myocardial infarction are the number one killers in the world. Epidemiological data demonstrate that the risk for coronary heart disease and other forms of atherosclerotic vascular disease rises with plasma cholesterol possible mode of action of flavonoids in regulating hypercholesterolemia can be attributed to blocking the entero-hepatic circulation of bile acids due to increased binding to flavonoids with bile acids conjugates and their subsequent excretion. Liver cells respond to this situation by increasing the number of LDL receptors and also by increasing the rate of cholesterol synthesis. But as the inhibition of bile acid recycling may far outweigh cholesterol synthesis, the decrease in both plasma total and LDL-C is noted in hypercholesterolemic rats treated with the flavonoids. The molecular structure of flavonoids plays a decisive role in its potential as a lipid lowering agent. Studies in HepG2 cells with two polymethoxylated citrus flavonoids (PMFs), tangertin and nobiletin have shown that flavonoids with a fully methoxylated A-ring lower blood cholesterol and triacylglycerol concentrations by suppressing hepatic apoB secretion²⁸. It is pertinent to note here that the leucodelphinidin derivative from ficus has two methoxy groups in its A-ring and further studies are warranted to relate its molecular structure with lipid lowering properties.

The leucodelphinidin derivative did not show any toxic effects even at a high dose of 4000 mg/kg in experimental animals. The methylation of many of the hydroxyl groups and absence of toxophore group (-CO-CH=CH-O-) in the structure of leucodelphinidin might have conferred on them non-toxicity. In contrast although quercetin also showed hypocholesterolemic and related effects its consumption for humans is debatable as it belongs to a class of toxic flavonoids²⁹. Acute toxicity studies revealed no mortality up to a dose of 4.0 g/kg body weight. Hence the LD₅₀ may be more than 4.0 g/kg body weight. The drug may be used at ordinary doses of 50-500 mg/ kg dose level.

Conclusion

Our observations suggest that leucodelphinidin may be useful as a naturally occurring non-toxic hypocholesterolemic agent. Further investigations are required to evaluate the mechanisms of action of this polymethoxylated ficus flavonoid in lowering the risk of cardiovascular diseases.

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