Chapter 1

Clerodendron glandulosum. Coleb leaf extract ameliorates high fat diet-induced alteration in lipid and cholesterol metabolism in rats.

INTRODUCTION

Cholesterol, a necessary component of human body is now a days a major concern for the society and scientific fraternity. Over the past few years, consumption of dietary cholesterol has increased greatly because of westernized dietary patterns. Saturated fats and/or cholesterol rich diet contribute to hypercholesterolemia and hypertriglycedemia (Kok *et al.,* 1996) that progress to CVDs (Reiner and Tedeschi-Reiner 2006). Epidemiological and experimental studies have established the role of elevated plasma cholesterol in the development of atherosclerosis and other cardiovascular diseases. Persistently high levels of plasma cholesterol (6-6.5 mmol/l) reportedly increase the vulnerability of CVDs by many folds (Ginsberg, 1990; Havel and Kane, 1995; Tripathi, 1995; Mary *et al.*, 2000).

Lipoproteins such as HDL, LDL and VLDL essentially transport endogenous lipids from the liver to the non-hepatic tissues while peripheral and hepatic chylomicrons transport dietary lipids from the intestine to the peripheral and hepatic tissues through endogenous pathways. Endogenous factors such as diet or genetic defects in the synthesis/degradation apparently elevate the levels of circulating plasma lipoproteins. Dyslipidemia or hypercholesterolemia characterized by a combination of elevated plasma levels of cholesterol, TG, LDL and VLDL along with lower level of HDL increases the risk of cardiovascular complications (Miek, 1999; Henry and Ginsberg, 2002).

Herbs, generally considered as a safe in strengthening and toning the human body systems are used as dried extracts or tinctures (alcoholextracts) or, in any other dosage form singly or in combination, as noted by Mukherjee (2001). Currently available hypolipidemic drugs like gemfibrozil, bezafibrate, lovastatin, and nicotinic acid are

unsafe particularly when used for prolonged periods. Viewed in this context, the hypolipidemic potentials of medicinal plants need critical study. Several plant species with anti-hypercholesterolemic potentials have found recognition. For example, garlic (*A. sativum*) has the effect of reducing cholesterol. It is most effective when included in the diet in the raw form or when taken as capsules. Herbs with an established scientific claim as hypocholesterolemic agents are many as listed here. Chickpea-*Cicer arietinium*, guggul-*Commiphora mukul,* turmeric-*Curcuma longa,* gooseberry-*Emblica officinalis,* pushkarmoola-*Inula racemosa, Terminalia* species of plants like arjuna-*Terminalia arjuna,* myrobalan-*Terminalia chebula,* fenugreek-*Trigonella foenum graecum*, milk thistle-*Silybum marianum*, dandelion root-*Taraxacum officinale*, burdock root-*Arctium lappa*, blue flag-*Iris versicolor*, greater celandine-*Chelidoniummajus*, blue vervain - *Verbena bastata*, ginger-*Zingier offiicinalis*, alfalfa -*Medicago sativa*, hawthorn berries-*Crataegusoxy anthoide etc.* These herbs help to lower high blood pressure, promote cholesterol metabolism and suppress cholesterol synthesis (Sharma and Dwivedi, 1997; Wang and Ng, 1999; Mukherjee, 2002).

Aim:-To evaluate effect of *C.glandulosum.*Coleb leaf aqueous extract on normolipidemic and hyperlipidemic rats.

MATERIALS AND METHODS

Plant material

CG leaves collected from Imphal district India in the month of June were shade dried. The Department of Botany, D.M.College of Science Manipur, Imphal identified the plant and a sample (voucher specimen No.405) deposited in the herbarium of the Department of Botany.

Preparation of extract

Hundred grams of fine powder (obtained by grinding shade dried leaves of CG) mixed with distilled water was boiled at 100°C for 3 hours and filtered. The filtrate was concentrated by heating to obtain a semisolid paste that was later freeze dried resulting in a total yield of 28% W/W. Different doses of freeze dried extract (CG) were prepared by dissolving a known weight of dried paste in 0.5% Carboxy methylcellulose (CMC).

Phytochemical analysis

The quantitative phytochemical analysis of CG leaves for saponins (Xi *et al.,* 2007), phytosterols (Goad and Akihisa, 1997), ascorbic acid (Barakat *et al.,* 1973), polyphenols (Yen and Hsieh, 1998), and flavanoids (Chang *et al.,* 2002) has been carried out.

HPLC chromatogram of aqueous extract of CG leaves

Mobile phase - Methanol:Water [50:50]

Flow rate- 0.5 ml/min

Total run rime- 40 min

Wavelengths- 254, 366 and 540 nm

Figure 1: HPLC chromatogram of CG leaf aqueous extract at 254 nm.

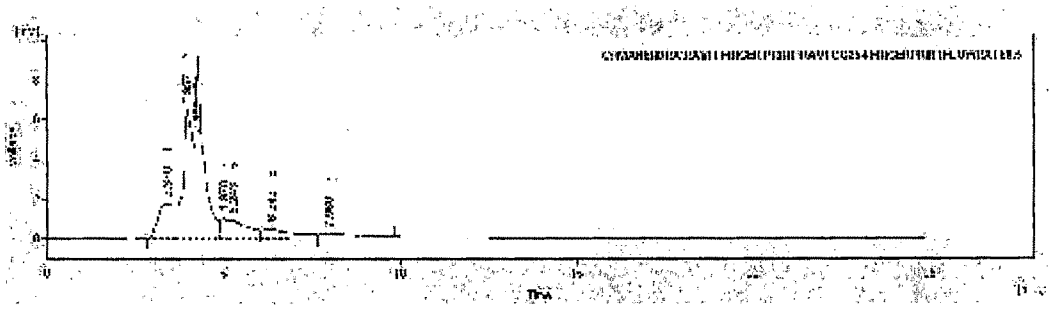


Table 1: Data analysis of HPLC chromatogram at 254nm

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	3.370	544.437	17.574	13.1	8.7	**0.53**
2	3.957	1215.980	64.683	29.3	32.2	**0.29**
3	4.257	1498.986	92.923	36.1	46.2	**0.20**
4	4.970	157.141	10.039	3.8	5.0	**0.28**
5	5.243	338.135	9.328	8.1	4.6	**0.71**
6	6.343	276.156	4.590	6.6	2.3	**0.94**
7	7.960	123.107	1.872	3.0	0.9	**1.21**
	Total	**4153.942**	**201.009**	**100.0**	**100.0**	

Figure 2: HPLC chromatogram of CG leaf aqueous extract at 366 nm.

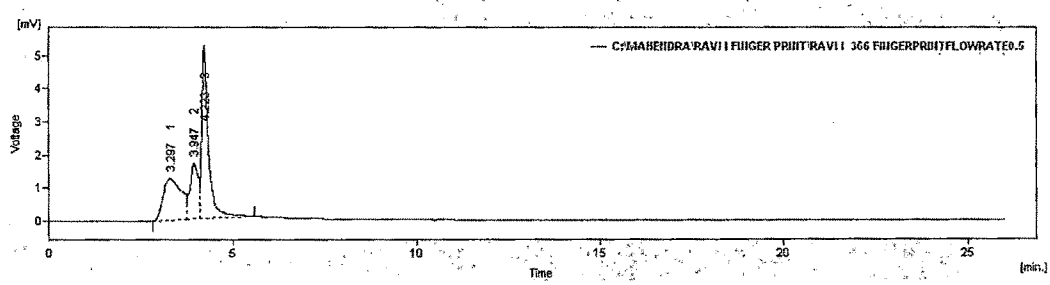


Table 2: Data analysis of HPLC chromatogram at 366 nm

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	3.297	436.788	12.713	33.2	15.5	0.68
2	3.947	240.089	16.872	18.2	20.6	0.25
3	4.223	640.359	52.250	48.6	63.8	0.15
	Total	1317.236	81.836	100.0	100.0	

Figure 3: HPLC chromatogram of CG leaf aqueous extract at 540 nm.

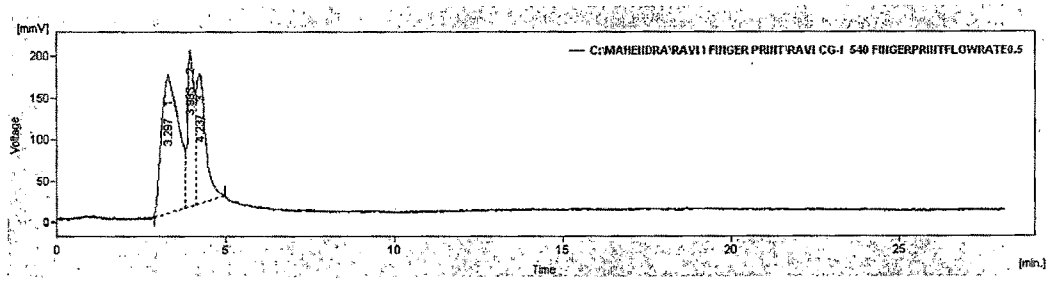


Table 3: Data analysis of HPLC chromatogram at 540 nm

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	3.297	57.258	1.643	51.0	32.6	0.67
2	3.963	25.110	1.858	22.4	36.9	0.23
3	4.237	29.975	1.540	26.7	30.5	0.29
		112.343	5.041	100.0	100.0	

Experimental Animals

Female *Charles foster* albino rats (180-220g) maintained in clean polypropylene cages under controlled room temperature (22±2°C) received normal diet (SLD) or high fat diet (HFD) (Rathi *et al.*, 1984) and water *ad libitum*. Experiments on animals were performed in accordance with the guidelines of the institutional animal ethical committee (Approval No.827/ac/04/CPCSEA).

Table 4: Composition of experimental diets.

Ingredients (g%)	Normal diet (SLD)	High fat diet (HFD)
Laboratory chow	50.0	33.9
Sucrose	20.0	20.0
Casein	20.0	20.0
Coconut oil	5.0	20.0
Salt mixture	4.0	4.0
Vitamin mixture	1.0	1.0
Cholesterol	-----	1.0
Cholic acid	-----	0.1

Experimental Design

Fifty-four animals were divided into 9 groups (n=6 per group). Groups I and V were fed with SLD and HFD respectively while, groups II, III, and IV were maintained on SLD and orally administered with 200,400 or 800 mg/kg CG extract daily. Groups VI, VII and VIII maintained on HFD received CG extract as mentioned above. Group IX fed with HFD received synthetic hypolipidemic agent, lovastatin (LVS; 5mg/kg BW). All the animals maintained for a total of 42 days received oral administration of vehicle, extract or LVS by gastric intubation.

Lipid profile

Plasma total cholesterol (TC), triglycerides (TG) and high-density lipoprotein (HDL) were analyzed using commercially available kits (Reckon diagnostics, Baroda, India). Application of Friedwald's formula (Friedwald *et al.*, 1972) provided the levels of very low-density lipoproteins (VLDL) and low-density lipoprotein (LDL).

VLDL= TG/5 and LDL=TC-HDL+VLDL

Hepatic and fecal lipids extracted in chloroform: methanol (2:1) mixture was dried. Gravimetric analysis yielded the total lipid (TL) content (Folch *et al.*, 1957). TC and TG contents were analyzed using kits as mentioned above from the dried lipid extract dissolved in 1% triton X 100 (Jong-Ho *et al.*, 2003).

Fecal cholic acid (CA) and deoxycholic acid (DCA)

Fecal samples from each experimental group collected on every 3rd day between days 31 and 42 of study were dried, eluted with absolute alcohol, filtered and processed for estimation of CA and DCA (Mosback *et al.*, 1954). The filtrate was subjected to hydrolysis with 2 ml of 5% NaOH at 15 LBS pressure in an autoclave for 1.5 hour,

neutralized with ether and evaporated to dryness. Later, supernatant obtained by centrifugation of residues dissolved in 1 ml acetone was dried. Then, 1.5 ml of 65% H_2SO_4 was added to the dried filtrate and heated at 60°C for 15 min, cooled at room temperature and the absorbance read at 320 nm for cholic acid and at 385 nm for deoxycholic acid.

Estimation of enzymes of lipid metabolism

- Lipoprotein lipase (LPL; EC 3.1.1.34) activity was estimated in fresh tissue homogenates (Dole, 1955; Mays and Felts, 1968) by mixing 0.5 ml of olive oil emulsion, 0.1ml of deoxycholate, 0.5ml of buffer and 0.5 ml of tissue homogenate and incubating at 37°C for 30 min along with control tubes to which enzymes were not added. After arresting the reaction by keeping the tubes at room temperature, 2.5 ml of chloroform and 5 ml of Doles mixture were added and mixed well. Then 3 ml of hexane and 2ml of distilled water were added, shaken well and centrifuged. To 2 ml of the supernatant, 2 ml of chloroform and 2 ml of copper reagent were added. From this, a 2ml aliquot was taken and 0.5 ml of diethyl thiocarbamate reagent added. The color was read at 420 nm and activity expressed as μ moles of free fatty acids liberated /hr/mg protein.
- Intravenous injection of Heparin (50 units/rats) two minutes before blood collection facilitated release of endothelium bound LPL (Qi *et al.*, 2006). Separated plasma was assayed for post-heparin lipolytic activity (PHLA) as per Dole (1955) and Mays and Felts (1968).

Estimation of Cholesterol metabolizing enzymes

- Assay of activity level of lecithin-cholesterol acyltransferase (LCAT, EC 2.3.1.43) in plasma was by the method of Legraud *et al.* (1979) with modifications by Hitz *et al.* (1983). From the incubation mixture containing 0.6 ml of substrate and 0.6 ml of tissue homogenate, 0.4 ml was transferred immediately to a tube containing 1ml of isopropanol to arrest the reaction. This gave the cholesterol content present at the beginning of the experiment. Incubation at 27°C was continued for 10 min and the reaction was arrested. After centrifugation, 2 ml of acetone and 1 ml of digitonin were added to the supernatant. It was left aside for 1 hr., centrifuged and the cholesterol content estimated. Plasma LCAT activity was expressed as µmol of cholesterol esterified /hr/mg protein
- HMG CoA reductase (EC 1.1.1.34) activity assayed by the method of Rao and Ramakrishnan (1975) was expressed as inversely proportional to the mevalonate content. Ten percent homogenate prepared in saline arsenate solution was mixed with equal volume of perchloric acid diluted (50 ml/l) and after 5 minutes, it was centrifuged for 10 min at 2000rpm. One ml of supernatant was divided into two tubes, followed by addition of 0.5 ml of acidic (for mevalonate) or basic (for HMG Co A) hydroxylamine reagent and ferric chloride. The mixture was then incubated for 10 min at room temperature and absorbance read at 540 nm.

Calculation: - $\frac{\text{Absorbance of HMG Co A}}{\text{Absorbance of Mevalonate}}$

- Cholesterol ester synthase (CES, EC 2.3.1.26) was assayed by the method of Kothari *et al.* (1973) in liver and intestine. An incubation mixture containing

0.5ml of acetate buffer, 0.2 ml substrate and 0.2 ml tissue homogenate was incubated at 37°C for 6 hr .The reaction was arrested by adding 5 ml acetone-ethanol mixture and centrifuged. Later, 1 ml digitonin was added to the supernatant, followed by 2 drops of acetic acid. After 16 hr of incubation in a dark chamber it was centrifuged and the precipitate was washed twice with acetone-ether mixture and finally with dry ether. The CES activity was expressed as moles of cholesterol esterified /hr/mg protein.

- Protein content of liver, adipose tissue and intestine was analyzed by the method of Lowry *et al.* (1951).

Statistical analysis

Statistical analysis of the data was done by one-way ANOVA followed by Bonferroni's multiple comparison test. The results are expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California, USA.

RESULTS

Phytochemical analysis

Quantitative phytochemical analysis of CG leaf extract recorded, 4.36 % Flavanoids, 6.39 % Sterols, 5.23 % Saponins, 3.22 % Polyphenols and 5.96 % Vitamin C.

Lipid profile

Rats fed with HFD for 42 days recorded significant elevation in plasma (Figure. 4&5, Table. 5&6) and hepatic lipids (Figure. 6, Table. 7) and decreased plasma HDL level compared to CON rats. CG extract did not record any significant effect in SLD fed rats in any of the doses tested herein. However, the extract had a dose dependent effect in lowering plasma TC, TG, LDL, and VLDL and hepatic TL, TG and TC compared to

HFD rats. A comparison of CG treated groups with LVS treated rats revealed a better response in the former with respect to plasma triglyceride and HDL levels (Table 5 & 6).

Lipid metabolizing enzymes

HFD rats recorded significant decrement in activity levels of PHLA, and LPL in adipose tissue and liver ($P<0.001$). HFD+CG 200mg/kg did not record any significant effect in PHLA and LPL activities. However, HFD+CG (400 and 800mg/kg) showed significant resistance towards decrease in PHLA and LPL activities compared to HFD rats ($P<0.001$). HFD+LVS rats registered non-significant effect in PHLA and LPL activities compared to HFD group (Figure 9, Table 10). The CG extract had no significant effect on these enzymes in tissues of SLD rats compared to CON rats (Figure 9, Table 10).

Cholesterol metabolizing enzymes

HFD rats recorded a significant decrement in plasma LCAT activity ($P<0.001$) and increment in hepatic and intestinal CES ($p<0.001$). Hepatic HMG Co A reductase activity registered a non-significant alteration in HFD rats. Higher doses of CG (400 and 800 mg/kg) depicted dose dependent decrement in the activity level of HMG Co A reductase ($p<0.05$ and $p<0.01$) and prevented decrease in plasma LCAT activity ($P<0.001$). HFD+CG rats recorded a dose dependent protective effect against decrement in hepatic and intestinal CES activity (Figure 10, Table 11). HFD+LVS rats recorded significant decrease in HMG Co A reductase activity ($P<0.001$) along with protective effect against the decrease in intestinal and hepatic CES activities ($P<0.01$) and a non-significant change in plasma LCAT activity compared to HFD rats. The CG extract had no significant effect in hepatic HMG Co A reductase, plasma LCAT and, CES activities in liver and intestine in SLD fed rats (Figure 10, Table 11).

Fecal lipid profile and bile acid content

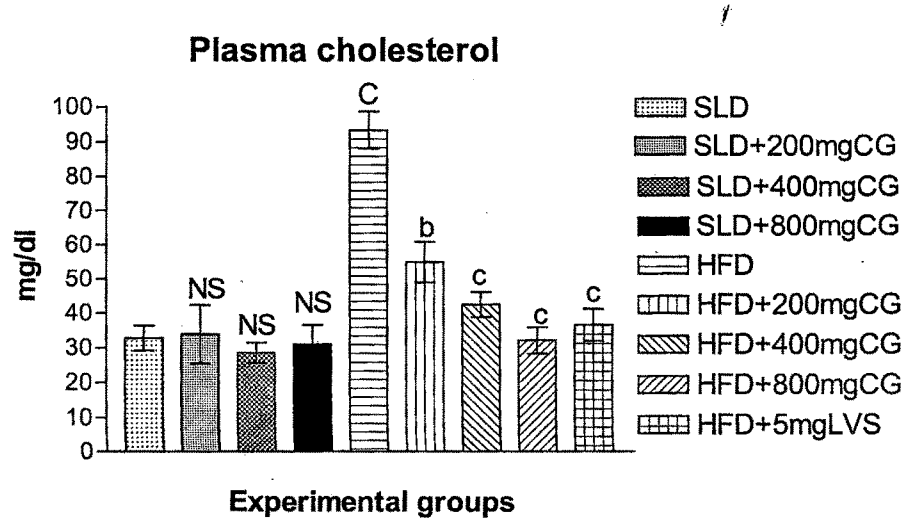
HFD rats did not show any significant changes in fecal TL, TC, TG, (Figure 7, Table. 8) cholic acid and deoxycholic acid contents (Figure 8, Table 9). The extract fed rats recorded a highly significant increment in fecal TL, TC and TG contents with all the three doses compared to HFD rats ($p<0.001$). HFD+CG recorded a dose dependent increment in fecal CA and DCA compared to HFD groups ($P<0.001$). HFD+LVS rats recorded highly significant increment in fecal TL and TC ($P<0.001$) and a non-significant increment in fecal TG, CA and DCA.

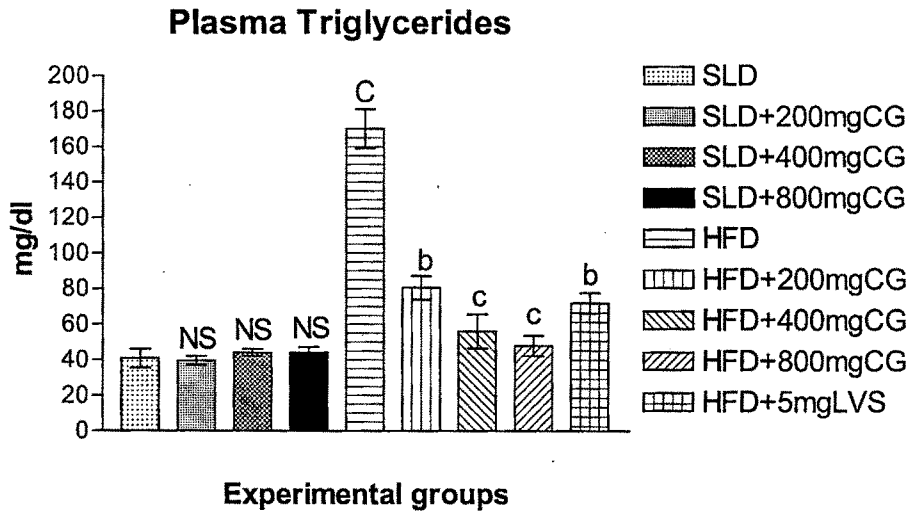
Table 5: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on plasma cholesterol and triglycerides.

Treatment	Cholesterol (mg/dl)	Triglycerides (mg/dl)
SLD	32.80±3.57	40.86±5.31
SLD+200mgCG	33.91±8.34^{NS}	39.63±2.45^{NS}
SLD+400mgCG	28.57±2.87^{NS}	44.26±2.01^{NS}
SLD+800mgCG	30.99±5.53^{NS}	44.48±3.00^{NS}
HFD	93.36±5.36^{C}	170.29±10.99^{C}
HFD+200mgCG	54.84±5.96^{b}	80.69±6.69^{b}
HFD+400mgCG	42.36±3.69^{c}	56.17±9.67^{c}
HFD+800mgCG	32.03±3.79^{c}	48.05±5.69^{c}
HFD+5mgLVS	36.43±4.60^{c}	72.05±5.65^{b}

Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Figure 4: Effect of *C.glandulosum*.Coleb leaf extract and lovastatin on plasma cholesterol and triglycerides.





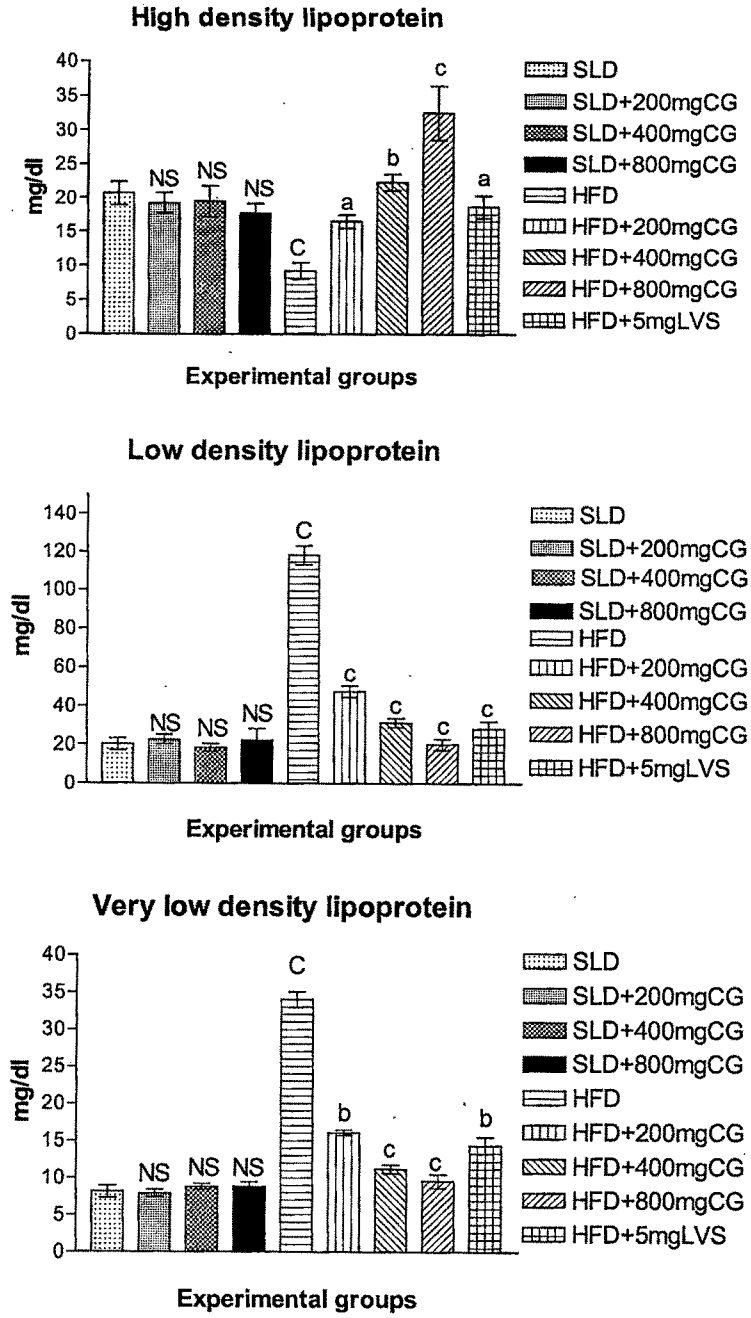
Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Table 6: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on plasma lipoproteins.

Treatment	High density lipoprotein (mg/dl)	Low density lipoprotein (mg/dl)	Very low density lipoprotein (mg/dl)
SLD	20.70±1.71	20.27±3.00	8.17±0.86
SLD+200mgCG	19.24±1.51 ^{NS}	22.59±2.49 ^{NS}	7.92±0.49 ^{NS}
SLD+400mgCG	19.48±2.31 ^{NS}	18.43±2.03 ^{NS}	8.85±0.40 ^{NS}
SLD+800mgCG	17.67±1.51 ^{NS}	22.22±6.01 ^{NS}	8.89±0.59 ^{NS}
HFD	9.20±1.21 ^{C}	118.21±5.06 ^{C}	34.05±1.02 ^{C}
HFD+200mgCG	16.49±1.00 ^{a}	47.50±3.07 ^{c}	16.13±0.34 ^{b}
HFD+400mgCG	22.31±1.21 ^{b}	31.28±2.36 ^{c}	11.23±0.56 ^{c}
HFD+800mgCG	32.51±3.95 ^{c}	20.15±2.67 ^{c}	9.61±0.93 ^{c}
HFD+5mgLVS	18.68±1.68 ^{a}	28.16±3.96 ^{c}	14.41±1.13 ^{b}

Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Figure 5: Effect of *C.glandulosum*.Coleb leaf extract and lovastatin on plasma lipoproteins



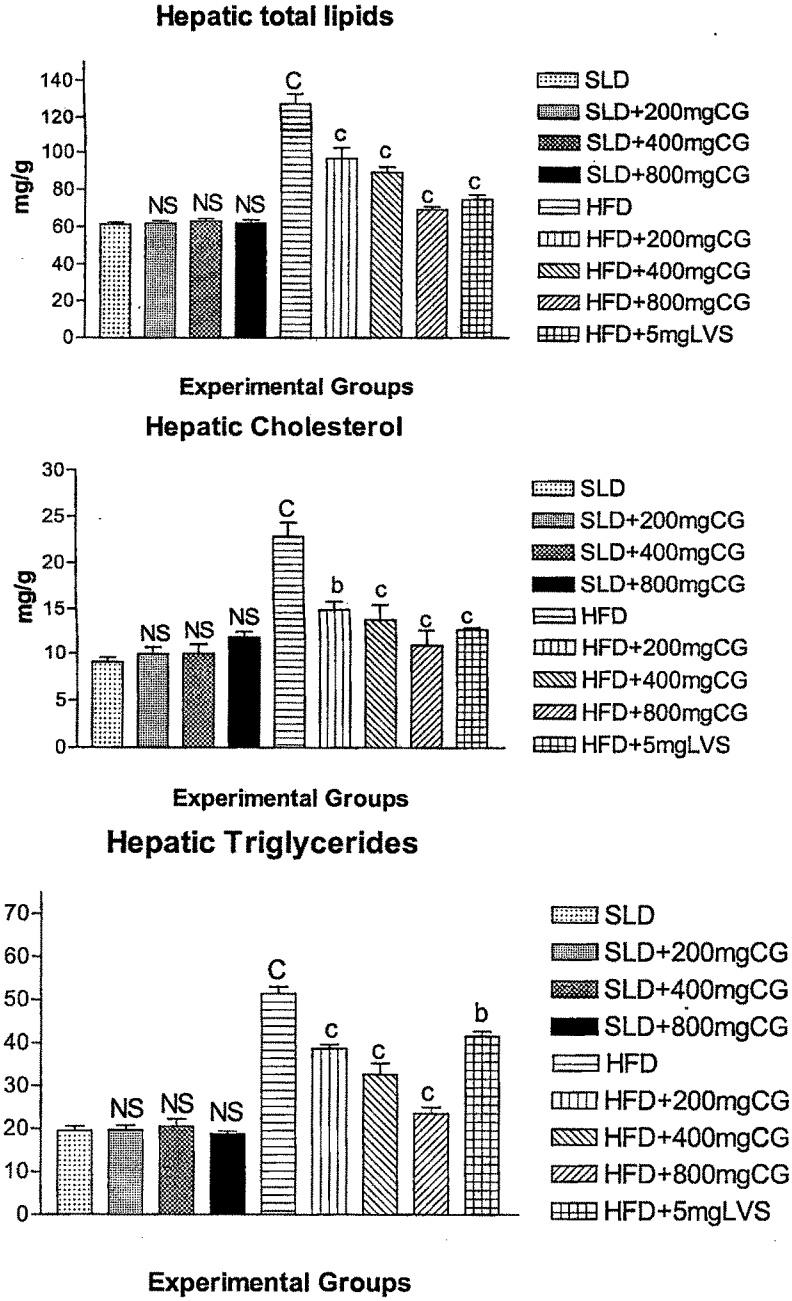
Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Table 7: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on hepatic lipids.

Treatment	Total lipids (mg/g)	Cholesterol (mg/g)	Triglycerides (mg/g)
SLD	61.30±1.30	9.04±0.49	19.50±1.11
SLD+200mgCG	61.70±1.41 ^{NS}	9.90±0.76 ^{NS}	19.70±1.19 ^{NS}
SLD+400mgCG	63.10±1.39 ^{NS}	9.96±1.08 ^{NS}	20.60±1.72 ^{NS}
SLD+800mgCG	62.00±1.94 ^{NS}	11.80±0.59 ^{NS}	18.90±0.54 ^{NS}
HFD	127.20±5.21 ^{C}	22.80±1.49 ^{C}	51.50±1.67 ^{C}
HFD+200mgCG	96.70±5.78 ^{c}	14.80±0.96 ^{b}	38.80±0.94 ^{c}
HFD+400mgCG	89.30±2.81 ^{c}	13.70±1.67 ^{c}	32.80±2.60 ^{c}
HFD+800mgCG	69.30±1.54 ^{c}	10.90±1.63 ^{c}	23.70±1.45 ^{c}
HFD+5mgLVS	74.80±2.53 ^{c}	12.60±0.20 ^{c}	41.70±1.07 ^{b}

Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Figure 6: Effect of *C.glandulosum*.Coleb leaf extract and lovastatin on hepatic lipids.



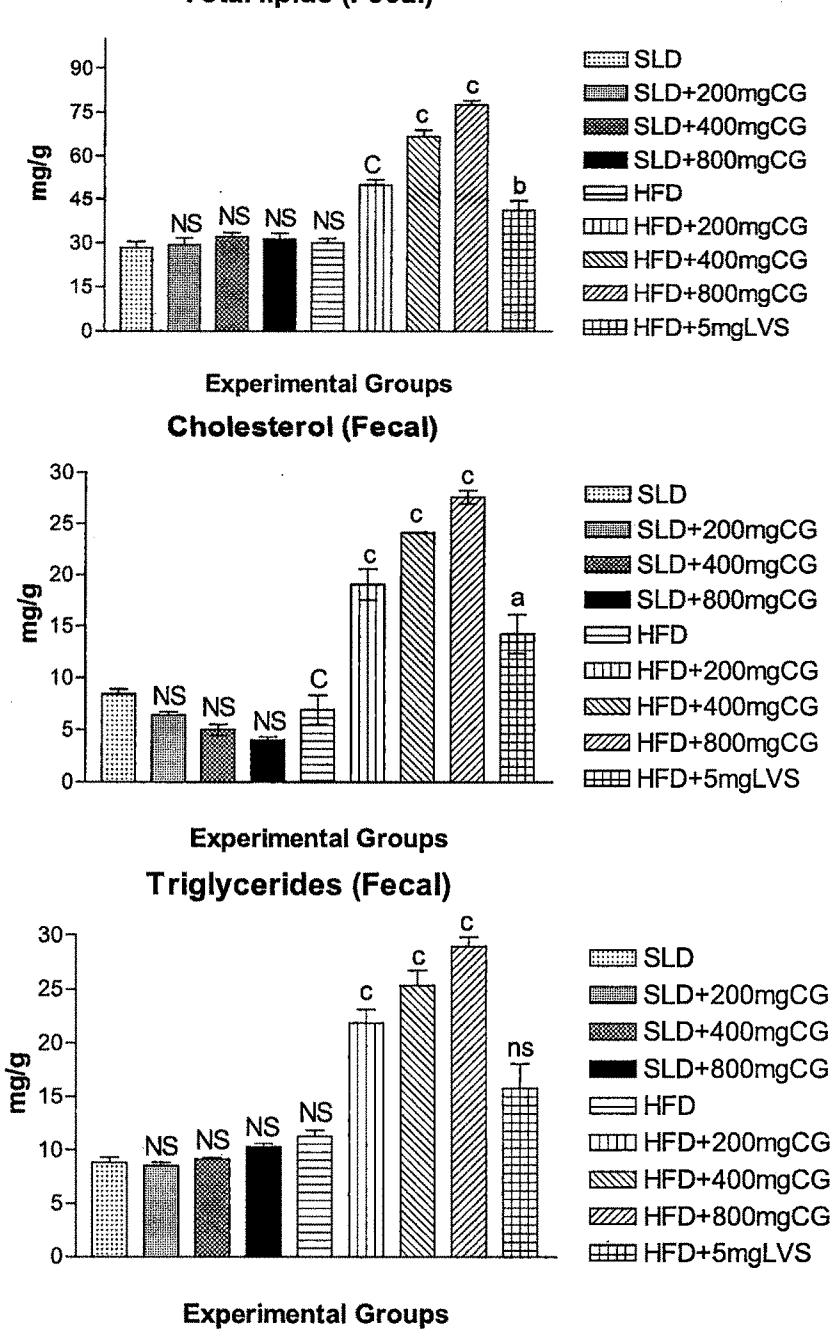
Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Table 8: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on fecal lipids.

Treatment	Total lipids (mg/g feaces)	Cholesterol (mg/g feaces)	Triglycerides (mg/g feaces)
SLD	28.30±2.07	8.43±0.45	8.82±0.49
SLD+200mgCG	29.30±2.16 ^{NS}	6.42±0.27 ^{NS}	8.52±0.31 ^{NS}
SLD+400mgCG	32.00±1.55 ^{NS}	5.00±0.54 ^{NS}	9.13±0.15 ^{NS}
SLD+800mgCG	31.20±2.20 ^{NS}	4.04±0.28 ^{NS}	10.30±0.34 ^{NS}
HFD	30.00±1.42 ^{NS}	6.93±1.39 ^{NS}	11.30±0.57 ^{NS}
HFD+200mgCG	50.20±1.78 ^{c}	19.08±1.52 ^{c}	21.90±1.23 ^{c}
HFD+400mgCG	66.70±2.15 ^{c}	24.15± 0.03 ^{c}	25.40±1.35 ^{c}
HFD+800mgCG	77.60±1.48 ^{c}	27.66±0.66 ^{c}	29.00±0.87 ^{c}
HFD+5mgLVS	41.10±3.43 ^{a}	14.25± 1.90 ^{a}	15.80±2.29 ^{ns}

Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Figure 7: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on fecal lipids.



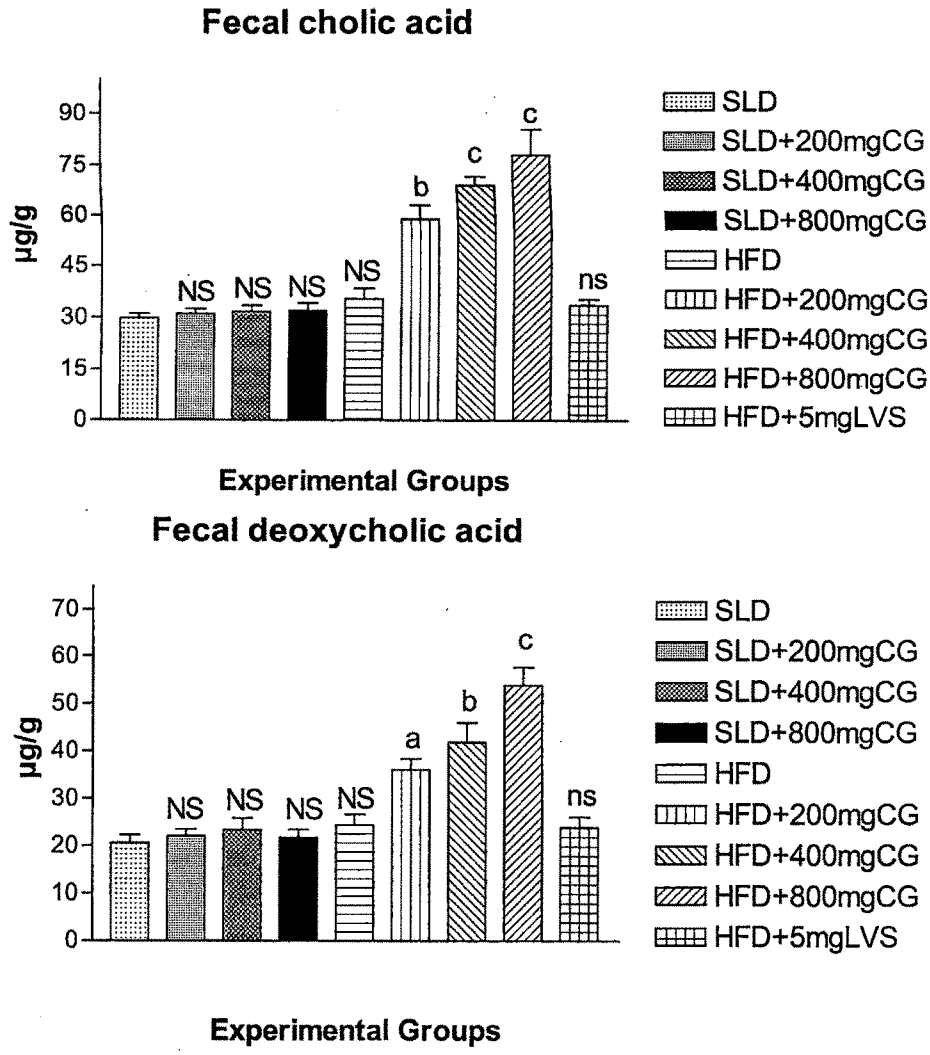
Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Table 9: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on fecal bile acids.

Treatment	Cholic acid (µg/g feaces)	Deoxycholic acid (µg/g feaces)
SLD	29.90±1.23	20.60±1.67
SLD+200mgCG	31.00±1.43 ^{NS}	22.00±1.55 ^{NS}
SLD+400mgCG	31.70±1.80 ^{NS}	23.40±2.46 ^{NS}
SLD+800mgCG	32.00±2.31 ^{NS}	21.80±1.71 ^{NS}
HFD	35.50±3.07 ^{NS}	24.40±2.30 ^{NS}
HFD+200mgCG	58.90±4.17 ^{b}	36.10±2.29 ^{a}
HFD+400mgCG	69.00±2.52 ^{c}	41.90±4.07 ^{b}
HFD+800mgCG	77.60±7.50 ^{c}	53.90±3.81 ^{c}
HFD+5mgLVS	33.80±1.74 ^{ns}	23.90±2.27 ^{ns}

Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Figure 8: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on fecal bile acids.



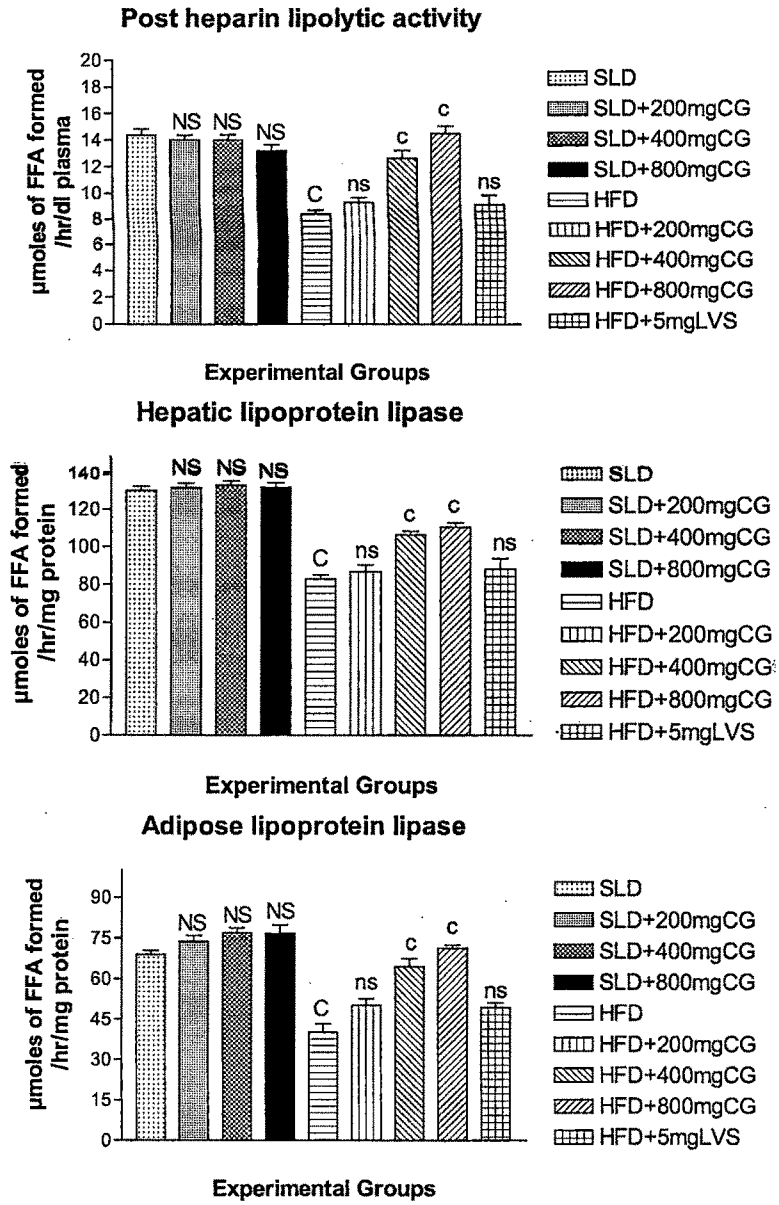
Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Table 10: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on enzymes of lipid metabolism.

Treatment	Post heparin lipolytic activity (µmoles of FFA formed /hr/dl plasma)	Hepatic lipoprotein lipase (µmoles of FFA formed /hr/mg protein)	Adipose lipoprotein lipase (µmoles of FFA formed /hr/mg protein)
SLD	14.41±0.42	130.20±2.18	68.93±1.46
SLD+200mgCG	14.00±0.40^{NS}	131.70±2.49^{NS}	73.73±2.17^{NS}
SLD+400mgCG	14.00±0.44^{NS}	133.10±2.19^{NS}	77.01±1.76^{NS}
SLD+800mgCG	13.20±0.44^{NS}	132.00±2.29^{NS}	76.77±3.21^{NS}
HFD	8.36±0.34^{C}	82.74±2.23^{C}	40.23±3.02^{C}
HFD+200mgCG	9.28±0.36^{ns}	86.76±3.49^{ns}	50.20±2.48^{ns}
HFD+400mgCG	12.60±0.59^{c}	106.30±2.06^{c}	64.50±2.91^{c}
HFD+800mgCG	14.50±0.54^{c}	110.50±2.54^{c}	71.27±1.15^{c}
HFD+5mgLVS	9.10±0.72^{ns}	88.36.0±5.55^{ns}	49.38±1.79^{ns}

Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Figure 9: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on enzymes of lipid metabolism.



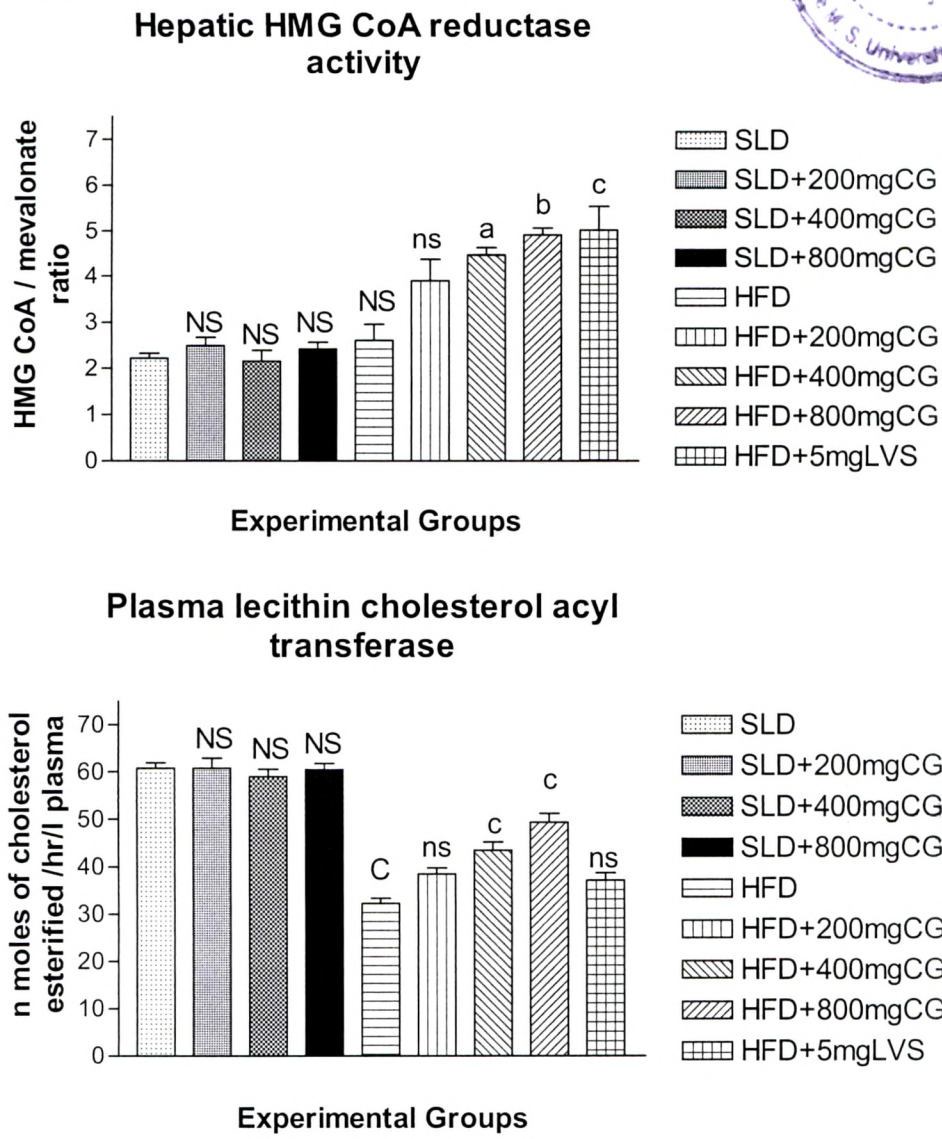
Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Table 11: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on enzymes of cholesterol metabolism.

Treatment	Hepatic HMG CoA reductase activity (HMG CoA / mevalonate ratio)	Plasma lecithin cholesterol acyl transferase (n moles of cholesterol esterified /hr/l plasma)	Hepatic cholesterol ester synthase (µ moles of cholesterol esterified/hr/mg protein)	Intestine cholesterol ester synthase (µ moles of cholesterol esterified/hr/mg protein)
SLD	2.22±0.11	60.80±1.14	11.20±.41	12.80±0.84
SLD+200mgCG	2.49±0.19 ^{NS}	60.80±2.08 ^{NS}	9.86±0.79 ^{NS}	14.70±1.72 ^{NS}
SLD+400mgCG	2.16±0.23 ^{NS}	59.00±1.62 ^{NS}	10.40±0.56 ^{NS}	14.00±0.70 ^{NS}
SLD+800mgCG	2.42±0.15 ^{NS}	60.50±1.30 ^{NS}	9.50±0.70 ^{NS}	12.90±0.45 ^{NS}
HFD	2.61±0.35 ^{NS}	32.30±1.09 ^{C}	29.60±2.05 ^{C}	35.00±2.08 ^{C}
HFD+200mgCG	3.90±0.48 ^{ns}	38.50±1.26 ^{ns}	15.40±1.19 ^{c}	24.10±1.91 ^{b}
HFD+400mgCG	4.47±0.17 ^{a}	43.50±1.77 ^{c}	12.20±0.56 ^{c}	24.00±0.75 ^{b}
HFD+800mgCG	4.92±0.15 ^{b}	49.40±1.89 ^{c}	10.20±1.37 ^{c}	21.00±1.40 ^{c}
HFD+5mgLVS	5.03±0.52 ^{c}	37.20±1.61 ^{ns}	17.70±2.15 ^{c}	19.80±2.68 ^{c}

Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

Figure 10: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on enzymes of cholesterol metabolism.



Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
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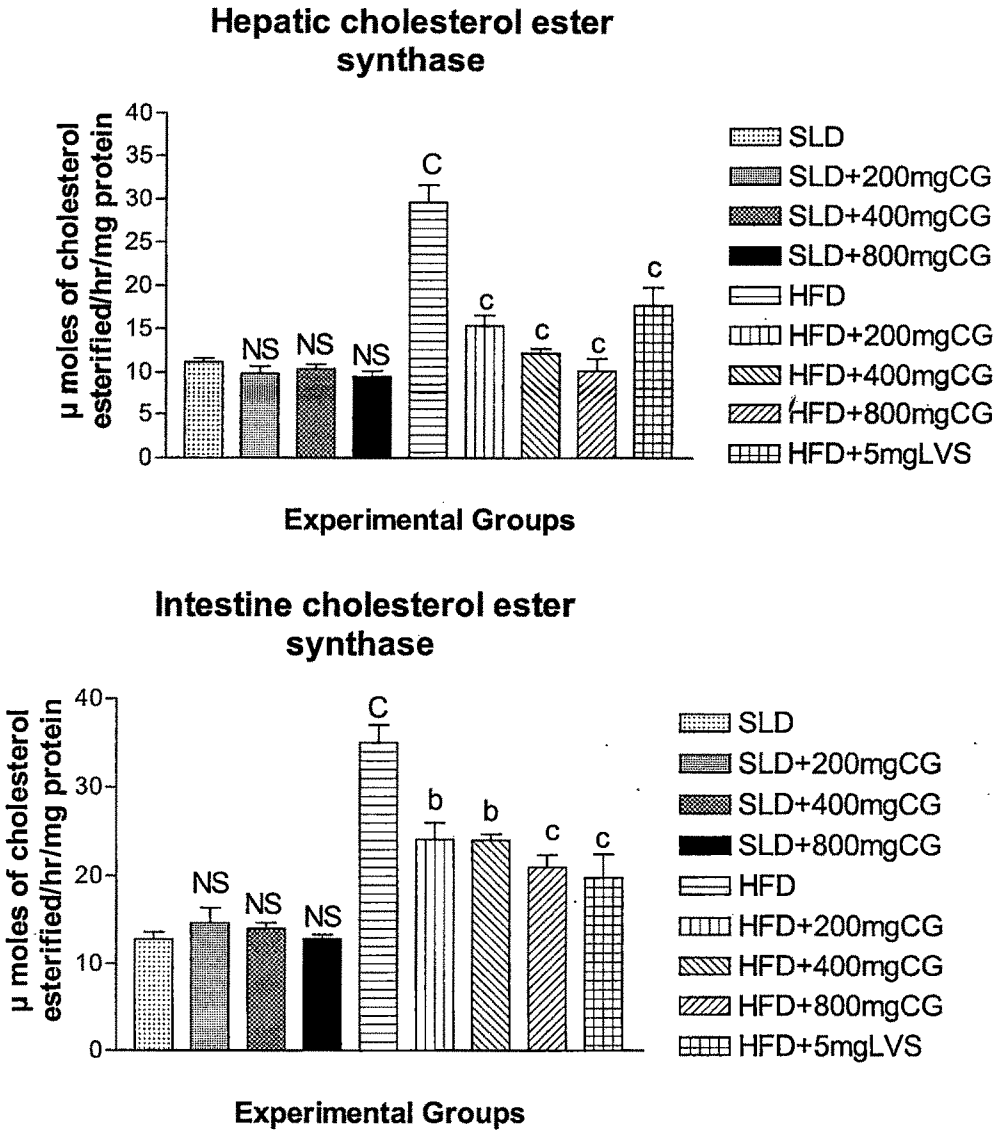
Figure 11: Effect of *C.glandulosum.*Coleb leaf extract and lovastatin on enzymes of cholesterol metabolism.



Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to SLD.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to HFD.

DISCUSSION

Cholesterol is an important molecule for eukaryotic organisms as it modulates membrane fluidity and serves as precursor for steroid hormones synthesis. In mammals, a complex system regulates biosynthesis and transport of cholesterol between cells in the body. Cholesterol is transported by lipoproteins in the blood. Lipoproteins are water-soluble protein-lipid complexes, which consist of a hydrophobic core containing triglycerides and cholesterol esters, and a hydrophilic monolayer shell composed of phospholipids, free cholesterol, and specific proteins (apolipoproteins). Several different lipoproteins can be distinguished based on their lipid and apolipoprotein composition, electrophoretic mobility, and size (Table 12).

Table 12: Classification of human lipoproteins (Ginsberg, 1998).

	Chylomicrons	VLDL	LDL	HDL
Diameter (nm)	75-1200	30-80	19-25	5-12
Density (g/ml)	<0.96	0.96-1.006	1.019-1.063	1.063-1.210
Mw (X 10^6 Da)	400	10-80	2.3	0.17-0.36
Mobility	origin	Preβ	B	A
Lipid composition				
Triglycerides	80-95	45-65	18-22	2-7
Free cholesterol	1-3	4-8	6-8	3-5
Cholesterol ester	2-4	6-22	45-50	5-20
Phospholipids	3-6	5-20	18-24	26-32
Apolipoproteins	A-I, A-II, A-IV	----	-----	A-I, A-II, A-IV
	B48	B100	B100	------
	C-I, C-II, C-III	C-I, C-II, C-III	----	C-I, C-II, C-III
	E	E	-----	E

Previous reports have documented a strong correlation between serum lipoprotein levels and the incidence of atherosclerosis and CVD. It has been shown in the clinical studies that, total non-HDL or LDL cholesterol is associated with a 30% to 35% higher incidence of CVD (Frost *et al.*, 1996). In addition, clinical data from the Lipid Research Clinics Program (LRCP) Follow-up Study have shown that the non-HDL cholesterol level is a good predictor of mortality due to CVD (Cui *et al.*, 2001). Further, Iso *et al.* (2001) in a 15.5-year prospective study showed that the non-fasting serum triglyceride level predicted the incidence of coronary heart disease among Japanese men and women with low mean values of total cholesterol. Based on the literature available it can be postulated that, circulating levels of cholesterol, triglycerides and lipoproteins should be maintained in order to reduce the risk of CVDs. In the present study, feeding of HFD to rats for 42 days resulted in significant increment in the plasma lipids and non-HDL lipoproteins and decrement in HDL level. However, concurrent presence of CG extract minimized these set of changes.

HDL is produced primarily by the liver through an interaction of lipid-poor ApoA-I with the ATP-binding cassette transporter A1 (ABCA1), which mediates lipidation of ApoA-I resulting in the formation nascent pre-β HDL particles (Schmitz and Langmann, 2001). The nascent HDL particles subsequently take up free cholesterol from peripheral cells via an ABC mediated efflux system, which is then converted to cholesterol esters by LCAT, leading to the formation of small spherical HDL3. HDL3 is subsequently converted to large α-migrating mature HDL2 by acquirement of phospholipids and apolipoproteins that are released during lipolysis of triglycerides from chylomicrons or VLDL. A low level of HDL is directly correlated with an increased risk

of CVD (Wilson *et al.,* 1988). Presently recorded significant elevation in the HDL level by CG extract could be of great relevance as synthetic hypocholesterolemic drugs have shown negligible effect on HDL level (Wilson, 1990). Increase in HDL level in HFD+CG groups stands well corroborated with an increased plasma LCAT activity. Previous studies have reported improvement in HDL levels by intake of dietary flavonoids and polyphenols (Daniel *et al.,* 2003). In this context, presence of flavonoids in CG extract (4.36%) could thus be responsible for the observed higher HDL levels in HFD+CG groups.

In addition to the uptake of cholesterol from lipoproteins, the liver can also increase its intra-hepatic cholesterol level via *de novo* synthesis of cholesterol from acetyl coenzyme A (acetyl-CoA). The biosynthesis of cholesterol (C27) from acetyl-CoA involves the formation of several carbon intermediates, including 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA; C6), mevalonate (C6), isopentenyl phosphate (C5), and squalene (C30). The rate-limiting step in cholesterol biosynthesis is the formation of mevalonate from HMG-CoA by the cytosolic enzyme HMG-CoA reductase. HMG-CoA reductase is therefore considered as the key rate limiting enzyme in cholesterol biosynthesis. However, the major site for cholesterol biosynthesis in mammals is the liver. Importantly, the expression and activity of HMG-CoA reductase are rapidly reduced by sterols and metabolites derived from mevalonate, a negative feedback effect (Nakanishi *et al.*, 1988). Furthermore, the essential role of HMG-CoA reductase in cholesterol biosynthesis has been evaluated using specific HMG-CoA reductase inhibitors. Treatment of both rats and mice with these inhibitors resulted in a >90% decrease in hepatic sterol synthesis, suggesting an essential role for HMG-

CoA reductase in hepatic cholesterol biosynthesis (Fears *et al.*, 1980; Tsujita *et al.*, 1986). Since inhibition of HMG-CoA reductase activity significantly affects the rate of cholesterol biosynthesis, specific inhibitors of HMG-CoA reductase have been developed for the primary treatment of dyslipidemia and atherosclerosis. Observed decrease in HMG CoA reductase activity in HFD+CG rats indicates diminished *de novo* cholesterol synthesis and efficacy of CG extract to act as a HMG-CoA reductase inhibitor. Further, decreased hepatic cholesterol levels are known to induce LDL receptor expression to promote LDL clearance from circulation in order to maintain hepatic cholesterol homeostasis (Ma *et al.*, 1986; Kovanen and Schneider, 1999). Thus, it can be speculated that CG decreases circulating LDL via increased LDL receptor expression and, a decreased plasma LDL level recorded in HFD+CG groups corroborates this hypothesis.

Cholesterol ester synthase is known to be involved in esterification and incorporation of free cholesterol into chylomicrons and VLDL (Klein and Rudel, 1983; Lawrence and Gregory, 2000). Recorded low activity of CES in intestine and liver of HFD+CG rats indicates decreased esterification of cholesterol resulting in impaired incorporation of cholestryl ester into chylomicrons and VLDL leading to low levels of circulating cholesterol along with high fecal cholesterol content. These observed effects are in accordance with other published reports on the effect of plant products on CES activity (Song *et al.*, 2002).

In addition to the conversion of free cholesterol to cholesterol esters, the intra-hepatic free cholesterol content can be decreased through catabolism of cholesterol to bile acids. CYP7A1 is a microsomal cytochrome P450 that catalyzes the first step in bile acid synthesis. In agreement with its function in bile acid formation, CYP7A1 is only

expressed in the liver (Jelinek *et al.*, 1990), the organ involved in the conversion of cholesterol to bile acids. The intracellular distribution profile of CYP7A1 is quite similar to the one found for HMG-CoA reductase, with a confined expression of both enzymes in the smooth, ribosome-poor endoplasmatic reticulum. The critical role for bile acid synthesis and thus CYP7A1 in the maintenance of cholesterol homeostasis has become clear from studies using transgenic mice. Young adult CYP7A1 gene knockout mice on a regular chow diet are hypercholesterolemic due a concomitant decrease in the expression of hepatic LDL receptors (Erickson *et al.*, 2003). In contrast, mice with transgenic over expression of human CYP7A1 had decreased plasma cholesterol levels on a regular chow diet (Miyake *et al.*, 2001). Importantly, recent data have also indicated an important role for CYP7A1 in the maintenance of cholesterol homeostasis in humans. Individuals with a homozygous deletion mutation in CYP7A1 resulting in loss of the active site and enzyme function (L413fsX414) have high LDL cholesterol levels, double the normal hepatic cholesterol content and a markedly deficient rate of bile acid excretion (Pullinger *et al.*, 2002). The cholesterol lowering property of CG could be due to increased excretion of cholesterol and bile acids (CA and DCA) through feces. These observed effects are attributable to the presence of phytosterol in CG, as phytosterols possess greater affinity for micelles than cholesterol and reduce incorporation of cholesterol into micelles (Ikeda and Sugano, 1998). The liver serves as the primary site for elimination of cholesterol from the body. In the liver, either free cholesterol is secreted directly into bile or it can be converted to oxysterols (Zhang *et al.*, 2001). Oxysterols stimulate expression of CYP7A1, thereby increasing conversion of cholesterol to bile acids (Lehmann *et al.*,

1997). High content of fecal bile acids (CA & DCA) in HFD+CG rats indicates that CG stimulates removal of excess cholesterol via increased bile acid excretion through feces.

Phytocompounds like saponins are known to inhibit pancreatic lipase in HFD fed animals, leading to greater fat excretion due to reduced intestinal absorption of dietary fats (Han *et al.,* 2002). Hence, increased elimination of TG through feces of HFD+CG rats seen in the present study can be related with the high saponin content (4.5 %) in CG. LPL hydrolyses TG from chylomicrons thereby generating non-esterified fatty acids (NEFA) for subsequent tissue uptake and metabolism (Sattler *et al.,* 1996). HFD rats in the present study recorded suppressed post heparin lipolytic activity levels (PHLA) while HFD+CG rats recorded significantly elevated levels. These observations are in accordance with the previous studies in hyperlipidemic rats fed with plant products (Khanna *et al.,* 1996). Similarly, LPL is related to hydrolysis of TG molecules and lipolytic activity in tissues. Elevated LPL activity in hepatic and adipose tissues of HFD+FESR rats could be held responsible for reducing tissue lipid load.

In recent times, therapeutic approach for treatment of hyperlipidemia has been shifted towards a combination therapy with synthetic drugs (i.e. niacin extended lovastatin release tablet) as, cholesterol lowering drugs have only a moderate effect on triglyceride levels (Richard, 2008). Another drawback of synthetic drugs is their inability to increase HDL levels (Wilson, 1990). Present study clearly indicates that treatments with *C.glandulosum*.Coleb is able to significantly lower plasma TG levels and elevate plasma HDL levels, and can therefore be a candidate for alternative therapy for treatment of hypercholesterolemia and hypertriglyceridemia.

Summary

Present study evaluated efficacy of aqueous extract of CG leaves on alteration in lipid and cholesterol metabolisms in high fat diet fed hyperlipidemic rats. Rats were orally administered with CG extract (200, 400 or 800 mg/kg bodyweight) and fed with the standard laboratory diet (SLD) or high fat diet (HFD) for 6 weeks. Alterations in the plasma and hepatic lipid profiles, lipid and cholesterol metabolizing enzymes in target tissues, feacal total lipids and bile acid contents were evaluated in all the experimental groups. The results were compared with synthetic hypolipidemic drug lovastatin. Results clearly indicate the all the doses of CG did not alter any of the parameters tested in this study. However, CG extract significantly prevented increment in plasma and tissue lipid profiles in HFD fed rats. CG extract feeding significantly suppresses activity levels of HMG Co A reductase (Hepatic) and cholesterol ester synthase (Hepatic and intestinal) and increase activity levels of plasma lecithin cholesterol acyl transferase and lipoprotein lipase (plasma, hepatic and adipose). Further, CG extract feeding to HFD diet fed rats increased excretion of triglycerides, cholesterol and bile acids through feaces. These results can be attributed to reduced absorption, effective elimination and augmented catabolism of lipids and cholesterol possibly due to high content of saponin and phytosterols in CG. Possibility of usage of CG extract as a potential therapeutic agent against hypercholesterolemia and hypertriglyceridemia was indicated.