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Research Article

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Study on Hypolipidaemic activity of *Litsea cubeba* leaves in rats

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Abstract Hyperlipidemia was induced in· rats by giving high cholesterol diet (2% cholesterol, 1% sodium cholate and 2% coconut oil) for seven days in· standard rat chow diet. The Methanolic extract of *Litsea Cubeba* leaves (200 & 400mg kg^{-1} b.wt.) was orally administered once a day to rats fed with a high cholesterol diet for seven days. High cholesterol fed diet rats exhibited significant in· crease in total serum cholesterol, triglycerides, low density lipoproteins, very low-density lipoprotein and significant decrease in· high density lipoproteins. Treatment with Methanolic extract of *Litsea Cubeba* leaves significantly decreased total serum cholesterol, triglycerides, low density lipoproteins, very low-density lipoprotein and increased the high-density lipoproteins in hyperlipidemic rats and was comparable with that of standard atorvastatin. Hence, it was concluded that significant Hypolipidemic activity of Methanolic extract of *Litsea Cubeba* leaves may be due to the presence of acidic compounds, flavonoids, phenols, saponins, tannins (Phenolic compounds) and triterpenoids found in the preliminary phytochemical screening.

Keywords Hypolipidaemic activity, *Litsea cubeba*, Leaves

Introduction

Antihyperlipidemic agents promote reduction of lipid levels in the blood. Some antihyperlipidemic agents aim to lower the levels of low-density lipoprotein (LDL) cholesterol, some reduce triglyceride levels, and some help raise the high-density lipoprotein (HDL) cholesterol. By reducing the LDL cholesterol, they can prevent both the primary and secondary symptoms of coronary heart disease. Cholesterol is found in each and every cell of our body. Cholesterol is a water repelling compound and so it does not get dissolved in the bloodstream. It is a waxy steroid metabolite. It is required for the proper functioning of the body. It is required for the production of vitamin D, hormones and bile juices. Low-Density Lipoprotein (LDL) or bad cholesterol and High-Density Lipoprotein (HDL) or good cholesterol are the two types of cholesterol. Simple blood test helps measure cholesterol levels in the body. The ideal LDL:HDL ratio and the ideal cholesterol levels are same for all ages. As you are looking for cholesterol range by age, here is the required information regarding normal cholesterol range.

Litsea cubeba (Lour.) Pers. is commonly known as mountain pepper, distributed throughout North and South America, Australia, New Zealand, South and South-East Asian countries including India, Myanmar, Thailand, Nepal, Bhutan, China, Japan, Taiwan, Vietnam and Indonesia (Liu and Yang, 2012; Si et al. 2012). Several local names were assigned to this species *viz. Bichengqie*, *Shancangzishu*, *Changzimu*, *Manshanxiang*, *Gangouzhang* and *Dongpizhuang* (in different provinces of China); *Kemukus* /*Ki lemo* or *Lemo* (West Java), *Krangean* (Central Java),

and *Lado-lado* (Sumatera) or *Attarasa* (North Sumatera), baleng la (East Kalimantan); *Medang ayer*, *Medang melukut* (in Malaysia); *Cay mang tang* (in Vietnam); *Chakhai-ton*, *Takhrai*, *Takhrai-ton* (in Thailand) (Kong et al. 2015; Suwandhi et al. 2014; Azah and Susiarti, 2016).

In India, the plant is well known from the North-Eastern Himalayan region and considered important in the indigenous health care practices. Several ethnic communities of this region utilize this plant in unique ways and different vernacular names have associated with this plant. The plant is known as *Tayer Rajil* (*Adi* tribe), *Santero* (*Apatani* tribe), *Ischi takke ame* (*Galo* tribe), *Earking /Jayar* (*Nyshi* tribe) and *Nengshing* (*Monpa* tribe) in Arunachal Pradesh (Bharali et al. 2017). In Mizoram, the plant is locally termed as *Ser-nam* (Kar et al. 2013). The plant is famous for its edible use in Manipur, where local people have termed it as *Ngairong* or *Tumila* or *Oosingsha mapaan* (Rajkumari et al. 2013; Devi et al. 2015; Konsam et al. 2016). In Darjeeling and Sikkim Himalayan region, the plant is quite popular as *Siltimmur*/ *Siltimbur* or *Tanghaercherkerng* (Chhetri et al. 2005; Uprety et al. 2016) and in Meghalaya as *Jinjok* among *Garo* tribal people (Ramashankar et al. 2012).

Materials and Methods

Chemicals: Cholesterol, sodium cholate and coconut oil were all purchased from SD-fine chemicals, India, atorvastatin. was procured form Ranbaxy labs. Ltd., Gurgaon, India. All other reagents used were of analytical grade.

Instrument: UV spectrophotometer (Shimadzu-UV-1 601), Centrifuge Machine (Eltek-research centrifuge-TC-4100D).

Animals

The study was carried out in rats of Wister strains of either sex weighing 150-200 gm. 2-3 months old. They were procured from animal house of the biological signature analytical laboratory, Gaziabad and were kept individually under standard laboratory condition. Food pellets and tap water were provided and libitum. Ethical clearance for experimental studies was obtained from institutional animal Ethical Committee, Accuprec research lab Ahmedabad under reg. **1709/Rc/S/13/CPCSEA.**

Collection and Authentication of Plant: Identification of the root of *Litsea Cubeba* Deputy conservator of Forest sikar Reference no.: **DCF/2022/21**

Plant is authenticated by Bhima ram Choudhary. Material was shade dried at room temperature and powdered mechanically and passed through a sieve #40.

PREPARATION OF EXTRACT

The collected leaves will be thoroughly washed with water and shade- dried at room temperature for about 15-20 days under well aerated condition. The dried leaves will crushed into fine powder with the help of a mechanical grinder. The powdered material will be stored in air-tight container at room temperature for future use. Sequential extraction of the dried powder was carried out using solvents of gradient polarity in a Soxhlet apparatus following the method of Jeyaseelan et al. (2012). 100 g of the dried powder was first extracted using 250 mL of hexane for 24-48h. The extract was filtered using Whatman filter paper no. 1 and the residue was air-dried. The air-dried residue was further sequentially extracted using other solvents *viz.* acetone, methanol, ethanol and water. After extraction, the pooled solvents were evaporated and concentrated using a rotary evaporator under reduced pressure. The percentage yield of each solvent was determined (Solvent extractive values) and the concentrated extracts were stored at 4 °C in a refrigerator. Solvent extracts were finally dissolved in dimethyl sulfoxide (DMSO) to obtain a final concentration of 10 mg/mL for further studies.

Preliminary phytochemical screening

Preliminary phytochemical screening of the gymnema leaf extract was carried out for the detection of the various plant constituents (Khandelwal, 2004).

ACUTE ORAL TOXICITY

Swiss albino mice of female sex weighing 20-25gms were used for the study. The animals were obtained from Mahaveer Enterprises Hyderabad and were housed in polypropylene cages. The animals were maintained under standard laboratory conditions ($25^0 \pm 2^0$C; 12hr light and dark cycle).

The animals were fed with standard diet and water *ad-libitum*. Ethical clearance (forhandling of animals and the procedures used in study) was obtained from the Institutional Animal Ethical Committee (IAEC) before performing the study on animals. Acute oral toxicity study for methanol extract of *Litsea Cubeba.* a leaf was carried out as per OECD guidelines 425 (Up and Down procedure). The test procedureminimizes the number of animals required to estimate the acute oral toxicity. The testallows the observation of signs of toxicity and can also be used to identify chemicalsthat are likely to have low toxicity. Animals were fasted (food but not water was withheld overnight) prior to dosing. The fasted body weight of each animal was determined and the dose was calculated according to the body weight.

The drug was administered in the dose of 2000mg/kg body weight orally to one animal. If the test animal survived. Then four other animals were dosed sequentially; therefore, a total of five animals were tested. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hour), and daily thereafter, for a total of 14 days. After the experimental period, the animals were weighed and humanely killed and their vital organs including heart, lungs, liver, kidneys, spleen, adrenals, sex organs and brain were grossly examined. (OECD Guidance; 2000)

Evaluation of hypolipidemic activity of extract of *Litsea cubeba* Leaves

Healthy Albino rats (Wistar strain) of either sex weighing 150-200g were used for the study. The animals were obtained from the animals were housed in polypropylene cages and were maintained under standard laboratory conditions (25±2 °C, 12 hr light and dark cycle). The animals were fed with standard pellet diet and water ad libitum.

Ethical clearance (for handling of animals and the procedures used in study) was obtained from Institutional Animal Ethical Committee (IAEC) before performing the study on animals.

Cholesterol Diet

Rats were made hyperlipidemic by the oral administration of high cholesterol diet (regular diet mixed with 2% w/w cholesterol, 1% w/w sodium cholate, 2.5% w/w coconut oil) to healthy rats for 30 days. (Pandya et al., 2006), (Rumi Ghosh et al., 2010)

Experimental Design

The rats with elevated cholesterol level were divided into 3 groups of 6 animals each and given drug/ vehicle treatment for 15 days. A group of 6 normal animals were included in the study.

Group I: Animals served as normal control fed with normal diet

Group II: Animals served as a Hypercholesterolemic Control (HC- C). Animals received vehicle (CMC 0.5%) 2 ml/kg P.O.

Group III: Hypercholesterolemic animals received methanol extract of Litsea Cubeba 200 mg/kg P.O once daily orally for 15 days.

Group IV: Hypercholesterolemic animals received methanol extract of Litsea Cubeba 400 mg/kg P.O once daily Group IV orally for 15 days.

At the end of experimental period, the animals were fasted overnight and fasting blood samples were collected by retro orbital vein puncture technique in clot activator tube. The samples were centrifuged at 4000-5000rpm to separate serum, which was subjected for the estimation of lipid profile.

Estimation of lipid profiles

The total cholesterol in plasma was estimated by cholesterol oxidase enzymatic method using Agappe Diagnostic kit (Siedel *et al.,* 1981). Plasma triglyceride (TGL) was estimated by Autoanalyser using enzymatic-GPO method (Rifai *et al.,* 1999). High density lipoprotein (HDL) was estimated by selective precipitation followed by cholesterol oxidase enzymatic method using HDL-cholesterol phosphotungstic acid of Erba diagnostic Mannheim gmbh kit (Burstein *et al.,* 1970). Low density lipoprotein (LDL) was estimated by direct measurement with the homogeneous method performed with the reagent LDL-C Select FS (DiaSys) (Caio Mauricio Mendes de Cordova *et al.,* 2004). Very low-density lipoprotein (VLDL) was calculated using the formula TGL/5 (Vijayabaskar *et al.,* 2008). Atherogenic Index (AI), which is a measure of the atherogenic potential of an agent, was calculated using the following formula and the results were tabulated (Rekha and Ekambaram, 2010).

Atherogenic Index (AI), which is a measure of atherogenic potential, was calculated using the following formula

$$Atherogenic\ Index = \frac{Total\ serum\ HDL}{Total\ serum\ cholesterol}$$

$$Percentage\ protection\ = \frac{AI\ of\ control - AI\ of\ treated\ group}{AI\ of\ control}\ X\ 100$$

Estimation of Biochemical Parameters

Total Cholesterol, HDL, LDL, VLDL, triglycerides and phospholipids were estimated in serum. All the biochemical parameters were estimated using semi- autoanalyser (photometer, Germany) with enzymatic kits.

Histopathological Techniques

Immediately after collection of blood the animals were sacrificed by cervical decapitation. The liver was separated, washed with pH 7.4 buffer, blotted with dry filter paper and the organ weight was recorded.

Histopathology

A small portion of the liver tissues from all the groups was excised immediately after sacrifice and fixed in 10% neutral formalin. The washed tissues were dehydrated in descending grades of isopropanol and cleared in xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 μm thickness and stained with hematoxylin and eosin and then viewed under light microscope for histopathological changes.

Statistical analysis

Results are expressed as mean ± SEM (standard error of mean). Results were analysed statistically by one-way ANOVA followed by Dunnett's test using Prism (Graphpad Software Inc., La Jolla, CA Trial version). The criterion for statistical significance was set at $p < 0.05$.

Result

Table 1: Phytoconstituents detected in methanol extract of *Litsea Cubeba* leaves

S. No.	Test	Inference
	Test for Alkaloids	
1.	a. Mayers test	+
	b. Wagner's test	+
	c. Dragen draft's test	+
	Test for Phytosterols and Triterpenoids	
2.	a. Leibermann's test	+
	c. Salkowaski test	+
	Test for Flavonoids	
3.	a. Alkaline reagent test	+
	b. Lead acetate test	+
4.	**Test for saponins**	
	Foam test	+
	Test for Proteins and Aminoacids	-
5.	a. Millon's test	-
	b. Ninhydrin test	-
	c. Biuret test	
6.	**Test for fixed oils and fats**	
	Oily spot test	+
7.	**Test for Phenolics and Tannins**	
	Ferric chloride test	+
	Test for carbohydrates	
8.	a. Molisch's test	+
	b. Fehling's test	+
	c. Benedict's test	+
	Test for Gylcosides	
9.	a. Modified Borntrager's test	+
	b. Legal's test	+
	c. Keller-Killiani test	+

Table 2: Acute oral Toxicity study of extract of *Litsea Cubeba* leaves. (Guideline 425)

Respiratory Blockage in Nostril	
Dyspnoea	Nil
Apnoea	Nil
Tachypnea	Nil
Nostril discharge	Nil
Motor activities	
Locomotion	Normal
Somnolence	Nil
Loss of righting reflex	Nil

Anaesthesia	Nil
Catalepsy	Nil
Ataxia	Nil
Toe walking	Nil
Prostration	Nil
Fasciculation	Nil
Tremor	Nil
Convulsion (Involuntray Contraction)	
Clonic/tonic/tonic-clonic convulsion	Nil
Asphyxial convulsion	Nil
Opistotones (titanic spasm)	Nil
Reflexes	
Corneal	Normal
Eyelid closure	Normal
Righting	Normal
Light	Normal
Auditory and sensory	Normal
Ocular Signs	
Lacrimation	Nil
Miosis	Nil
Mydriasis	Nil
Ptosis	Nil
Chromodacryorrhea	Nil
Iritis	Nil
Conjunctivitis	Nil
Salivation	
Saliva secretion	Nil
Piloerection	
Contraction of erectile tissue	Nil
Analgesia	
Decrease in reaction to induced pain	Nil
Muscle Tone	
Hypo or hypertonia	Nil
Git Sign	
Solid dried / watery stool	Nil
Emesis	Nil
Red urine	Nil
Skin	
Oedema	Nil
Erythema	Nil

Table 3: Effect of *Litsea Cubeba* extract on body weight in high fat diet induced hypercholesterolemic rats

Groups	**Treatment**	**Initial weight gm(s)**	**Final weight gm(s)**	**Weight gain**	**% increase in Bodyweight**
Group I	Normal diet	121.7±11.88	130.9 ±1.33	9.23±0.649	7.58 %
Group II	Extract 200mg/kg +	135.7±12.16^{nsa}	140.8 ±5.509^{nsa}	5.16 ±1.218^{nsa}	3.82 %

	Normal diet				
Group III	Extract 400mg/kg + Normal diet	130.0 ±6.952^{nsa}	138.8 ±7.463^{nsa}	8.862±1.254**^{a}	6.81 %
Group IV	High fat diet (HFD)	165.0 ±5.77^{nsb}	195.8 ±5.449^{nsb}	30.81±1.217***^{b}	18.67 %
Group V	Extract 200mg/kg +HFD	158.3 ±6.67^{nsc}	167.5 ±8.827^{nsc}	9.216±0.654***^{c}	5.81 %
Group VI	Extract 400mg/kg +HFD	144.2 ±8.7^{nsc}	148.5 ±5.881^{nsc}	4.350±0.427***^{c}	3.01 %

All the values are expressed as mean ± SEM, n = 6 in each group.
^{a}Values are significantly different from group I. Non significant (ns); *p < 0.05; **p < 0.01; ***p < 0.001 ^{b}Values are significantly different from group I. Non significant (ns); *p < 0.05; **p < 0.01; ***p < 0.001 ^{C}Values are significantly different from group IV. Non significant (ns); *p < 0.05; **p < 0.01; ***p < 0.001

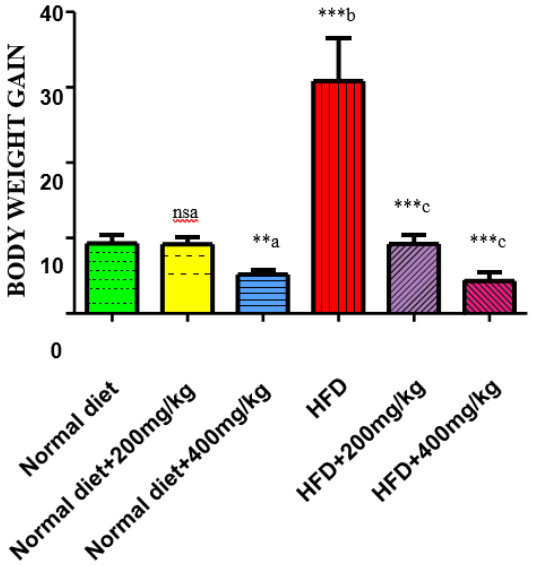

Figure 1: Effect of Litsea cubeba Leaves Extract on Body Weight Gain

Table 4: Effect of methonalic extract of *Litsea Cubeba* leaves on serum lipid profiles in rats fed with high fat diet

Group	**Treatment**	**Total cholesterolmg / dl**	**HDL mg / dl**	**LDL mg / dl**	**Triglyceride mg / dl**	**VLDL mg / dl**
Group I	Normal diet	175.0±8.851	27.83 ±8.851	73.17 ±5.294	110.0 ±10.25	19.33±1.476
Group II	High fat diet (HFD)	212.5 ±8.839**^{b}	13.33±0.988***^{b}	100.8 ±5.23**^{b}	167.5 ±14.82***^{b}	23.33±2.246^{nsb}
Group III	Normal diet + 200mg/kg	150.0 ± 7.303^{nsa}	16.17±1.424**^{a}	70.50 ±3.97^{nsa}	108.7 ±3.180^{nsa}	18.0±0.9309^{nsa}
Group IV	Normal diet + 400 mg/kg Extract	139.2 ±6.509**^{a}	22.50±2.895^{nsa}	65.83 ±4.16^{nsa}	95.83 ± 4.16^{nsa}	16.33±1.382^{nsa}
Group V	Extract 200mg/kg +HFD	150.8 ±7.12***^{c}	20.17±2.786^{nsc}	74.0 ±6.583**^{c}	102.5 ±4.425***^{c}	17.5±1.607*^{c}
Group VI	Extract 400 mg/kg	117.8 ±5.002***^{c}	27.17 ±1.641***^{c}	67.83±4.324***^{c}	97.50 ± 3.81***^{c}	15.17±0.833**^{c}

	+ HFD

All the values are expressed as mean ± SEM, n = 6 in each group.

[a]Values are significantly different from group I. ns; *p < 0.05; **p < 0.01; ***p < 0.001

[b]Values are significantly different from group I. ns; *p < 0.05; **p < 0.01; ***p < 0.001

[c]Values are significantly different from group IV. ns; *p < 0.05; **p < 0.01; ***p < 0.001

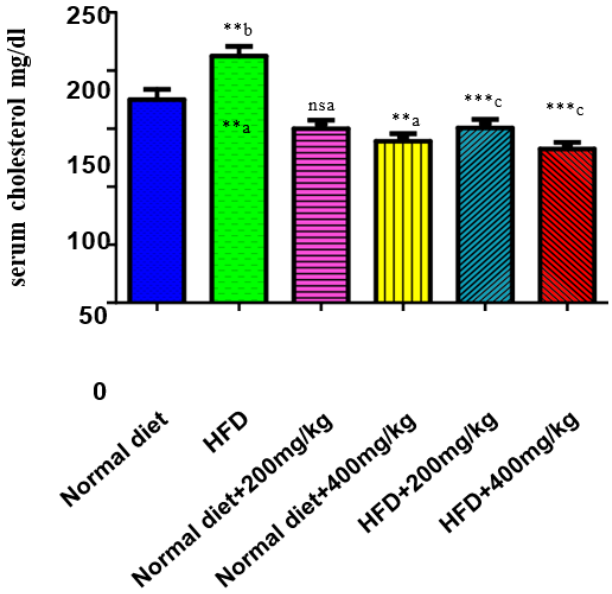

Figure 2: Effect of Litsea cubeba Leaves Extract on Serum Cholesterol

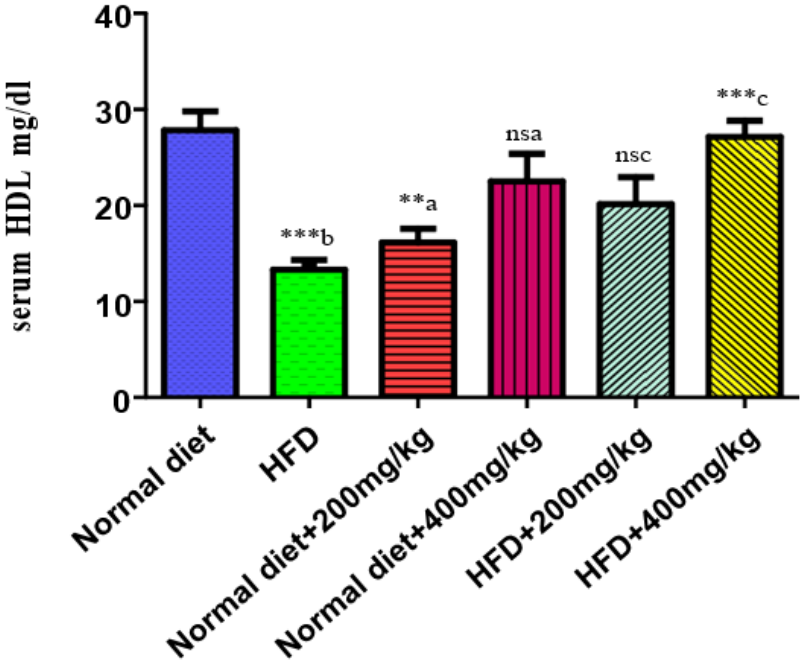

Figure 3: Effect of Litsea cubeba leaves extract on serum HDL

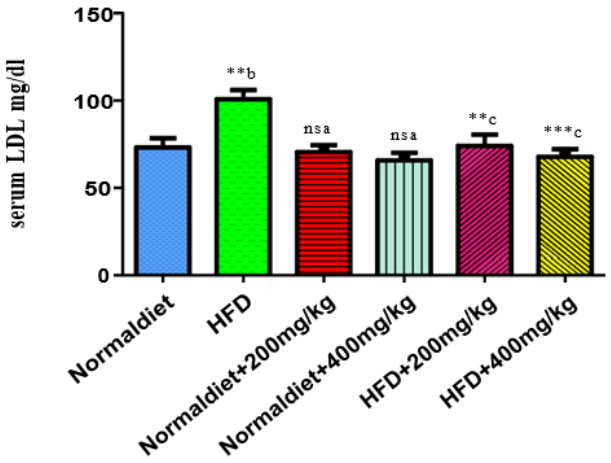

Figure 4: Effect of Litsea cubeba Leaves Extract on Serum LDL

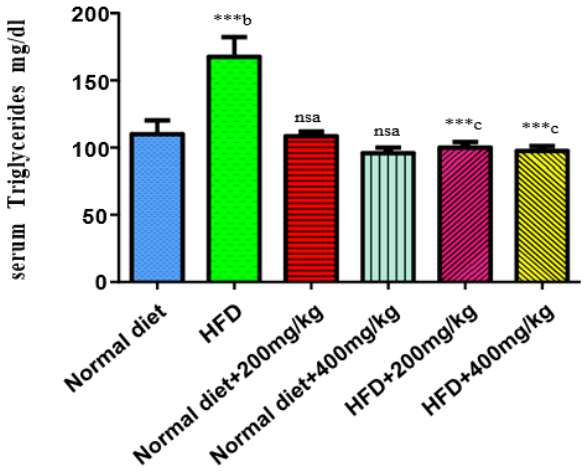

Figure 5: Effect of Litsea cubeba Leaves Extract on Serum Triglycerides

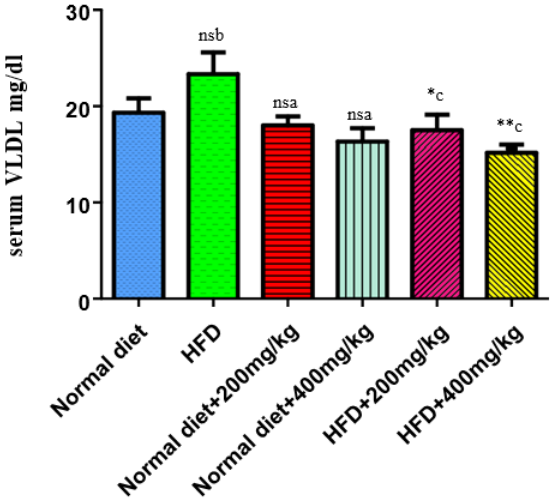

Figure 6: Effect of Litsea cubeba Leaves Extract on Serum VLDL

Table 4: Atherogenic index of *Litsea Cubeba* extract

Group	Treatment	Atherogenic index	Percentage (%)Protection
Group I	Normal diet	3.95 ±0.328	--
Group II	High fat diet (HFD)	12.56 ± 0.921***[b]	--
Group III	Extract (200 mg/kg) +Normaldiet	6.722 ± 0.527[nsa]	33.56%
Group IV	Extract (400 mg/kg)+ Normaldiet	4.25 ± 0.358[nsa]	55.23%
Group V	Extract (200 mg/kg)+HFD	5.08 ± 0.625***[c]	52.70%
Group VI	Extract (400 mg/kg)+HFD	3.58 ± 0.268***[c]	70.62%

All the values are expressed as mean ± SEM, n = 6 in each group.
[a]Values are significantly different from group I. ns;*p<0.05;**p < 0.01;***p<0.001 [b]Values are significantly different from group I. ns;*p <0.05;**p<0.01;***p< 0.001 [c]Values are significantly different from group IV. ns;*p <0.05;**p<0.01;***p< 0.001

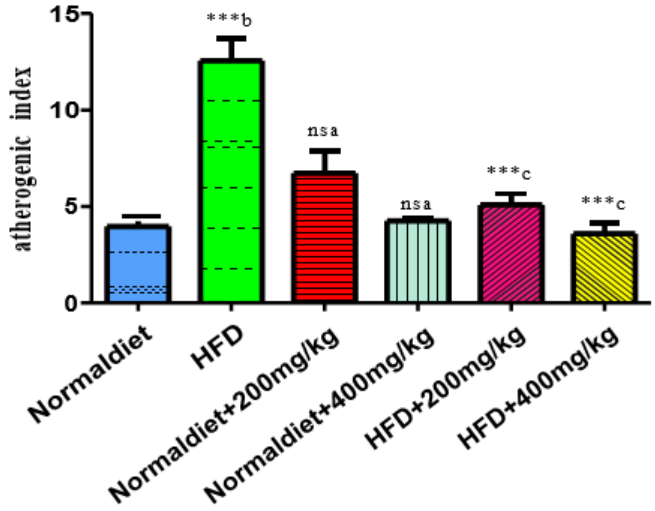

Figure 7: Effect of Litsea cubeba Leaves Extract on Atherogenic Index

Histopathological examination of Liver

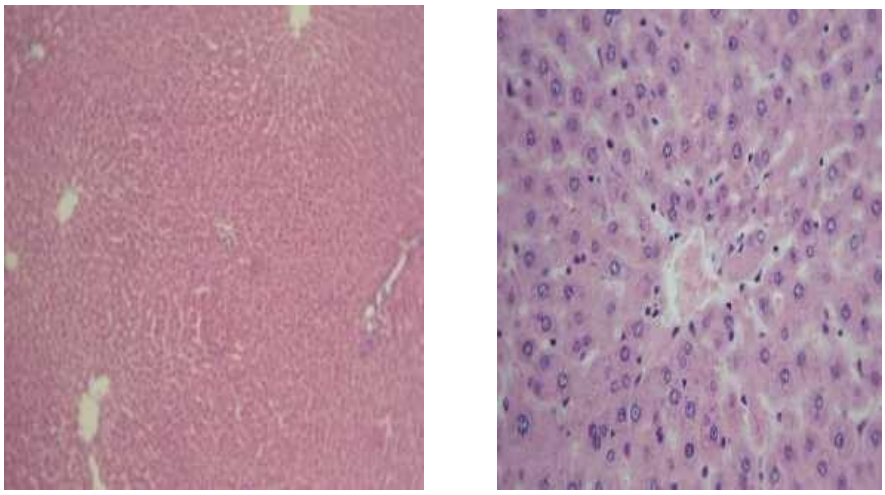

Figure 8(a): Section of liver of control group rat on normal diet. (H&E 10x)
Normal liver with intact lobular architecture, normal central veins and sinusoids

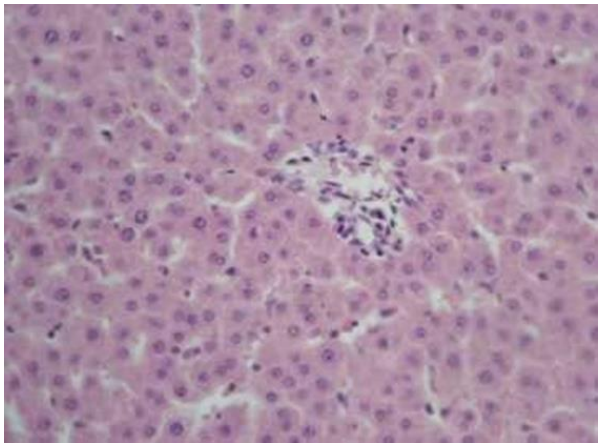

Figure 8(b): Normal portal tracts without inflammation

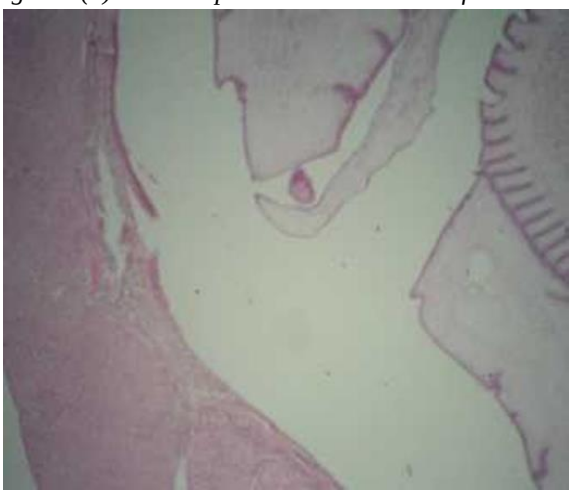

Figure 9(a): Section of liver of rat fed with high fat diet showing large dilated space with fibrous wall and amorphous material. (H&E 10x)

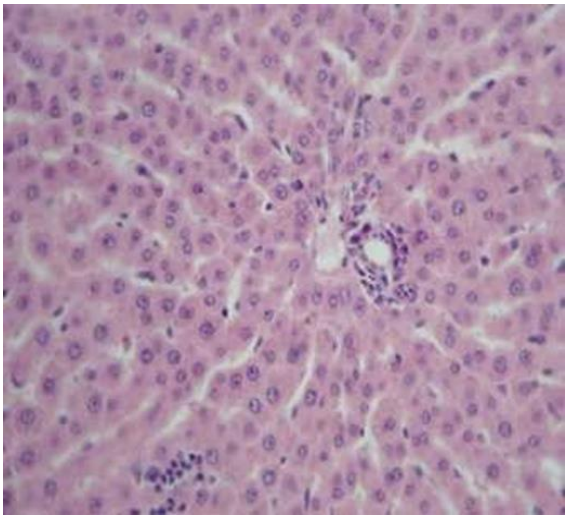

Figure 9(b): Section of liver of rat fed with high fat diet showing normal portal tracts and lobular inflammation. (40X)

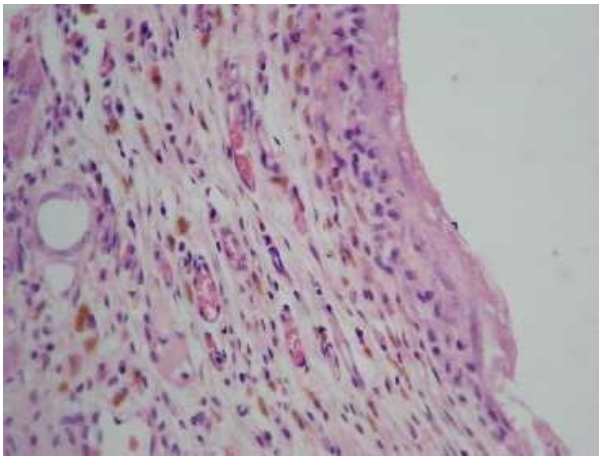

Figure 10(a): Section of liver of rat given extract of Litsea cubeba 200mg/kg along with high fat diet showing fibrous wall with inflammation and macrophages (H&E 10x)

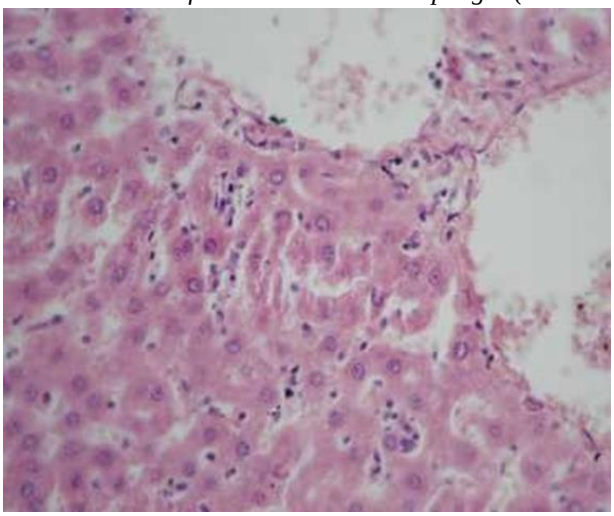

Figure 10(b): Section of liver of rat given methonal extract of Litsea Cubeba 200mg/kg along with high fat diet showing dilated central vein (H&E 10x)

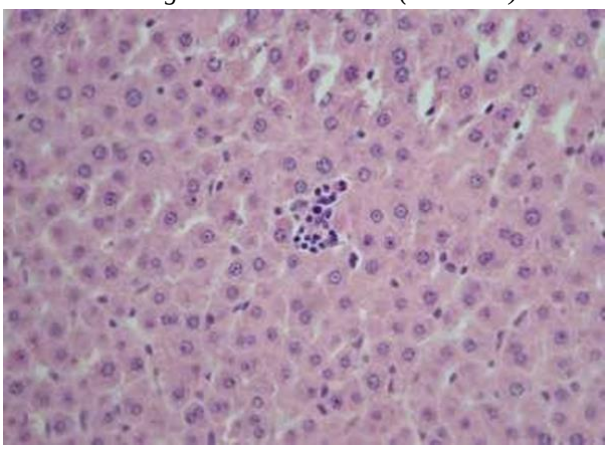

Figure 11(a): Section of liver of rat given extract of Litsea cubeba 400mg/kg along with high fat diet showing lobular inflammation (H&E 40x)

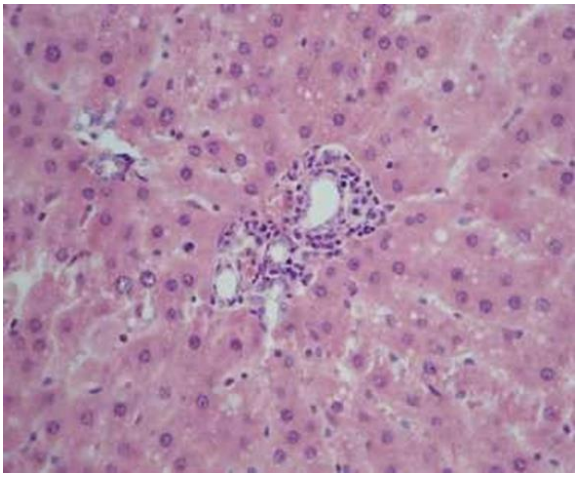

Figure 11(b): Section of liver of rat given extract of Litsea cubeba 400mg/kg along with high fat diet showing portal tract with mild inflammation (H&E 40x)

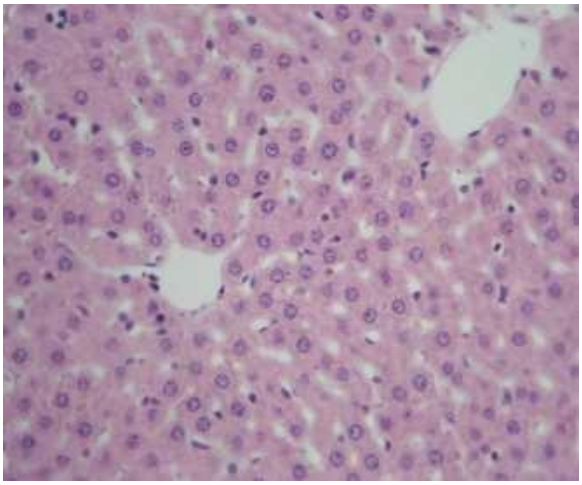

Figure 12: Section of liver of rat given extract of Litsea cubeba 200mg/kg along with normal diet showing normal sinusoids and central vein (H&E 40x)

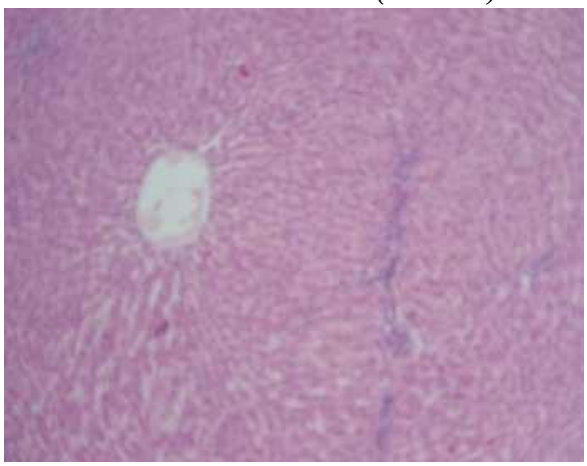

Figure 13(a): Section of liver of rat given extract of Litsea cubeba 400mg/kg along with normal diet showing dilated central vein (H&E 40x

Discussion

Hyperlipidemia is a well-known risk factor for cardiovascular diseases, especially to cause atherosclerotic coronary artery disease (CAD). Plaque formation, thrombosis, andvessel occlusion can follow, leading to CAD. CAD involves one or more specific cardiovascular pathologies, including myocardial infarction, ischemia, and angina. Between 13 and 14 million people in the United States are believed to suffer from this complex and life-threatening condition, and over 25 million people worldwide are expected to die from cardiovascular-related pathologies by the year 2020.It has been established that nutrition plays an important role in the etiology of hyperlipidemia and atherosclerosis.

A significant decline in serum triglyceride level observed in *Litsea Cubeba* extract treated rats supports the cardiovascular protective influence. Increase in LDL cholesterol has been pointed out as one of the risk factors for the development of atherosclerosis and related cardiovascular diseases. *Litsea Cubeba* extract lowered the LDL cholesterol, which can afford a beneficial role in reducing cardiovascular complications. One of the possible mechanisms of lowering body weight by *Litsea Cubeba* extract is by decreased adipocytic lipogenesis excreted by phenolic glycosides. *Litsea Cubeba* contains plenty of different flavonoid glycosides e.g. quercetin-3-glycoside, quercetin-3- galactoside, kaempferol-3-galactoside, kaempferol-3-glycoside, kaempferol-3- malonylhexoside, isorhamnetin-3-glycoside and isorhamnetin-3- malonylhexoside, phenolic acid derivatives and other compounds. These evidences strongly support that the *Litsea Cubeba* extract shows hypolipidaemic activity through multiple mechanisms.

Conclusion

The deleterious effects of high blood cholesterol and beneficial effects of lowering blood cholesterol in reducing morbidity and mortality from cardiovascular diseases are well established. Non pharmacological measures like dietary restriction and exercise may help in lowering blood cholesterol levels. When such therapy fails, and in patients with abnormally high blood cholesterol levels, drug therapy is indicated. The available drugs like statins, fibrates and nicotinic acid though very effective, have a spectrum of adverse effects and are costly.

A growing attention has been recently focused on the improvement of human health by consumption of herbal plant like *Litsea Cubeba*, which has excellent historical health benefits due to presence of plenty of phytoconstituents. A myriad of nutritional benefits has been attributed to these phytochemicals. Since phytoconstituents are concentrated largely in leaves and considering it as a health benefits in treating cardiovascular diseases, a study was carried out to evaluate the effect of *Litsea cubeba* extract on rats fed with high fat diet.

The results of the study demonstrated that oral administration of *Litsea cubeba* extract evokes a beneficial effect on the hyperlipidemia. This finding supports its use for the treatment and management of cardiovascular diseases. This implies that *Litsea cubeba* leaves extract consumption can prevent or be helpful in reducing the complication of dyslipidemia associated disorders.

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