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ANTIHYPERLIPIDEMIC ACTIVITY OF RUELLIA TUBEROSA ROOT EXTRACT

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Keywords:
Ruellia tuberosa, acute hyperlipidaemia, High Fat Diet, Triton X-100 model.

Abstract

The *Ruellia tuberosa* L. have both Chronic hyperlipidemia was induced by feeding female rats with High Fat Diet for 30 days. Acute hyperlipidemia was induced by administration of Triton X-100 (100mg/kg, i.p., at once) in female rats. Administration of Ruellia tuberosa Ethanolic extract (RTEE) (250/500/1000mg/kg) for 30 days in High Fat Diet (HFD) model and RTEE (250/500/1000mg/kg) for 7 days in triton models respectively, successfully prevented the elevation of serum triglycerides(TG), total cholesterol(TC),Low density lipoproteins(LDL), Very Low density lipoproteins (VLDL), Serum glutamate oxalo transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT),Alkaline Phosphatase and decrease of serum High density lipoproteins(HDL) in High Fat Diet (HFD) and Triton X-100 model rats in a dose dependent manner. The study find out that RTEE is a potent antihypercholesterolemic drug lowering the LDL, VLDL and increasing HDL levels in two hyperlipidemic models such as HFD, Triton X-100. The mechanism of action of this compound inhibits the cholesterol and triglyceride synthesis in animal models.

Introduction

Ruellia tuberosa L is a low-growing perennial herb with tuberous roots, growing to a height of a foot or more. Leaves are opposite, elliptic, short petioled, abruptly narrowed at the base, with undulate margins and up to 12 cm long. Fruit is a pod with 7 to 8 seeds, bursting open and hurtling the seeds when it gets wet. It is found in open waste places in the Philippines [1].

Chemical constituents

Ruellia tuberose L, Leaves contain apigenin and luteolin. Seed oil yields myristic, capril and lauric acids. Study yielded flavonoids, glycosides, phenols, saponins and essential minerals with good nutritive value and secondary metabolites.

Hyperlipidaemia

Hyperlipidaemia means abnormally high levels of fats in the blood. These fats include cholesterol and triglycerides. These are important for our bodies to function but when they are high, they can cause heart disease and stroke. Hyperlipidaemia is manifested as hypercholesterolemia and/or hypertriglyceridemia. However, hypercholesterolemia is the most common hyperlipidaemia. The lipids that are involved in hypercholesterolemia are cholesterol, an essential component of cell membrane and a precursor of steroid hormone synthesis and triglycerides, an important energy source. They are transported in blood as lipoproteins. The consequence of hyperlipidaemia is that with time it can cause atherosclerosis, and thus the risk of coronary heart disease and stroke is increased. However, according to the newer scientific view, the cholesterol level alone is not the whole story. The risk of heart disease in future also depends on many other factors that influence the health of a person's blood vessels and circulation.

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"Hyperlipidaemia is an abnormally high level of fatty substances called lipids, largely cholesterol and triglycerides, in the blood. It is also called hyperlipiproteinemia, because these fatty substances travel in the blood by attaching to proteins forming large molecules called lipoproteins" [3-4].

Materials and Methods

Materials

Cholesterol, Triton-x 100, Atorvastatin, Serum triglyceride diagnostic kit, Serum LDL cholesterol diagnostic kit, Serum total cholesterol diagnostic kit, Serum HDL cholesterol diagnostic kit, Thiobarbituric acid, Hydrogen Peroxide, EDTA, Sodium azide, Sodium dihydrogen phosphate, Disodium hydrogen phosphate and Nacl extra pure was used.

Equipments and instruments used

Centrifuge, semi auto analyser, Spectrophotometer, Tissue Homogenizer was used.

Methods

Plant material

Roots of *Ruellia tuberosa* was collected from the moderate deciduous plants found in Talakona forest of Andhra Pradesh, India. The plant was identified and authenticated by Dr. K. Madhavachetty, Assistant professor, Department of botany, Sri Venkateswara University, Tirupati, A.P, India.

Preparation of plant extract

The collected plant was washed thoroughly with water and dried in the shade. Ethanolic extract was obtained by extracting powder with 95% ethanol by soxhlet extraction method for 72hr. After completion of the extraction the solvent was removed by rotary evaporator method. The ethanolic extract was used for further study. The yield obtained from the above process was found to be % w/w.

Phytochemical screening

The different qualitative tests were performed for establishing profile of the given extract for its chemical composition. The following tests were performed on extracts to detect various phytoconstituents present in them.

1. **Detection of alkaloids**

Solvent free extracts of 50 mg was stirred with few ml of dilute Hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloid reagents as follows

To a few ml of the filtrate, a drop or two of Mayer's / Wagner / Hager's / Dragendroff's reagent were added by the side of the test tube.

A) Mayer's reagent

Mercuric chloride (1.358 g) was dissolved in 60 ml of water and potassium iodide (5.0 g) was dissolved in 10 ml of water. The two solutions were mixed and made up to 100 ml with water.

B) Wagner's reagent

Iodine (1.27 g) and potassium iodide (2 g) was dissolved in 5 ml of water and made up to 100 ml with distilled water.

C) Dragendroff's reagent

Stock solution: Bismuth carbonate (5.2 g) and sodium iodide (4 g) were boiled for a few minutes, with 50 ml glacial acetic acid. After 12 h, the precipitated sodium acetate crystals were filtered off using a sintered glass funnel. Clear, red-brown filtrate, 40 ml was mixed with 160 ml ethyl acetate and 1 ml water and stored in amber-colored bottle.

Working solution: 10 ml stock solution was mixed with 20 ml of acetic acid and made up to 100 ml with water [5-10].

2. **Detection of carbohydrates**

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The extracts (100 mg) were dissolved individually in 5 ml of water and filtered. The filtrate was subjected to the following tests.

a. Molisch's test

To a few ml of the filtrate two drops of alcoholic solution of alpha napthol was added, the mixture was shaken well and 1 ml of conc. sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

b. Fehling / Barfoed / Benedict test

1 ml of the filtrate was boiled on water bath with 1 ml of Fehling's solution A and B / Barfoed's reagent / Benedict's reagent was added and heated to boiling for 2 minutes.

c. Barfoed's reagent: Copper acetate, 30.5 g was dissolved in 1.8 ml of glacial acetic acid.

3. Detection of glycosides

For detection of glycosides, 50 mg of the extract was hydrolyzed with concentrated hydrochloric acid for 2 h on a water bath, filtered and the hydrolysate was subjected to the following.

a. Legal test

Fifty mg of the extract was dissolved in pyridine; sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide. Presence of glycoside was indicated by pink colour.

4. Detection of saponins by foam test

The extract (50 mg) was diluted with water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 min. A two cm layer of foam indicated the presence of saponins.

5. Detection of proteins and amino acids

The extract (100 mg) were dissolved in 10 ml of distilled water and filtered through Whatmann No.1 filter paper and the filtrates were subjected to tests for proteins and amino acids[11-14].

a. Millon's test

To 2 ml of the filtrate, few drops of Millon's reagent were added. A white precipitate indicated the presence of proteins.

b. Biuret test

An aliquot of 2 ml of the filtrate was treated with one drop of 2% copper sulphate solution. To this, 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. Pink colour in the ethanolic layer indicated the presence of proteins.

c. Ninhydrin test

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) were added to 2 ml of aqueous filtrate. A characteristic purple colour indicated the presence of amino acids.

6. Detection of phytosterols

a. Libermann- Buchard's test

The extracts of the formulations (50 mg) were dissolved in 2 ml acetic anhydride. To this, one or two drops of conc. sulphuric acid were added slowly along the sides of the test tube. An array of colour changes showed the presence of phytosterols.

7. Detection of phenolic compounds and tannins

a. Ferric chloride test

The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride were added. A dark green colour indicated the presence of phenolic compounds.

b. Lead acetate test

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The extract (50 mg) was dissolved in distilled water and to this; 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds [15-19].

Phytochemical screening

Table: 2

s.no	Chemical constituent	Rullea tuberosa ethanolic extract (RTEE)
1	Alkaloid	-ve
2	Glycoside	-ve
3	Saponins	-ve
4	Carbohydrates	-ve
5	Tannins	+ve
6	Flavanoids	+ve
7	Amino acids	-ve
8	Phenols	+ve

Methodology

Experimental work

Experimental animals

Albino rats (130±20gms) were procured from Mahaveer Enterprises, Hyderabad, India and used for the experiment. Rats were maintained in an air conditioned room (25±2°C) with a normal night and day cycle. Rats were feed with standard pellet diet and demineralised drinking water ad libitum. The rats were allowed to acclimatize to the laboratory environment for a week before the start of the experiment. All experimental procedures were conducted in conformity with Animal Ethics committee (Reg. No. number 769/2010/CPCSEA) for the care and use of animals and were strictly followed throughout the study [20-24].

Figure No: 8 Experimental animals.

Acute toxicity studies

Acute toxicity study for the ethanol extract of Ruellia tuberosa roots (RTEE) was done according to the OECD guidelines No: 423 and dose was selected for treatment.

Table : 5

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S.NO	Drug	LD_{50}	Administered dose
1.	Ruellia Tuberosa root extract	200mgkg.B.W	20mg/kg
2.		500mg/kgB.W	50mg/kg
3.		1000mg/kgB.W	100mg/kg

Method

Depending on the mortality and or moribund status of the animals, on the average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substance. This procedure results in the use of a minimum number of animals while allowing for acceptable data-based scientific conclusion. The method used defined doses (5, 50, 300, 2000 mg/kg body weight) and the results allow a substance to be ranked and classified according to the Globally Harmonized System (GHS) for the classification of the chemical, which cause acute toxicity.

Three mice weighing between 25 – 30gm were used for study. The dose level of extracts was increased up to 2000mg/kg (b.w, p.o). The dose was administered orally to mice, which were fasted over night with water *ad libitum*, food was withheld for further 3 – 4 hrs.

Body weight of the mice before and after treatment were noted and any changes in skin and fur, eyes and mucous membranes and also autonomic, central nervous systems, somatomotor activity were observed and also signs of tremors, convulsions, salivation, lethargy sleep and coma were noted. The onset of toxicity and signs of toxicity was also to be noted. The mice were then observed for another 14 days [25-30].

Techniques for inducing hyperlipidemia

Various techniques are available for induction of hyperlipidemia in animals. There are many numbers of animal models of hyperlipidemia including [31-33]

I. Induction of experimental hyperlipidemia

1. Cholesterol diet induced hyperlipidemia.
2. Hereditary hypercholesterolemia in rats.
3. Transgenic animals.

II. Influence on lipid metabolism

1. Hypolipidemic activity in rats.
2. Triton induced hyperlipidemia in rats
3. Fructose induced hyperlipidemia in rats.
4. Intravenous lipid tolerance test in rats.

III. Inhibition of cholesterol biosynthesis

1. Determination of HMG-CoA reductase inhibitory activity

IV. Inhibition of cholesterol absorption

1. Inhibition of ACAT (acyl coenzyme A; cholesterolacyl transferase)
2. Lymph fistula model for cholesterol absorption

V. Interruption of bile acid recirculation

1. Cholestyramine binding

VI. Inhibition of lipid peroxidation

1. Inhibition of lipid peroxidation of isolated plasma (LDL)

High fat diet and triton models to evaluate the hypolipidemic activity

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High fat diet induced hyperlipidemia in rats

In rats, hyperlipidemia can be induced by daily oral administration of high fat diet (cholesterol 25mg/kg in oil) to healthy rats for 30days. The test compounds were administered simultaneously along with cholesterol diet. High intake of saturated fat and cholesterol increases serum LDL, probably by decreasing the amount of and activity of LDL receptors in the liver. Elevated and modified LDL is one of the principal factors in the development of atherosclerosis. Feeding the high fat diets causes fatty liver with accumulation of TG and TC.

High fat diet induced hyperlipidemia

Evaluation of hypolipidemic activity

Hyperlipidemia was induced by feeding a high fat diet (cholesterol 25mg/kg in oil) to healthy rats for 30days. Rats were divided into five groups containing six animals each.

Group 1 received normal diet (normal)
Group 2 received high fat diet (control).
Group 3 received RTEE 250 mg/kg, p.o.
Group 4 received RTEE 500 mg/kg p.o.
Group 5 received RTEE 1000 mg/kg p.o.
Group 6 received Atorvastatin 10 mg/kg p.o.

A homogeneous solution of the extracts and standard drug atorvastatin was freshly prepared individually using 10% v/v dimethyl sulfoxide (DMSO). Rats were fed daily with standard diet supplied by cholesterol in oil was given by oral route at 10 am and Ruellia tuberosa extracts or Atorvastatin was given by oral route at 3 pm daily, to respective groups, for a period of 30 days. The normal control group was treated with vehicle instead of drugs. Initial and final body weights and food intake of rats were monitored. At the end of the experimental study, animals were fasted for 12 hr and blood samples were collected by retro-orbital puncture technique in a coagulant-free vessel, and were kept at room temperature for 1 h. Samples were centrifuged at 4000–5000 rpm to separate serum, which was subjected for the estimation of lipid profile [34-36].

The main parameters assessed in hyperlipidemic model were as follows

Biochemical lipid parameters

The main biochemical parameters recommended by the National Cholesterol Education Program guidelines for lipid screening i.e. Total Cholesterol, Low Density Lipoprotein Cholesterol, Very Low Density Lipoprotein Cholesterol, High Density Lipoprotein Cholesterol and Triglycerides, Serum glutamate oxalo transaminase. Serum glutamate pyruvate transaminases, Serum alkaline phosphatase were evaluated from the serum.

Cardiac risk indicators the cardiac risk ratios recommended by NCEP guidelines were estimated by calculating the TC: HDL ratio (Atherogenic Index) and LDL: HDL ratio.

Preparation of liver Homogenate:

Liver homogenate was prepared using 0.9% saline by homogenizing with tissue homogenizer. This homogenate was centrifuged at 7000 rpm for 15 min. The obtained supernatant is used to assay the following in-vivo antioxidant parameters.

Evaluation of antioxidant activity

A) Estimation of Lipid peroxidation assay

Lipid peroxidation in the homogenate was determined by measuring the amounts of malondialdehyde produced primarily. 0.2 ml of tissue homogenate, 0.2 ml of 8.1 % of sodium dodecyl sulphate, 1.5 ml of 20 % acetic acid and 1.5 ml of 8 % TBA were added. The volume of mixture was made up to 4 ml with distilled water and then heated at 95^{o}C on water bath for 60 min using glass ball as a condenser. After incubation, tubes were cooled to room temperature and final volume was made to 5 ml in each tube. 5 ml of butanol: pyridine (15: 1) mixture was added

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and the contents were vortexed thoroughly for 2 min. After centrifugation at 3000 rpm for 10 min, the upper organic layer was taken and its absorbance was read at 532 nm against an appropriate blank without the sample. The values were expressed as nm of MDA formed /mg of protein values are normalized to protein content of tissues. [37]

B) Estimation of super oxide dismutase

0.5ml of sample was diluted with 0.5ml distilled water, to this 0.25 ml ethanol, 0.5ml of chloroform (all chilled reagents) were added. The mixture was shaken for 1min and centrifuged at 200 rpm for 20 min. The enzymatic activity of supernatant was determined. To it 0.05ml of carbonate buffer (0.05M pH10.2) and 0.5ml of EDTA (0.49M) was added. The reaction was initiated by addition of 0.4 ml epinephrine (3mM) and the change in absorbance was measured at 480nm. SOD was expressed as unit/mg protein.

C) Estimation of catalase

A 0.1 ml of supernatant was added to curette containing 1.9 ml of 50 mm phosphate buffer (pH 7). Reaction was started by the addition of 1 ml of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240nm. Activity of catalase was expressed as unit/mg protein

Procedures for tested parameters

Estimation of serum of triglycerides

Chemicals and equipments kit was used for estimation of triglycerides, which followed end point colorimetry enzymatic test using glycerol-3-phosphate oxidase.

Standard: The concentration of standard triglyceride used was 200mg/dl.

Assay & Procedure: Fresh clear and unhaemolysed serum was used for the estimation.

Table : 6 Reaction parameters:

S.NO	Reaction type	End point
1	Wave Length	505 nm
2	Optical length	1 Cm
3	Temperature	37^0C
4	Measurement	Against reagent blank

Summary of assay details

Table No: 6.1

S.NO	Pipette into the test tube	Blank	Standard	Test
1.	**Reagent**	1.0ml	1.0ml	1.0ml
2.	**Standard**	-	10µl	-
3.	**Sample**	-	-	10µl

The reaction mixtures were mixed well and incubated for 10 min at 37^0C. The absorbance of sample and standard were measured against reagent blank at 505 nm. The absorbance was measured by using a semi auto analyser.

Calculation

$$\text{Serum triglycerides (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc.of Standard}$$

Estimation of serum total cholesterol

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Chemicals and equipment skit was used for the estimation of total cholesterol, which followed cholesterol oxidase/peroxidase (CHOD-POD) method [36-38].

Standard: The concentration of standard cholesterol used was 200mg/dl.

Assay &Procedure: Fresh clear and unhaemolysed serum was used for the estimation.

Reaction parameters

Table No: 6.2

S.NO	Reaction type	End point
1	Wave Length	505 nm
2	Optical length	1 Cm
3	Temperature	37^0C
4	Measurement	Against reagent blank

Summary of assay details

Table No: 6.3

S.NO	Pipette into the test tube	Blank	Standard	Test
1.	Reagent	1.0ml	1.0ml	1.0ml
2.	Standard	-	10µl	-
3.	Sample	-	-	10µl

The reaction mixtures were mixed well and incubated for 10 min at 37^0C. The absorbance of reaction mixtures at 505nm against reagent blank was taken. The absorbance was measured by using a semi auto analyser.

Calculation

$$\text{Serum total cholesterol(mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc.of standard}$$

Estimation of serum high-density lipoprotein cholesterol

Chemicals and equipments kit was used for estimation of HDL cholesterol, which followed Cholesterol oxidase / peroxidase (CHOD-POD) method [38-40].

Principle

HDL is measured in the supernatant after the precipitation of the lipoproteins including chylomicrons, very low-density lipoproteins, low-density lipoproteins, intermediate-density lipoproteins directly from serum polyanions like phosphotungstic acid and along with $MgCl_2$ are added to an aliquot of serum an immediate heavy precipitation is formed. The precipitate then is sedimented by centrifugation and HDL cholesterol is measured in the clear supernatant, which is estimated by enzymatic method as described earlier in estimation serum of TC.

Assay & Procedure

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Fresh clear and unhaemolysed serum was used for the estimation.

Standard: The concentration of standard high density lipoprotein used was 50mg/dl

Reaction parameters

Table No:6.4

S.NO	Reaction type	End point
1	Wave Length	505 nm
2	Optical length	1 Cm
3	Temperature	37^0C
4	Measurement	Against reagent blank

Standard of assay details

1. 0.5ml of serum was taken into test tube and 0.5ml of precipitating reagent was added, mixed well and kept at room temperature for 15 min.
2. Centrifuged for 15 min at 4000 rpm.
3. The clear supernatant was separated and immediately used to determine the cholesterol content as follows:

Table No:6.5

S.NO	Pipette into the test tube	Blank	Standard	Test
1.	Reagent	1000µl	1000µl	1000µl
2.	Standard	-	50µl	-
3.	Sample (supernatant)	-	-	50µl

The reaction mixtures were mixed well and incubated for 10 min at 37^0C. The absorbance of test and standards was measured against the reagent blank at 505nm. The absorbance was measured by using a Semi auto analyser.

Calculation

$$\text{Serum HDL cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc.of standard}$$

Estimation of serum low-density lipoprotein cholesterol

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Using the data obtained including total cholesterol, HDL cholesterol and VLDL, the LDL cholesterol levels were calculated using the empirical equation of Friede Wald. [50]

Calculation
Serum LDL cholesterol = Total cholesterol - (HDLc holesterol + VLDL cholesterol)

Estimation of serum very low-density lipoprotein cholesterol
Using the data obtained including triglycerides, the VLDL cholesterol levels were calculated using empirical equation of Friede Wald[40-43].

Calculation

$$\text{Serum VLDL cholesterol} = \frac{\text{Triglycerides}}{5}$$

Estimation of serum glutamate pyruvate transaminase
Assay and procedure: Fresh, clear and unhaemolysed serum was used for estimation.

Reaction parameters

Table No: 6.6

S.NO	Reaction type	Kinetic with factor
1.	Wavelength	340nm
2.	Temperature	37^0
3.	Measurement	against reagent blank

Reagent composition
R1 - Tris buffer, L-Alanine, LDH, α-Ketoglutarate.
R2 – β NADH
Reagent preparation
Prepare the working reagent by mixing 4 parts of R1 with 1 part of R2 per assay tube.
Procedure:
Pipette into test tube:
Blank: Take distilled water as a blank solution.
Test: Pipette 500µl of working reagent, to this add 25µl sample.
Mix well and aspirate.
Read absorbance of all the tubes against distilled water.

Calculations

SGPT (ALT) activity in IU/L = (ΔA) /min × Factor (3376)

Where (ΔA): Difference in absorbance.

Estimation of serum glutamate oxaloacetate transaminase
Principle: Kinetic determination of the aspartate aminotransferase (AST) activity:

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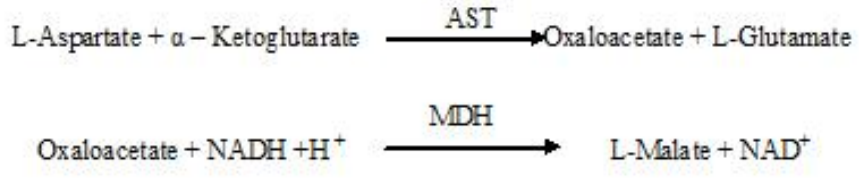


Assay and procedure
Fresh, clear and unhaemolysed serum was used for estimation. [44-45]

Reaction parameters

Table No:6.7

S.NO	Reaction type	Kinetic with factor
1.	Wavelength	340nm
2.	Temperature	37^0
3.	Measurement	against reagent blank

Reagent composition
R1 - Tris buffer, L- Aspartame, LDH, 2-Oxoglutarate.
R2 – NADH
Reagent preparation: Prepare the working reagent by mixing 4 parts of R1 with 1 part of R2 per assay tube.
Procedure:
Pipette into test tube:
Blank: Take distilled water as a blank solution.
Test: Pipette 500µl of working reagent, to this add 25µl sample.
Mix well and aspirate.
Read absorbance of all the tubes against distilled water.

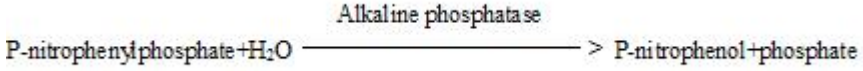
Calculations

SGOT (AST) activity in IU/L = (ΔA) /min × Factor (3376)

Where (ΔA): Difference in absorbance

Estimation of serum alkaline phosphatises

Principle
Alkaline Phosphatase in a sample hydrolyses para-nitrophenyl phosphate into paranitrophenol and phosphate, in the presence of magnesium ions. The rate of increase in absorbance of the reaction mixture at 405nm due to liberation of paranitrophenol is proportional to the alkaline phosphatase activity. [46-48]



Reagent
Dissolve one tablet of alkaline phosphatise in 1.1 ml of DEA buffer to make buffer substrate.

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Reaction parameters

Table No: 6.8

S.NO	Type of reaction	Kinetic
1.	Wave length	405nm
2.	Temperature	37^0c
3.	Path length	1cm
4.	Measurement	Against blank reagent

Procedure

Table No: 6.9

Pipette into test tubes	Test
1. Buffered substrate	1.0ml
2. Sample	0.02ml

Mix and read absorbance at30, 60, 90 and 120 seconds at 405nm.

Calculation

Alkaline phosphatises activity (IU/L) =ΔA/min×F

Where F =2713(calculated on the basis of molar extinction coefficient for P-nitro phenol and ratio of total assay volume to sample volume.

Statistical analysis:

The results were expressed as mean ± S.E.M. Statistical analysis was carried out by ANOVA followed by Dunnet's multiple comparison tests using Graph pad PRISM software version. P values ≤ 0.001, ≤ 0.01 were considered as statistically significant. [49-50]

Results and Discussion

High fat diet induced hyperlipidaemias in rats:

Effect of administration of RTEE (250/500/1000mg/kg, p.o., once daily)/Atorvastatin (10mg/kg, p.o., once daily) on serum lipid Parameter levels in rats fed with HFD for 30 days.

Table : 7

	GROUP	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	LDL : HDL
1	NORMAL	43.88± 0.1*	17.42±0.1*	19.62±0.1*	0.404
2	HFD	19.59±0.1*	94.63±0.2	22.48±0.2	4.83
3	HFD+ RTEE DOSE I	42.81±0.1*	65.67±0.01	21.83±0.1	1.534
4	HFD+ RTEE DOSE II	49.30±0.001***	54.12±0.02	21.25±0.2	1.097

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5	HFD+ RTEE DOSE III	52.60±0.01**	37.14±0.01**	20.8±0.2	0.706
6	HFD+ STD	53.38±0.001***	34.15±0.001***	17.95±0.001***	0.639

LDL- Low density lipoprotein, HDL- High density lipoprotein, and VLDL- Very low density lipoprotein. n = 6 animals in each group. Values are expressed as mean ± SEM. Statistically significant at P**≤0.01, P***≤0.001.Variation analysis done by One way ANOVA followed by Dunnet's test. Comparison of RTEE with Control group and of Control group with Normal group.
RTEE DOSE I: 250mg/kg body weight.
RTEE DOSE II: 500mg/kg body weight.
RTEE DOSE III: 1000mg/kg body weight.
STD (Atorvastatin): 10mg/kg body weight.

Table :8 Effect of administration of RTEE (250/500/1000mg/kg, p.o., once daily)/Atorvastatin (10mg/kg, p.o., once daily) on TC, TG levels in rats fed with HFD for 30 days.

S.NO	GROUP	TG (mg/dl)	TC (mg/dl)	ATHEROGENIC INDEX
1	NORMAL	98.12 ± 0.2	80.12 ± 0.2	0.82
2	HFD	112.4 ±0.01**	136.7 ±0.01***	5.98
3	HFD+ RTEE DOSE I	109.16 ±0.01**	130.3 ±0.01**	1.54
4	HFD+ RTEE DOSE II	106.28 ±0.1**	124.67±0.001***	1.21
5	HFD+ RTEE DOSE III	104.02 ±0.001***	110.54±0.01**	0.98
6	HFD+ STD	89.75 ±0.001***	105.48±0.001***	0.68

TC-Total Cholesterol, TG-Triglycerides. n=6 animals in each group. Values are expressed as mean ± SEM. Statistically significant at P**≤0.01,P***≤0.001.Variation analysis done by One way ANOVA followed by Dunnet's test Comparison of RTEE 2012 with Control group and of Control group with Normal group.
RTEE DOSE I: 250mg/kg body weight.
RTEE DOSE II: 500mg/kg body weight.
RTEE DOSE III: 1000mg/kg body weight.
STD (Atorvastatin): 10mg/kg body weight.

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Table: 9 Effect of administration of RTEE (250/500/1000mg/kg, p.o., once daily)/Atorvastatin (10mg/kg,p.o., once daily) on SGOT, SGPT, ALKALINE PHOSPHATASE levels in rats fed with HFD for 30 days.

S.NO	GROUP	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)
1	NORMAL	25.65 ±0.83	24.65 ± 1.71	86.45 ±1.13
2	HFD	54.75±0.001***	62.57±0.001***	350.66 ±0.01***
3	HFD+ RTEE DOSE I	31.56 ±0.01**	33.45 ±0.01**	201.41±0.01**
4	HFD + RTEE DOSE II	28.25 ±1.70**	29.55 ±0.80**	189.62±0.001***
5	HFD + RTEE DOSE III	24.14 ±0.01**	25.93±0.001***	178.08±0.001***
6	HFD + STD	20.34±0.001***	22.61 ±0.01**	170.81±0.001***

Serum glutamate oxalo transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), Serum alkaline phosphatase(SAP), n = 6 animals in each group. Values are expressed as mean ± SEM. Statistically significant at P**≤0.01, P***≤0.001.Variation analysis done by One way ANOVA followed by Dunnet's test. Comparison of RTEE with Control group and of Control group with Normal group.
RTEE dose I: 250mg/kg body weight.
RTEE dose II: 500mg/kg body weight.
RTEE dose III: 1000mg/kg body weight.
Standard drug (Atorvastatin): 10mg/kg body weight.

Table: 10 Effect of administration of RTEE (250/500/1000mg/kg, p.o., once daily)/Atorvastatin (10mg/kg, p.o., once daily) on LPO, SOD, CAT levels in rats fed with HFD for 30 days.

Table No: 10

S.NO	GROUP	LPO	SOD units/mg	CAT units/mg
1	NORMAL	30.45±0.61	7.92±0.56	65.24±2.01
2	HFD	48.74±0.001***	4.35±0.001***	40.55±0.01**
3	HFD+ RTEE DOSE I	45.66±0.01**	4.82±0.01**	53.27±1.01**

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4	HFD + RTEE DOSE II	43.85±0.001***	4.98±0.01**	56.26±0.001***
5	HFD + RTEE DOSE III	41.57±0.01**	5.13±0.01***	58.96±0.01***
6	HFD + STD	39.24±0.001***	6.24±0.01***	67.26±0.01**

LPO- Lipid peroxidation, CAT- Catalase, SOD- Superoxide dismutase. n = 6 animals in each group. Values are expressed as mean ± SEM. Statistically significant at P**≤0.01, P***≤0.001.Variation analysis done by One way ANOVA followed by Dunnet's test Comparison of RTEE with Control group and of Control group with Normal group.
RTEE DOSE I: 250mg/kg body weight.
RTEE DOSE II: 500mg/kg body weight.
RTEE DOSE III: 1000mg/kg body weight.
STD (Atorvastatin): 10mg/kg body weight.

Discussion
The RTEE indicates the presence of tannins, flavonoids, and phenols.

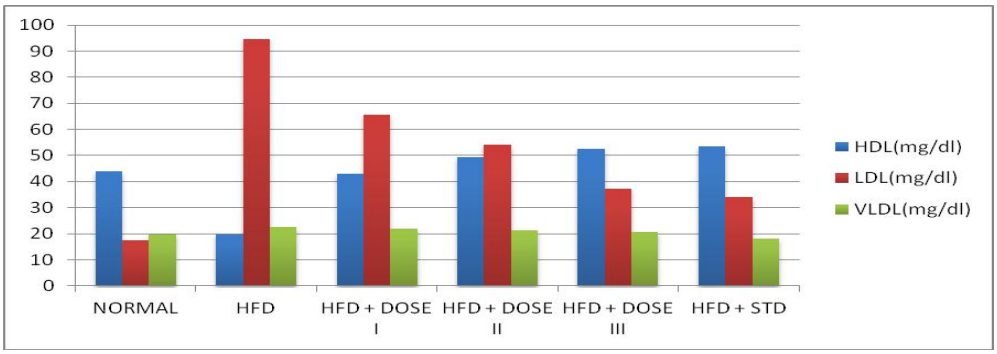

Figure:1 Effect of administration of RTEE (250/500/1000mg/kg)/Atorvastatin (10mg/kg) on serum lipid parameter levels in rats fed with HFD for 30 days

Table no 7,8, 9, and 10, shows that RTEE, the total lipids i.e. total cholesterol and triglycerides in plasma as well as LDL and VLDL cholesterol were significantly reduced at three doses of feed supplementation. However, HDL cholesterol level increased in drug treated groups significantly. This observation indicates that, as a feed component is effective in reducing serum LDL and VLDL levels. It is well known that increased HDL levels have a protective role.

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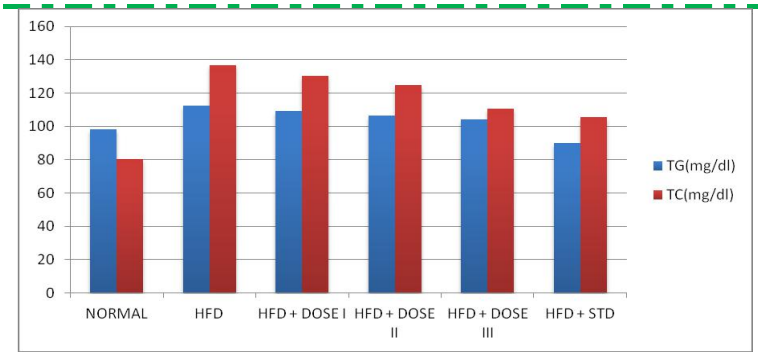

Figure: 2 Effect of administration of RTEE (250/500/1000mg/kg)/Atorvastatin (10mg/kg) on TC, TG levels in rats fed with HFD for 30 days.

That RTEE, SGOT, SGPT, Alkaline phosphatase in serum was significantly reduced at three doses of feed supplementation. However, HDL cholesterol level increased in drug treated groups significantly. This observation indicates that, as a feed component is effective in reducing serum SGOT, SGPT, Alkaline phosphatase levels.

Fig: 3

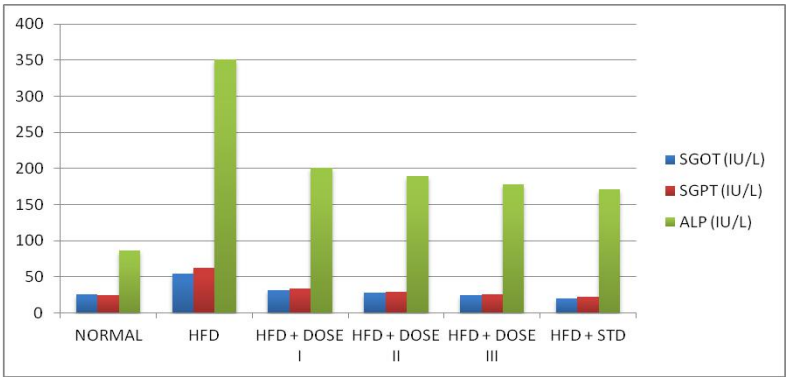

Figure: 3 Effect of administration of RTEE (250/500/1000mg/kg)/Atorvastatin (10mg/kg) on SGOT, SGPT, and ALP levels in rats fed with HFD for 30 days.

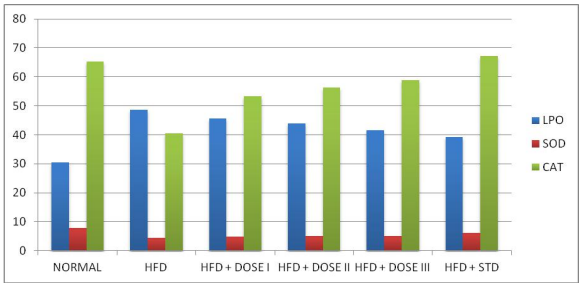

Figure: 4 Effect of administration of RTEE (250/500/1000mg/kg)/Atorvastatin (10mg/kg) on LPO, SOD, and CAT levels in rats fed with HFD for 30 days.

The RTEE treated groups have higher levels of anti oxidative parameters (catalase, superoxide dismutase) and decreased level of lipid peroxidation indicating its efficacy to reduce the LDL oxidation. The results of our study showed that administration of high fat diet induced significant production of MDA in liver, and administration of

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RTEE significantly decreases the MDA production in liver. RTEE also resulted in a significant increase in the liver CAT, SOD as compared to the control animals, which suggests its antioxidant activity.

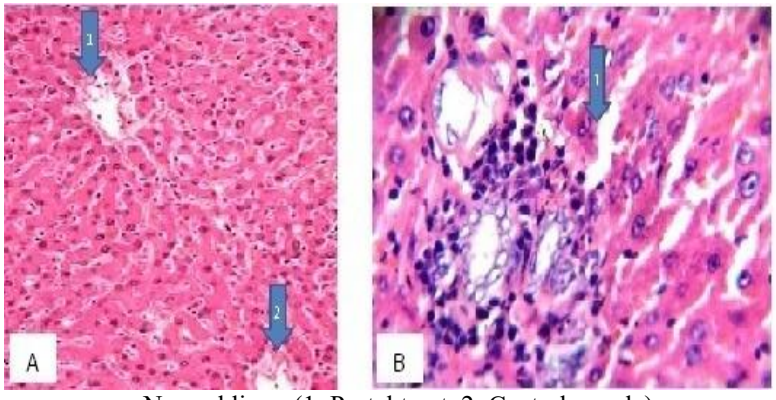
Biological membranes are often rich in unsaturated fatty acids and bathed in oxygen-rich metal containing fluid. Lipid peroxidation is a free radical mediated process, which has been accepted to be one of the principle causes of cholesterol-induced diseases, and is mediated by the production of free radical derivatives. Therefore, it is not surprising that membrane lipids are susceptible to per oxidative attack. The biochemical mechanisms involved in the development of hypercholesterolemia have long been investigated. MDA, a stable metabolite of the free radical mediated lipid peroxidation cascade, is widely used as marker of lipid peroxidation. Lipid peroxide levels in tissue were found to be significantly elevated in hypercholesterolemic rats. The antioxidant enzymes, mainly superoxide dismutase and catalase are first-line defensive enzymes against free radicals. The qualitative analysis of RTEE indicated the presence of tannins, flavonoids and phenols. It is well known that tannins, flavonoids and phenols are natural antioxidants but have also been reported to significantly increase SOD and catalase activities. Further, it was shown that these compounds act as promoters for SOD and catalase and cause the expression of SOD and catalase. The currently noted elevated levels of SOD and catalase with RTEE due to the influence of tannins, flavonoids and phenols.

It shows that RTEE, SGOT, SGPT, Alkaline phosphatase in serum was significantly reduced at three doses of feed supplementation. However, HDL cholesterol level increased in drug treated groups significantly. This observation indicates that, as a feed component is effective in reducing serum SGOT, SGPT, Alkaline phosphatase levels.

In triton induced hyperlipidemic model, the groups treated with the RTEE 2012 and Atorvastatin demonstrated a significant decrease in the serum TC, LDL, VLDL, TG, besides an increase in serum HDL levels when compared to triton induced hyperlipidemic control group. The groups treated with the RTEE 2012 and Atorvastatin demonstrated significant decrease in the Atherogenic Index and LDL: HDL risk ratios. The present investigation shows that all triton induced rats displayed hyperlipidemia as shown by their elevated levels of serum and liver cholesterol, triglyceride, VLDL, LDL and the reduction in the HDL level.

The groups treated with the RTEE 2012 also showed decrease in body weights when compared to triton induced hyperlipidemic control group. In triton induced hyperlipidemic model, the histopathological studies were conducted in the liver sections of rats and the histopathological changes were observed. These figures illustrate the protective action of the RTEE against fatty infiltration and granular degeneration due to hyperlipidemia closely comparable to that with Atorvastatin. The RTEE 2012 showed a significant antihyperlipidemic activity in the animal model and the best activity was shown by RTEE.

Histopathology of liver section of rats



Normal liver. (1: Portal tract, 2: Central venule)

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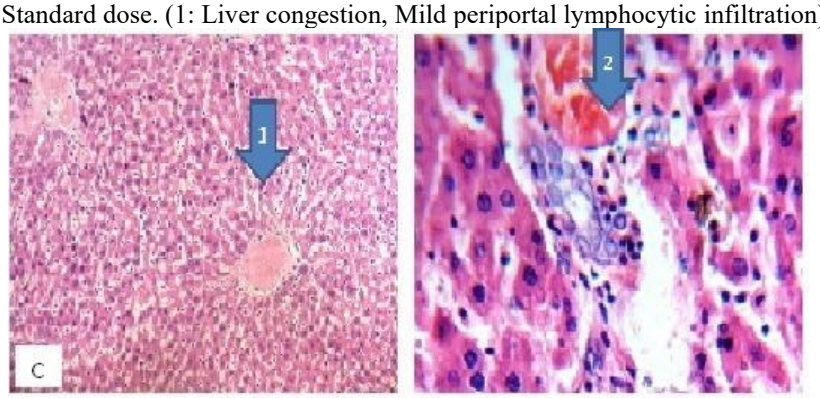
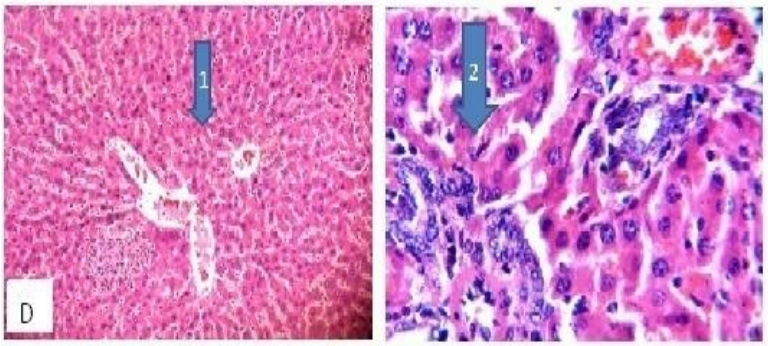
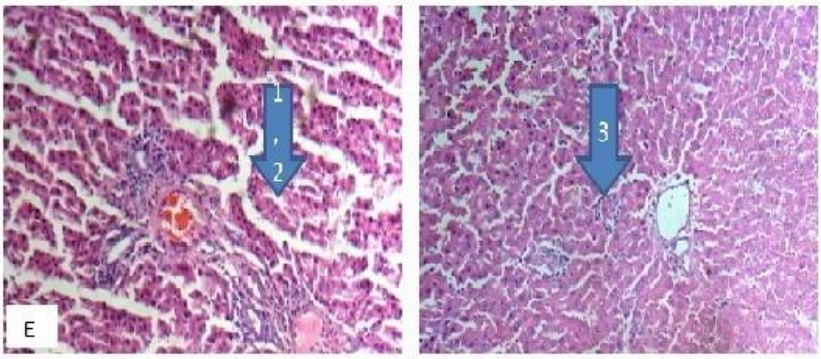
Standard dose. (1: Liver congestion, Mild periportal lymphocytic infiltration)



Control dose. (1: Necrosis of liver cells in small groups around central veins, 2: Moderate periportal inflammation).



RTEE dose1. (1: Focal necrosis of cells near central vein with inflammatory infiltrate, mild edema, necrosis, 2: Mild periportal inflammation)



RTEE dose 2. (1: Focal necrosis of hepatocytes around central veins, 2: Congestion of sinusoids, 3: Mild periportal inflammation)

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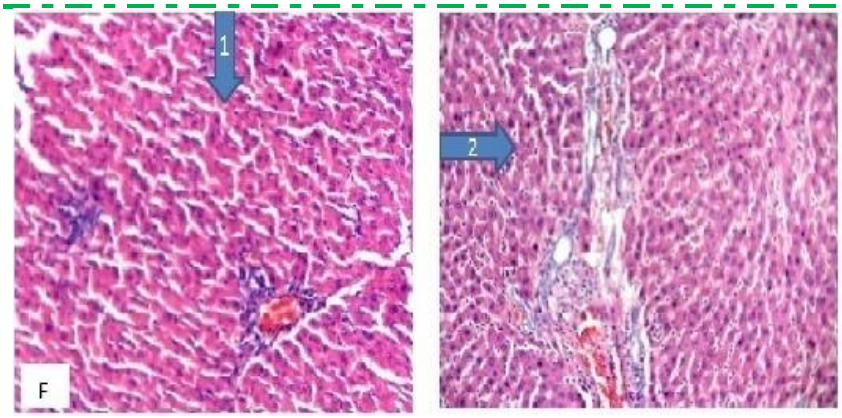

RTEE dose3. (1: Liver within normal limits, 2: Mild Periportal inflammation)

Conclusion

This plant ***Ruellia tuberosa*** **L.** has antimicrobial activity for both Gram-positive and Gram negative bacteria. However, very few chemical constituents and pharmacological activities have been reported for this species. Chronic hyperlipidemia was induced by feeding female rats HFD for 30 days. Acute hyperlipidemia was induced by administration of Triton X-100 (100mg/kg, i.p., at once) in female rats. Administration of RTEE (250/500/1000mg/kg) for 30 days in HFD model and RTEE(250/500/1000mg/kg) for 7 days in triton models respectively, successfully prevented the elevation of serum TG, TC, LDL, VLDL, SGOT, SGPT, Alkaline Phosphatase and decrease of serum HDL in HFD and Triton X-100 model rats in a dose dependent manner [51-56].

Treatment with RTEE for 30 days successfully prevented the elevated liver homogenate LPO levels indicating its efficacy to reduce the LDL-c oxidation and decreases of in-vivo antioxidant enzyme catalase, superoxide dismutase.

Hypolipidemic activity was observed with standard drug Atorvastatin at a dose of 10 mg/kg of body wt. cause decreased serum cholesterol, triglyceride, LDL and VLDL levels, whereas HDL was increased more as compared to both doses of the test drug.

In conclusion, the findings of the study suggest that RTEE is a potent antihypercholesterolemic drug lowering LDL, VLDL and increasing HDL levels in two hyperlipidemic models such as HFD, Triton X-100. The mechanism has point towards inhibiting cholesterol and triglyceride synthesis.The drug also demonstrated for antioxidant properties in in-vivo antioxidant models

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