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PHYTOCHEMICAL SCREENING AND ISOLATION OF RUTIN FROM *CEDRELA TOONA* ROXB. LEAVES.

Dr. KINJAL H. SHAH[1*]

[1]**Associate Professor, B. Pharmacy College, Rampura, Gujarat, India**

ABSTRACT

Air-dried, powdered material of the leaves of *Cedrela toona* Roxb. was successively extracted with the solvents of increasing polarity in a soxhlet apparatus and subjected to various qualitative chemical tests to determine the presence of various phytoconstituents like alkaloids, glycosides, carbohydrates, phenolics and tannins, phytosterols, fixed oils and fats, proteins amino acids, flavonoids, saponins. Total flavonoids content was measured with the 'Aluminum chloride colorimetric assay method'. Total Phenolic content in the aqueous and methanol extracts of leaves of *Cedrela toona* Roxb. were determined using the Folin-Denis method and expressed as mg of tannic acid equivalents per gram dry weight of extract. The various extracts of leaves of *Cedrela toona* Roxb prepared by successive solvent extraction were subjected to thin layer chromatographic studies to identify the number and nature of the chemical constituents present. The HPTLC was carried out to identify and to check the purity of raw herbal extracts as well as finished products.

KEYWORDS: ***Cedrela toona*, HPTLCD, Total flavonoids , Phytoconstituent**, Rutin.

Dr. KINJAL H. SHAH
Associate Professor, B. Pharmacy College, Rampura, Gujarat, India

***Corresponding Author**

INTRODUCTION

The Litreture survey reveals that *Cedrela toona* Roxb. is medium sized to large deciduous tree with brown to gray scaly bark. Leaves 15 – 45 cm long usually paripinnate but sometimes with a terminal leaflet in juvenile growth, leaflets mostly 8-20, ± ovate, often falcate, 4-15 cm long, 15-50 mm wide, apex acuminate, base strongly asymmetric, margins entire, mostly glabrous, domatia present as small hair – tuffs; petiole 4-11 cm long, petiolules 5-12 mm long. Penicles 20-40 cm long. Petals 5-6 mm long, white. Capsule ellipsoid, 10-20 mm long, 6-8 mm diameter; seeds winged at both ends[1-4]. Traditionally the bark is astringent, antidysentric, antiperiodic[5]. Flowers are emmenagogue, leaf is spasmolytic, hypoglycaemic and antiprotozoal[6]. Bark and heartwood yielded tetraterpenoids, including toonacillin. Heartwood also gave a coumarin gerarnyl gernalol as its fatty esters. Toonacillin and its 6 – hydroxyl derivatives are antifeedent[5].

MATERIALS AND METHODS

Plant Material

The leaves of the plant were collected from the Paritosh Herbals, Dehradun in the month of October 2011. The plant was identified and authentified as *Cedrela toona* Roxb. (Family: Meliaceae) by Dr. M. S. Jangid, Department of Botany at Sir P. T. Science College, Modasa, Gujarat, India where a voucher specimen has been deposited.

Preliminary phytochemical screening [5-6]

100g of each of air-dried powdered material of leaves, stems and fruits of *Cedrela toona* Roxb. was successively extracted with the following solvents of increasing polarity in a soxhlet apparatus.

- petroleum ether (60º - 80ºc)
- hexane
- chloroform/acetone
- ethanol/methanol
- distilled water

All the extracts were concentrated by distilling the solvents and the extracts were dried in an oven at 50^0C. Each time before extracting with the next solvent, the marc was dried in an air oven below at 50^0C.The marc was finally macerated with water for 24 hours to obtain the aqueous extract. The completion of the extraction was confirmed by evaporating a few drops of extract from the thimble on a watch glass to observe that no residue remained after evaporation of the solvent. The liquid extracts obtained with different solvents were collected. The consistency, odour, colour, appearance of the extracts and their percentage yield were noted. The extracts were then subjected to various qualitative tests using reported methods, to determine the presence of various phytoconstituents such as alkaloids, glycosides, flavonoids, carbohydrates, amino acids, saponins, sterols and terpenoids, anthraquinone glycosides, coumarins, carotenoids, tannins, phenolic compounds, fixed oils, fats etc.

Qualitative chemical identification of Cedrela toona Roxb. [6-10]

The extracts were subjected to various qualitative chemical tests to determine the presence of various phytoconstituents like alkaloids, glycosides, carbohydrates, phenolics and tannins, phytosterols, fixed oils and fats, proteins amino acids, flavonoids, saponins, etc. using reported methods.

Alkaloids

Extracts were dissolved individually in dilute hydrochloric acid and filtered. The filtrates were tested carefully & treated with alkaloid reagents.

i. Mayer's Test

Filtrates were treated with Mayer's reagent (potassium mercuric iodide). The formation of a yellow cream precipitate indicated the presence of alkaloids.

ii. Wagner's Test

Filtrates were treated with Wagner's reagent (iodine in potassium iodide) and observed. The Formation of brown or reddish brown precipitate indicated the presence of alkaloids.

iii. Dragendorff's Test

Filtrates were treated with Dragendorff's reagent (solution of potassium bismuth iodide). Formation of a red precipitate indicated the presence of alkaloids.

iv. Hager's Test

Filtrates were treated with Hager's reagent (saturated picric acid solution). Formation of a yellow colored precipitate indicated the presence of alkaloids.

Proteins and Amino acids

i. Millon's Test

The extracts were treated with 2 ml of Millon's reagent. The formation of a white precipitate, which turned to red upon heating, indicated the presence of proteins and amino acids.

ii. Biuret Test

The extracts were treated with 1ml of 10% sodium hydroxide solution and heated. A drop of 0.7% copper sulphate solution to the above mixtures was added. The formation of a purplish violet color indicated the presence of proteins.

iii. Ninhydrin Test

To the extracts, 0.25% ninhydrin reagent was added and boiled for few minutes. Formation of a blue color indicated presence of amino acid.

Carbohydrates

Extracts were dissolved individually in 5ml of distilled water and filtered. The filtrates were used to test the presence of carbohydrates.

i. Benedict 's Test

Filtrates were treated with Benedict's reagent and heated on water bath. Formation of an orange red precipitate indicated the presence of reducing sugars.

ii. Molisch's Test

Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml concentrated sulphuric acid is added carefully along the sides of the test tube. Formation of violet ring at the junction indicated the presence of carbohydrates.

iii. Fehling's Test
Filtrates were hydrolyzed with dilute hydrochloric acid, neutralized with alkali and heated with Fehling's A and B solutions. A red precipitate was formed which indicated the presence of carbohydrates.
iv. Barfoed's Test
Filtrates were treated with Barfoed's reagent and heated on a water bath. Formation of an orange red precipitate indicated the presence of reducing sugars.

Flavonoids

i. Shinoda test
1-5mg of dried extract was extracted with 10ml of ethanol (95%v/v) for 15 min on a boiling water bath and filtered. To the filtrate, added a small piece of magnesium ribbon and 3 to 4 drops of concentrated hydrochloric acid. Formation of red colour was observed.
ii. Fluorescence test
1-2 mg of dried extract was extracted with 15 ml methanol for 2 min., on a boiling water bath, filtered while hot and evaporated to dryness. To the residue 0.3 ml boric acid solution (3% w/v) and 1 ml oxalic acid solution (10% w/v) were added. The mixture was evaporated to dryness and the residue was dissolved in 10 ml ether. The ethereal layer was observed under U.V. light.
iii. $FeCl_3$ test
To the test solution, added $FeCl_3$ solution. A change of colour from green to black was observed.
iv. Lead acetate solution test
To the test solution, added 10% lead acetate solution. A yellow precipitate was observed.

Phenols
A drop of ethanolic extract was spotted on a filter paper and a drop of phosphomolybdic acid reagent was added on it. The spot was then exposed to ammonia vapor. Blue coloration of the spot indicated the presence of phenols.

i. Glycosides
Extracts were hydrolyzed with dilute hydrochloric acid and the hydrolysate was subjected to glycosides tests.
ii. Modified Borntrager's Test
The extracts were treated with ferric chloride solution and heated on a boiling water bath for about 5 mins. The mixture was cooled and shaken with equal volume of benzene. The benzene layer was separated and treated with half of its volume of ammonia solution. The formation of rose pink or cherry red color in the ammonical layer indicated the presence of anthranol glycoside.
iii. Legal's Test
The extracts were treated with sodium nitroprusside in pyridine and methanolic alkali. The formation of pink to red color indicated the presence of cardiac glycosides.
iv. Balget Test
The extract of the drug was treated with sodium picrate and the formation of a yellowish orange color confirmed the presence of cardiac glycosides.
v. Killer killani Test
Take 0.5g of dried extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solutions. This was then under laid with 1 ml of concentrated H_2SO_4. A brown ring obtained in the presence of a cardenolides.

Saponins

i. Froth's Test
The extracts (alcoholic and aqueous) were diluted with 20 ml of distilled water separately and further shaken for 15 mins in a graduated cylinder. A layer of foam measuring about 1 cm was formed which indicated the presence of saponins.

Tannins (Phenolic compounds)

i. Ferric chloride Test
The extract was treated with few drops of neutral ferric chloride solution (5%). The formation of bluish black color indicated the presence of phenolic nucleus.
ii. Lead acetate Test
The extracts were treated with few drops of 10% lead acetate solution. The formation of yellow precipitate confirmed the presence of flavonoids.
iii. Alkaline reagent Test
The extracts were treated with few drops of sodium hydroxide separately. Formation of intense yellow color, which turned colorless on addition of few drops of dilute acid, indicated the presence of flavonoids.
iv. Shinoda Test
The extracts were treated with few fragments of magnesium metal separately, followed by drop wise addition of concentrated hydrochloric acid. The formation of magenta color indicated the presence of flavonoid.
v. Vanillin hydrochloric Test
The extracts were treated with few drops of vanillin hydrochloride reagent. The formation of pinkish red color indicated the presence of tannins.

Steroids and Triterpenoid

i. Libermann- Burchard's test
To one ml of ethanolic extract of drug, one ml of chloroform and 2 to 3 ml of acetic anhydride was added. To the above mixture, 1 to 2 drops of concentrated Sulphuric acid was added. Dark green coloration of the solution indicated the presence of steroids and dark pink or red coloration of the solution indicated the presence of triterpenoids.
ii. Salkowski test
Treat extract in chloroform with few drops of concentrated Sulfuric acid, shake well and allow standing for some time, red color appears in the lower layer indicates the presence of sterols and formation of yellow colored lower layer indicating the presence of triterpenoids.

Fixed Oils and Fats

i. Stain test
Small quantity of extracts was pressed between two filter papers separately. An oily stain on filter paper indicated the presence of fixed oil.
ii. Saponification test
The extracts were heated on water bath with 0.5 N alcoholic potassium hydroxide solutions. Formation of

soap indicated the presence of fixed oils and fats.

Coumarins

i. WithAmmonia

Took a drop of ammonia on a filter paper; to this added a drop of aqueous extract. Fluorescence was observed.

ii. With Hydroxylamine hydrochloride

Took ethereal extract; treated it with one drop of saturated alcoholic hydroxylamine hydrochloride and a drop of alcoholic KOH. Heated it, cooled and acidified with 0.5 N hydrochloric acid and added a drop of 1 %w/v $FeCl_3$. Violet color was observed.

Anthraquinone glycosides

i. Borntrager's test

Boiled the test solution with dilute sulphuric acid, filtered and added chloroform to the filtrate. Shook well and collected the organic layer. A few drops of strong ammonia solution was added, and shaken slightly and the test tube kept aside for a few minutes. The colour of lower ammonical layer was observed.

ii. Modified borntrager's test

To the test solution of drug ferric chloride and dilute HCl were added; heated it, cooled it and filtered. Filtrate was shaken with ether or any other organic solvent. The organic layer was shaken with strong ammonia solution and the test tube kept aside. The colour of lower ammonical layer was observed.

Determination of total flavonoids content of leaves of Cedrela toona Roxb[11-12]

Aluminum chloride colorimetric assay method

Total flavonoids content was measured with the 'Aluminum chloride colorimetric assay method'. Aqueous and methanol extracts of leaves of *Cedrela toona* Roxb. that has been adjusted to come under the linearity range i.e. (400µg/ml) and different dilution of standard solution of Quercetin and Rutin (10-100µg/ml) were added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3ml of 10% $AlCl_3$ was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10ml with distill water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Total flavonoid content of the extracts was expressed as percentage of Quercetin and Rutin equivalent per 100 g dry weight of sample.

Determination of total phenolic content of leaves of Cedrela toona Roxb[12-14]

Folin – Denis Method

Total phenolic content in the aqueous and methanol extracts of leaves of *Cedrela toona* Roxb. were determined using the Folin-Denis method and expressed as mg of tannic acid equivalents per gm dry weight of extract. The absorbance values of the test extracts after subtraction of control (y) were translated into total phenolic content (mg/gm of TAEs) using the tannic acid calibration plot with the following formula:

[Total phenolic content (mg/gm of TAEs) = y – 0.0004 / 0.0012]

Principle

The polyphenolic compounds are estimated by spectrophotometric method known as Folin-Denis method. The method is based on the oxidation of the molecules containing a phenolic hydroxyl group. The tannins and tannin like compounds reduce phospotungstomolybdic acid in alkaline solution to produce a highly blue coloured solution, the intensity of which is proportional to the amount of tannins and phenolic compounds and can be estimated against standard tannic acid solution.

Preparation of Reagents

1) *Folin-Denis Reagent*

Sodium tungustate (10 gm) and phosphomolybdic acid (2 gm) were dissolved in distilled water (75 ml) along with phosphoric acid (5 ml). The mixture was refluxed for 2 hours and volume was made with water up to 100 ml.

2) *Sodium carbonate solution*

sodium carbonate (35 gm) was dissolved at 70-80°C in distilled water and volume was made up to 100 ml. It was filtered through glass wool after allowing it to stand overnight.

3) *Working standard solution of tannic acid*

Accurately weighed standard tannic acid (100 mg) was dissolved in distilled water in a volumetric flask. 5 ml of this solution was diluted with water to 100 ml in another volumetric flask to give 50 µg/ml tannic acid solutions.

4) *Preparation of solution*

A series of calibrated 10 ml volumetric flasks were taken and appropriate aliquots of standard tannic acid solution ranging from 1.6, 2, 2.4, 2.8, 3.4, 4, 5, 6 ml were added. To these solutions Folin-Denis reagent (0.5 ml) and Sodium Carbonate solution (1 ml) and Distilled water (up to 10 ml) were added. And the absorbance was measured at 700 nm within 30 min of the reaction. The calibration curve was prepared and concentration of total phenolic compound was found out from methanol and water extract by taking their absorbance respectively.

5) *Preparation of test solution*

a) Methanol Extract: Take 2 gm dried powder obtains from methanolic extract. Solubalize it into 50 ml methanol and take 0.5 ml of this solution and dilute up to 10 ml with methanol. Take 1 ml of this solution and dilute up to 10 ml with methanol and take this solution as a test solution.

b) Aqueous Extract: Take 5 gm dried powder obtains from Water extract. Solubalize it into 20 ml water and take 0.1 ml of this solution and dilute up to 10 ml with methanol. Take 1 ml of this solution and dilute up to 10 ml with methanol and take this solution as a test solution.

Determination of total tannin content of leaves of Cedrela toona Roxb.[15-16]

Take 0.1 gm of both extracts (aqueous and methanol) of *Cedrela toona* Roxb., dissolved in 10 ml of distilled water separately, add 10ml of indigo carmine dye, add 300 ml of distilled water, heat it at 60-70°C and titrate with 0.1 M $KMnO_4$. Carry out same experiment by omitting substance.

Chromatographic Examination of Various Extracts [17-19]

The vrious extracts of leaves and fruits of *Cedrela toona* Roxb prepared by successive solvent extraction procedure were subjected to thin layer chromatographic studies to identify the number and nature of chemical constituents present. The R_f values of different phytoconstituents present in various extracts of leaves and fruits of *Cedrela toona* Roxb were recorded.

Preparation of TLC plate

Absorbent used :Silica gel G.
Vehicle used for preparation of slurry :Distilled water
Method of preparation :Pour plate method
Activation of plate :In oven at 110˚c for 30 minutes
Application of sample :About 10 to 15 µl of sample was applied with the help of glass capillary.
Mobile phase :Take required quantity of solvents in TLC chamber, shake well and utilized for chamber saturation.
Chamber saturation :30 minute
Parameters observed :Color of the spot, $R_f$value

HPTLC finger-printing of ethanolic extract and water extract of Cedrela toona Roxb and quantitative determination of rutin. [20, 21]

HPTLC is the most simple separation technique available today which gives better precision and accuracy with extreme flexibility for various steps (stationary phase, mobile phase, development technique and detection). The HPTLC was carried out using a Hemilton 100 µl HPTLC syringe, Camag Linomat V automatic spotting device, Camag twin trough chamber, Camag TLC Scanner-3, WINCAT integration software, aluminium sheet precoated with Silica Gel 60F254(Merck), 0.2 mm thickness. HPTLC finger printing technique is useful to identify and to check the purity of raw herbal extracts as well as finished product. Hence forth it is very useful tool in standardizing process of raw herbal extracts and finished products.

Steps involved in HPTLC analysis

- ***Selection of plate and adsorbent***

Pre-coated aluminium plates with Silica Gel 60F254 (E. Merck, India) of 10 x 10 cm and 0.2 mm thickness, were used for the detection. The plates were pre-washed by methanol and activated at 60°C for 5 min prior to chromatography.

- ***Sample solution***

Accurately weighted 20 mg of both extracts of *Tephrosia purpurea* Linnleaves was taken, dissolved in methanol and transferred to a 10 ml volumetric flask. The volume made up to the mark with Methanol.

- ***Application of sample***

Sample application is the most critical step for obtaining good resolution for quantification in HPTLC. The automatic application devices are preferable. The most recent automatic device “CAMAG LINOMAT V” was used to apply 1 band of 6 mm width with different concentration of all the extracts and marker solution also.

- ***Development***

The plate was developed in CAMAG glass twin-through chamber (10-10 cm) previously saturated with the solvent for 60 min (temperature 25.2 °C, relative humidity 40%). The development distance was 8 cm. Subsequently scanning was done. The mobile phase or solvent system for all the raw herbs, raw ingredients, marker compound which is given in the Table 4.1.

Table 1
Solvent system for HPTLC of Cedrela toona Roxbextract

Sr. No.	Sample	Solvent system
1	Rutin, Aqueous extract and Ethanolic extract	Ethyl acetate : n-Butanol : Formic acid : Distilled water (5:3:1:1)

Detection

The plate was scanned at UV 366 nm and 254 nm using CAMAG TLC Scanner-3 and LINOMAT-V. Rf value of each compound which were separated on plate and data of peak area of each band was recorded.

Isolation and Identification of rutin from leaves of Cedrela toona Roxb.

Isolation of rutin [22]

Twenty grams of the powder of leaves was extracted by soxhlet apparatus with 250 ml of 80% ethanol till exhaustion. The extract was filtered and concentrated by evaporation under vacuum to about 10ml then mixed with 25ml distilled water, and extracted with petroleum ether (50ml x 3), then with chloroform (50 ml x 3). After extraction, the aqueous layer was collected and left to stand in a cold place for 72 hours; a yellow precipitate separated out of the solution. The precipitate was filtered and washed with a mixture of chloroform: ethylacetate: ethanol (50:25:25). The un-dissolved part of the precipitate was dissolved in hot methanol and filtered, the filtrate was evaporated to dryness to give 115 mg yellow powder (rutin), and its melting point was measured

Identification of isolated rutin [22]

- ***Chemical identification of Constituents***

Little amount of the isolated constituent are dissolve in methanol and perform the following test.

1. ***Shinoda Test (Magnesium Hydrochloride reduction test)***

To the test Solution, add few fragments of Magnesium ribbon and add concentrated Hydrochloric acid drop wise and observe the color.

2. ***Zinc Hydrochloride Reduction Test***

To the test solution add a mixture of Zinc dust and conc. Hydrochloric acid. Heat the solution and observe the color.

3. ***Alkaline Reagent Test***

To the test solution add few drops of sodium hydroxide solution and observe the colour formation.

TLC and paper chromatography

Isolated rutin was also compared with standard rutin using TLC method; a pre-coated aluminum sheet with silica gel GF254 with the following mobile phases: ethyl acetate: butanone: formic acid: water (50:30:10:10), ethyl acetate: formic acid: acetic acid: water (100: 11: 11:27). In paper chromatography, watman No.1 filter paper was used as a stationary phase and mobile phases of acetic acid: water (15:85) and isopropyl alcohol: water (60:40).

Spectrophotometric analysis

1. *Ultra Violet spectra*

The Ultra Violet spectra of the constituents (isolated from TLC and dissolved in methanol) are taken in double beam Shimadzu spectrophotometer (UV-1700) in between range 200 nm to 700 nm. Methanol was taken as reference solvent.

2. *Infrared Spectra*

The IR spectrum of isolated constituents as KBr disc has been determined on a Perkin–Elmer Infrared Spectrophotometer. The structural assignments have been correlated for the characteristic bands as mentioned in results.

3. *Proton NMR Spectra*

The proton NMR spectra was taken by dissolving the sample in DMSO – D6 and run on a 60-MHz NMR Spectrometer. All chemical shifts reported are in reference to tetra methyl silane (TMS) at 0 ppm.

4. *Mass Spectra*

The mass spectrum of rutin obtained by desorption chemical ionization (DCI) using ammonia as a reactant gas show a molecular ion M at m/e 611 amu. The prominent fragments and their relative intensities are mention in results.

RESULT AND DISCUSSION

Preliminary phytoprofiles

The presence of different chemical constituents in the crude drug can be detected by subjecting them to successive extraction using solvents in the order of increasing polarity. The extracts obtained were then dried completely and kept in vacuum desiccator. They were then subjected to qualitative chemical tests in order to detect the various chemical constituents present in them. The colour, consistency and percentage yield of extracts were determined which are shown in (Table 2).

Table 2
Preliminary phytoprofiles of leaves of Cedrela toona Roxb.

Sr. No.	Solvent	Color and consistency after drying	Average value of extractive (% w/w)
1	Petroleum ether (60 – 80 ºc)	Dark green sticky mass	1.30
2	Hexane	Dark green sticky mass	2.45
3	Chloroform	Greenish yellow sticky mass	2.68
4	Ethanol	Greenish brown sticky mass	6.37
5	Aqueous	Reddish brown sticky mass	8.54

Qualitative chemical analysis of various extracts of Cedrela toona Roxb

Qualitative chemical examination of various extracts of powder of leaves of *Cedrela toona* Roxb. indicated the presence of carbohydrates, flavonoids, phytosterols, cardiac glycosides, phenolic compounds, triterpenoids, tannins and saponins. The results obtained by chemical examination of various extracts are shown in (Table 3).

Table 3
Qualitative chemical analysis of various extracts of leaves of Cedrela toona Roxb.

Sr. No.	Tests of phytoconstituents	P. ether extract	Hexane extract	Chloroformextract	Ethanol extract	Water extract
1	Tests for alkaloids					
	a) Mayer's reagent	-ve	-ve	-ve	-ve	-ve
	b) Dragendorff's reagent	-ve	-ve	-ve	-ve	-ve
	c) Hager's reagent	-ve	-ve	-ve	-ve	-ve
	d) Wagner's reagent	-ve	-ve	-ve	-ve	-ve
2	Tests for flavonoids					
	a) Shinoda test	-ve	+ve	+ve	+ve	+ve
	b) Fluorescence test	-ve	+ve	+ve	+ve	+ve
	c) FeCl3 test	-ve	+ve	+ve	+ve	+ve
	d) Lead acetate test	-ve	+ve	+ve	+ve	+ve
3	Tests for saponins					
	a) Froth test	-ve	-ve	+ve	+ve	+ve
	b) Hemolytic zone	-ve	-ve	+ve	+ve	+ve
4	Tests for carbohydrates					
	a) Molisch's test:	-ve	-ve	-ve	+ve	+ve
	b) Fehling's solution test	-ve	-ve	-ve	+ve	+ve
	c) Benedict's test:	-ve	-ve	-ve	+ve	+ve
5	Tests for cardiac glycoside					
	a) Legal's test	-ve	-ve	+ve	+ve	-ve
	b) Keller Killiani's test	-ve	-ve	+ve	+ve	-ve
	c) Baljet test	-ve	-ve	+ve	+ve	-ve
6	Tests for fixed oil and fat					

Sr. No.	Tests of phytoconstituents	P. ether extract	Hexane extract	Chloroformextract	Ethanol extract	Water extract
	a) Spot test	-ve	-ve	-ve	-ve	-ve
	b) Saponification test	-ve	-ve	-ve	-ve	-ve
7	Tests for sterols and triterpenoids a) Libermann-burchard`s test	+ve	+ve	+ve	+ve	+ve
	b) Salkowski reaction	+ve	+ve	+ve	+ve	+ve
8	Tests for anthraquinone glycosides					
	a) Borntrager's test	-ve	-ve	-ve	-ve	-ve
	b) Modifying borntrager's test	-ve	-ve	-ve	-ve	-ve
9	Tests for phenolic compounds					
	a) Test with $FeCl_3$	-ve	-ve	+ve	+ve	+ve
	b) Test with folinciocalteureagent	-ve	-ve	+ve	+ve	+ve
10	Tests for coumarins					
	a) With ammonia	-ve	-ve	-ve	-ve	-ve
	b) With hydroxylamine hydrochloride	-ve	-ve	-ve	-ve	-ve
11	Tests for tannins					
	a) Test with gelatin	-ve	-ve	-ve	+ve	+ve
	b) Reaction with lead acetate	-ve	-ve	-ve	+ve	+ve

+ve: Present, -ve: Absent

The Results showed that the alcohol extract of *Cedrela toona* leaves contained carbohydrates, sterols & triterpenoids, flavonoids, saponin glycosides, carbohydrates and tannins and phenolics. Petroleum ether extract of *Cedrela toona* leaves contained steroids only. Chloroform extract of *Cedrela toona* leaves contained steroids, triterpenoids, flavonoidsand glycosides. Hexane extract of *Cedrela toona* leaves contained flavonoids and steroids. In methanol extract contain carbohydrates, triterpenoids, saponins, glycosides, alkaloids, tanins and phenolics. Aqueous extract contain of *Cedrela toona* leavescarbohydrates, saponins, glycosides, tanins and phenolics, steroids and triterpenoids and flavonoids.

Total flavonoids content of Cedrela toona Roxb. Leaves

Total flavonoids content in the methanol and aqueous extracts of leaves *Cedrela toona* Roxb. were determined according to the 'Aluminum chloride colorimetric assay method'. The total flavonoids content of methanol and aqueous extracts of *Cedrela toona* Roxb. are shown in (Table 4 & 5).

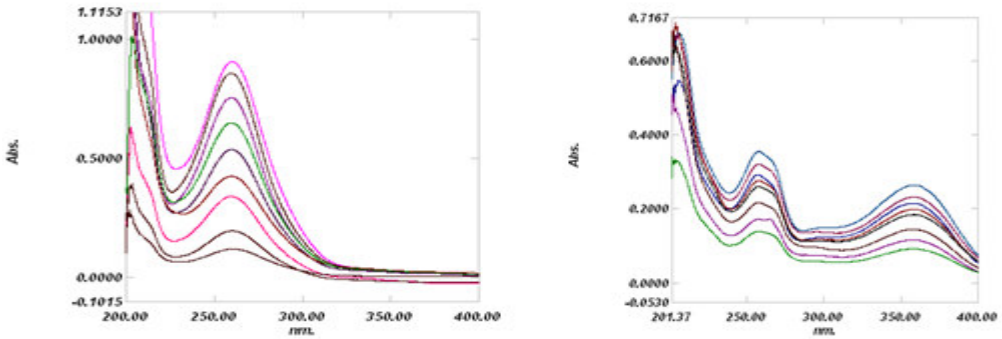

Figure 1
Overlay spectra of Quercetin and Rutin in Cedrela toona Roxb. extract

Table 4
Quercetin content in Cedrela toona Roxb. Extracts

Cedrela toonaRoxb. Extracts	Concentration µg/ml	Absorbance	Total Flavonoid %w/w
Quercetin	20	0.26	-
	30	0.313	-
	40	0.371	-
Quercetin	60	0.456	-
	80	0.561	-
	100	0.668	-
Aqueous	50	0.185	0.91
Ethanol	50	0.201	1.56

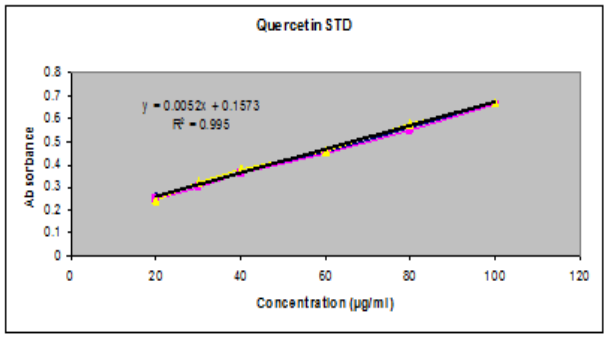

Figure 2
Calibration curve of Quercetin

The Result indicated that Quercetin gave maximum absorbance at λmax 257 and linear relationship with concentration and absorbance between the concentration applied. (Y= 0.081X+0.1573; R^2 =0.995). Aqueoues extract of *Cedrela toona* Roxb. was found to contain total Flavonoid 0.91%w/w equivalent to Quercetin. Ethanol extract of *Cedrela toona* Roxb. contained total Flavonoid 1.56%w/w equivalent to Quercetin. Flavonoid content was higher in the alcoholic extract of *Cedrela toona* Roxb. leaves extract than the water extract.

Table 5
Rutin content in Cedrela toona Roxb.

Cedrela toona Roxb.	Concentration (µg/ml)	Absorbance	Total Flavonoid %w/w
Rutin	20	0.256	-
	30	0.342	-
	40	0.431	-
	60	0.578	-
Rutin	80	0.789	-
	100	0.898	-
Aqueous	50	0.189	1.85
Ethanol	50	0.207	2.54

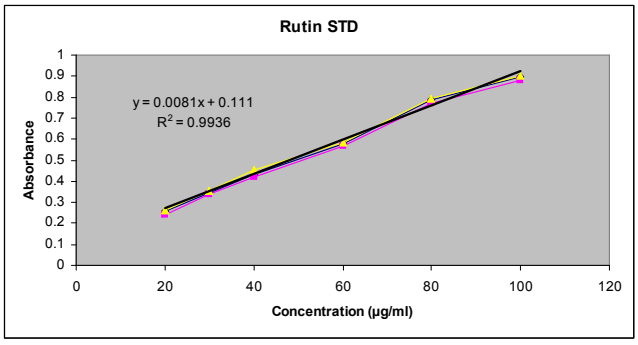

Figure 3
Calibration curve of Rutin

The Result indicated that Rutin gave maximum absorbance at λmax 254 and linear relationship with concentration and absorbance between the concentration applied. (Y= 0.081X+0.1573; R^2 =0.995). Aqueoues extract of *Cedrela toona* Roxb. was found to contain total Flavonoid 1.85%w/w equivalent to Rutin. Ethanol extract of *Cedrela toona* Roxb. contained total Flavonoid 2.54%w/w equivalent to Rutin. Flavonoid content was higher in alcoholic extract of *Cedrela toona* Roxb. leaves extract than water extract.

Total phenolics content of Cedrela toona Roxb. leaves extracts

Total phenolics

Total Phenolic content in the aqueous and ethanol extracts of leaves of *Cedrela toona* Roxb. were determined using the 'Folin-Denis method' and expressed as mg of tannic acid equivalents per gm dry weight ofextract (Table 6). The absorbance values of the test extracts after subtraction of control (*y*) were translated into total phenolic content (mg/gm of TAEs) using the tannic acid calibration plot with the following formula:

Total phenolic content (mg/gm of TAEs) = y – 0.0004 / 0.0012

Table 6
Total phenolics content of *Cedrela toona* Roxb. leaves extracts

Cedrela toona Roxb. extract	Concentration µg/ml	Absorbance	Total Phenolic content %w/w
Tannic acid	5	0.22	-
	10	0.313	-
	15	0.412	-
	20	0.472	-
	25	0.588	-
	30	0.645	-
Water	50	0.236	9.44
Ethanol	50	0.31	18.44

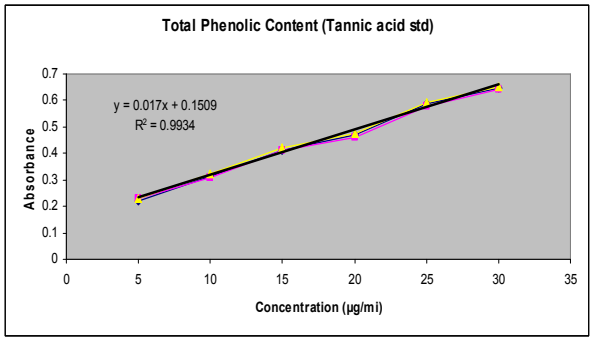

Figure 4
Calibration curve of tannic acid

Total phenolic content calculated in terms of tannic acid in the aqueous and methanol extracts of leaves of *Cedrela toona* Roxb.were found to be 9.44 %w/w and 18.44 % w/w respectively.

Total tannin content in Cedrela toona Roxb. leaves extracts

Total tannin content was determined by titrimetric method. Results indicated that total tannin content inaqueous and ethanol extracts of leaves of *Cedrela toona* Roxb. were 24.55 %w/w and 28.31 % w/w respectively equivalent to tannic acid.

Thin layer chromatographic studies of various extracts of Cedrela toona Roxb.

The successive extracts of leaves of *Cedrela toona* Roxb. were then subjected to thin layer chromatographic studies (TLC) in order to separate and detect the presence of different phytoconstituents. Silica gel G was used as adsorbent and different solvent mixtures depending upon the polarity were used as mobile phase. Several available detecting reagents for different categories of chemical constituents were tried and those which gave best results were used for further studies. The R_f values of different components were recorded. They are indicated in (Table 7).

Table 7
Thin layer chromatography of leaves extracts of Cedrela toona Roxb.

Solvent System	*P.ether Extract (R_f and Colour of spot)*	*Hexane Extract (R_f and colour of spot)*	*Chloroform Extract (R_f and colour of spot)*	*Ethanol Extract (R_f and colour of spot)*	*Water Extract (R_f and colour of spot)*	*Spraying reagent*	*Phytochemical constituents*
Chloroform: Glacial acetic acid: methanol: water (64:32:12:8)		R_{f-} 0.89 (blue) R_{f-} 0.88 (brown)	R_{f-} 0.89 (brown)	R_{f-} 0.87 (blue) R_{f-} 0.85 (brown)	R_{f-} 0.71 (yellow) R_{f-} 0.70 (pink)	Vanillin-sulphuric acid reagent	Saponins may be present.
Ethyl acetate: Formic acid: Glacial acetic acid: Water (80:10:10:20)	---	---	R_{f-} 0.68 (brown) R_{f-} 0.55 (brown) R_{f-} 0.18 (blue)	R_{f-} 0.76 (yellow) R_{f-} 0.68 (brown) R_{f-} 0.53 (brown) R_{f-} 0.40 (green) R_{f-} 0.18 (blue)	R_{f-} 0.78 (yellow) R_{f-} 0.68 (brown)	NP-PEG reagent	Flavonoids (UV-365nm) Flavone/Flavonol glycosides may be present.

Solvent System	*P.ether Extract (R_f and Colour of spot)*	*Hexane Extract (R_f and colour of spot)*	*Chloroform Extract (R_f and colour of spot)*	*Ethanol Extract (R_f and colour of spot)*	*Water Extract (R_f and colour of spot)*	*Spraying reagent*	*Phytochemical constituents*
Toluene: Ethyl acetate: Acetone: Glacial acetic acid (20: 40: 40: 10)	---	---	R_{f-} 0.74 (blue) R_{f-} 0.68 (brown)	R_{f-} 0.76 (blue) R_{f-} 0.68 (brown) R_{f-} 0.53 (green) R_{f-} 0.28 (blue)	R_{f-} 0.64 (blue) R_{f-} 0.68 (brown)	NP-PEG Reagent	Flavone/Flavonol aglycone may be present.
Ethyl acetate: Glacial acetic acid: Formic acid: Water (10:11:11:26)	R_{f-} 0.81 (reddish violet)	R_{f-} 0.14 (violet)	R_{f-} 0.39 (purple) R_{f-} 0.32 (Reddish violet)	R_{f-} 0.87 (greenish violet) R_{f-} 0.39 (violet blue) R_{f-} 0.32 (green) Rf.o.42 (brown)	R_{f-} 0.67 (green) R_{f-} 0.39 (violet)	Anisaldehyde sulphuric acid reagent	Tri-terpenoids may be present.
Hexane: Ethyl acetate (65 : 35)	R_{f-} 0.82 (purple) R_{f-} 0.74 (violet)	R_{f-} 0.79 (purple) R_{f-} 0.68 (blue)	R_{f-} 0.89 (blue) R_{f-} 0.21 (blue) R_{f-} 0.89 (purple) R_{f-} 0.21 (purple)	R_{f-} 0.74 (violet) R_{f-} 0.68 (blue)	R_{f-} 0.89 (purple) R_{f-} 0.21 (purple)	Anisaldehyde sulphuric acid reagent	Phytosterols may be present.
Chloroform: Methanol (85 : 15)	Rf.54 (violet)	R_{f-} 0.57 (blue) R_{f-} 0.65 (violet)	R_{f-} 0.68 (blue) R_{f-} 0.65 (violet)	R_{f-} 0.69 (violet) R_{f-} 0.54 (violet)	R_{f-} 0.74 (violet) R_{f-} 0.57 (blue)	Libermann burchard's reagent	Steroids may be present.
n-butanol : acetic acid : water (5:1:4)	---	---	---	R_{f-} 0.42 (brown) R_{f-} 0.38 (brown)	R_{f-} 0.47 (sandy brown)	Ninhydrin reagent	Sugars may be present.

HPTLC finger-printing of Ethanolic extract and water extract of Cedrela toona Roxb and quantitative determination of rutin.

CAMAG TLC scanner 3 and LINOMAT-V densitometry evaluation system with WINCAT software was used for scanning of thin layer chromatogram objects in reflectance or transmission mode by absorbance or by fluorescence at 254 or 366 nm, respectively. R_f value of sample was evaluated using following formula:

$$R_f = \frac{Distance\ travelled\ by\ sample\ from\ baseline}{Distance\ travelled\ by\ solvent\ from\ baseline}$$

HPTLC photograph, chromatogram 3-D image of ethanol extract and water extractofCedrela toona Roxb. leaves are given in Fig. 5,6 and table 8.

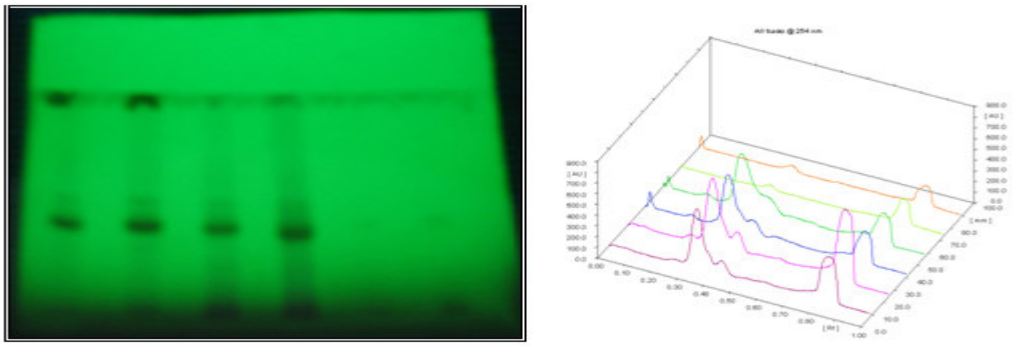

Figure 5

HPTLC plate of Rutin and extacts of Cedrela toona3 D- image

[Track 1: 100 µg/ml of Ethanol extract; Track 2: 100 µg/ml of Ethanol extract; Track 3: 100 µg/ml of Aqueous extract; Track 4: 100 µg/ml of Aqueous extract; Track 5: 10 µg/ml of Rutin; Track 6: 10 µg/ml of Rutin]

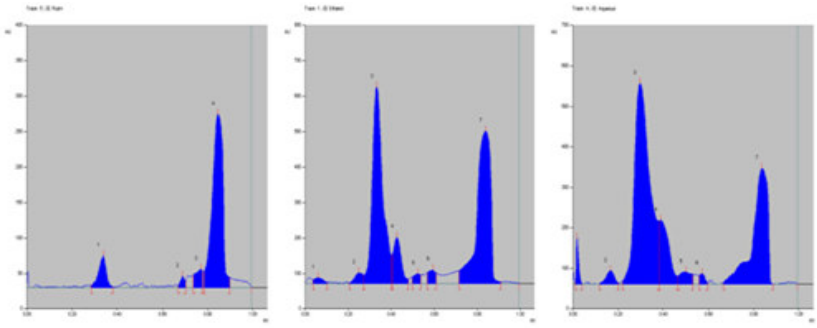

Figure 6
HPTLC chromatogram of Rutin, Ethanol and aqueousextacts of Cedrela toona Roxb leaves

Table 8
Chromatogram of Rutin, ethanol and water extract of Cedrela toona Roxb

µg/ml	Rf	Substance
100	0.372	Ethanol extract of *Cedrela toona*
100	0.381	Ethanol extract *Cedrela toona*
100	0.342	Aqueous extract *Cedrela toona*
100	0.359	Aqueous extract *Cedrela toona*
10	0.352	Rutin
10	0.364	Rutin

The Result showed that the Rf value of standard rutin was 0.36. Ethanol and aqueous extract of Cedrela toona Roxb. leaves also shown a substance at Rf. 0.37 and 0.36 respectively. So it was suggested that the compound separated at Rf 0.37 in ethanol extract and at Rf 0.36 in water extract of Cedrela toona Roxb. leaves may Rutin.

Isolation and identification of Rutin from the leaves of Cedrela toonaRoxb.

1. General and Physical Property

Colour : Pale yellow needles which gradually darken on exposure to light.
Taste : Tasteless
Odour : Odourless
Solubility : Soluble in pyridine, formamide, methanol and alkaline solution.
Melting Point : 189^{0}c

2. *Chemical identification of Constituents*

Table 9
Chemical test of isolated rutin

Test	Observation	Result
Shinoda Test (Magnesium Hydrochloride reduction test)	Green to blue color appears after few minutes	Flavonol are present
Zinc Hydrochloride Reduction Test	It gives red color after few minutes	Flavonol are present
Alkaline Reagent Test	Formation of an intense yellow color, which turns to colorless on addition of few drops of dilute acid.	Flavonol are present

3. *TLC and paper chromatography*

Table 10
Comparison between the R_f values of isolated and standard rutin in different mobile phases

Solvent systems in TLC and Paper Chromatography	$R_f$value of isolated rutin	$R_f$value of standard rutin
Ethyl acetate: butanone: formic acid: water	0.32	0.28
Ethyl acetate: formic acid: acetic acid: water	0.33	0.35
Acetic acid: water	0.61	0.58
Isopropyl alcohol: water	0.76	0.79

4. Spectrophotometric analysis

i) Uv spectroscopy: The UV spectrum of rutin in metabolic solution shows two major absorption bands at 359 nm and 257 nm, which indicates the presence of flavonol structure.

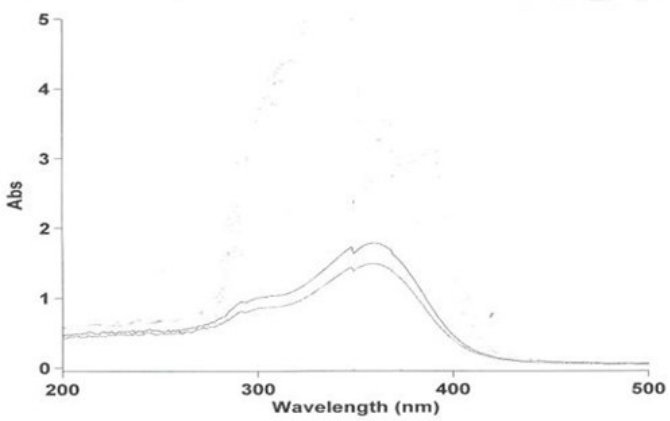

Figure 7
UV spectrum of rutin. Lower spectrum isolated rutin; upper spectrum: standard rutin

ii) IR Spectra

3330 OH (bonded) , 2920 CH stretch, 1660 C=O, 1620 C=C, 1600 Aromatic structure, 1510 C=C aromatic, 1360 C-O-C, 1295 C-O-C, 1200 C-O-C, 1060 C-O-C, 810 Substituted aromatics other fingerprint bands charcterstics to rutin are seen following 970, 880, 730 and 700.

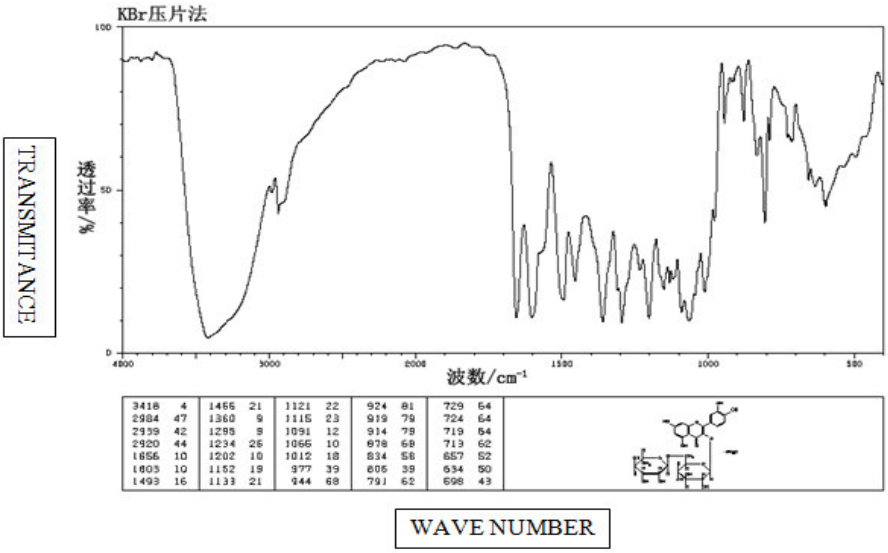

Figure 8
IR spectrum of rutin.

iii) *NMR Spectra*

Chemical shifts of Rutin in DMSO-D6 with respect to TMS.

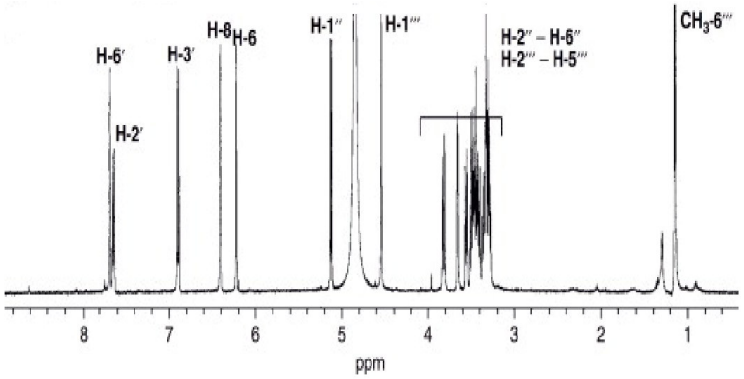

Figure 9
NMR spectrum of rutin.

Table 11
NMR values of isolated rutin

Group	Position	Chemical Shift
CHMe	Rhamnosyl Me	103(d)
Rhamnoglucosyl		3.20;3.40 (bS)
H-1 Rhamnosyl		4.40(S)
H-1 Glucosyl		5.30 (bS)
H-6	Aromatic	6.17 (SL)
H-8	Aromatic	6.36 (SL)
H-5	Aromatic	6.80 (d)
H-6	Aromatic	7.50 (bS)

(S)= Singlet; (d)= doublet; (bS)= broad singlet; (SL)= Singlet showing long range coupling

iv) Mass Spectrum

The mass spectrum of rutin obtained by desorption chemical ionization (DCI) using ammonia as a reactant gas show a molecular ion M at m/e 611 amu. The prominent fragments and their relative intensities are shown below

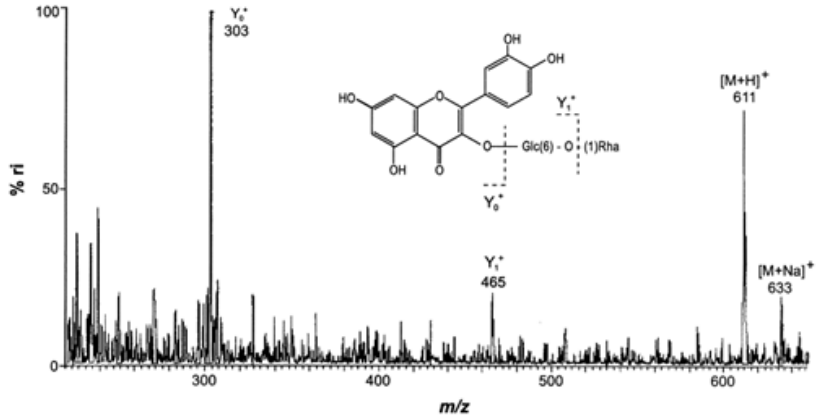

Figure 10
MASS spectrum of rutin.

Table 12
Values of mass spectra of isolated rutin

M/e	Relative intensity
303	100.0
611	28.88
628	2.22
164	57.77
180	66.66
304	44.44
308	22.22
320	24.44
326	42.22
449	11.11
465	33.33

General description and spectral analysis shows that the isolated constituent is rutin. Based on the melting point and other related data (UV, IR, 1HNMR and 13CNMR) the structure of the isolated compound was proposed as;

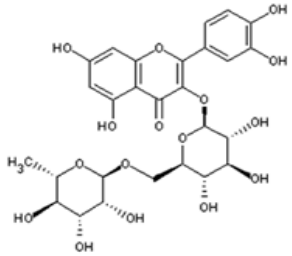

Figure 11
Structure of rutin.

CONCLUSION

The present study suggested that *Cedrela tone* Roxb. leaf possesses rutin which is utilized by scientist for further research and used to establish its beneficiary effect to society. Rutin inhibits platelet aggregation, as well as decreasing capillary permeability, making the blood thinner and improving circulation. It also shows anti inflammatory activity, aldose reductase activity, antioxidant activity, can reduce the symptoms of haemophilia and reduce the cytotoxicity of oxidized LDL cholesterol which lowers the risk of heart disease. There is also some evidence that rutin can be used to treat hemorrhoids, varicosis and microangiopathy.

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CONFLICT OF INTEREST

Conflict of interest declared as None.

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