International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.960

Screening of secondary metabolites in root extracts of *Couroupitaguianensis* and its *in-vitro* pharmacological activity study

[1]Pavani Peddi, [2]Dr. T. Raja Rajeswari,[3]Dr.N.Usha Rani

[1,3]Assistant Professor Dept. of Chemistry, PVP Siddhartha Institute of Technology,Kanuru, Vijayawada, India.

[2]Associate Professor, Dept. of Chemistry, Y.A. Govt. College for Women, Chirala,

Abstract

The aim of this study to evaluate the antioxidant activity, Anti-inflammatory activity, Anti-arthritic activity and screening the phytogenic chemical compounds present in the root extract of *Couroupitaguianensis*. Standard methods were used for preliminary phytochemical analysis.The total phenolic content, flavonoid and alkaloid content in the extracts were determined with the Folin-Ciocalteu's reagent (FCR).aluminium chloride ($AlCl_3$) and Dragendroff'smethodrespectively. Antioxidant activity was analyzed by using 2,2-diphenyl-1-picryl-hydrazyl-hydrate assay.α- amylase inhibition activity was conducted to determine the antidiabetic activity and Anti-inflammatory Activity was evaluated by Albumin denaturation Assay. Measurement of Antioxidant Activity using DPPH method, Antidiabetic activity by *in vitro* study α- amylase inhibition activity, anti-inflammatory Activity by Albumin denaturation Assay and In vitro anti-arthritic activity by inhibition of protein denaturation.The results obtained with screening studies were found that among three extracts more phytochemicals are reported in the methanolic extract. Quantitative analysis results reveal that Flavanoids have been found high in quantity (58.91mg/gram) than other to compounds.Dose dependent antioxidant activity was reported with three extracts and aqueous extract (93.98%) and methanol (90.11%) exhibit more activity than ethyl acetate extracts (59.59%). Anti diabetic activity with three extracts reported that aqueous extract (74.56%) exhibit more activity than ethyl acetate (58.69%) and methanol extracts (69.52%). Also aqueous extract (85.96%) presented potent anti inflammation activity than methanol extract (78.89%) and ethyl acetate found less activity (61.78%).As of now there is no report has been established with root part of the plant the present study is novel approach for screening, estimation of phytochemicals and evaluation of their antioxidant, ant diabetic and anti inflammatory activity with root extract of *Couroupitaguianensis.*

Keywords:*Couroupitaguianensis,* Alkaloids, flavanoids, phenolic compounds, antioxidant activity, ant diabetic activity, anti inflammation activity.

Introduction:

*Couroupitaguianensis*Aubl.belong to family Lecythidaceae and commonly known as cannon ball tree, locally known as „ "Kailashpati". It is distributed throughout India as ornamental tree and its native to Central and South America (Brazil, Colombia, French, Guyana, Peru and Venezuela). The plantgrows in the thick humid forests, often along the river belts and low altitude. It is a large deciduous tropical tree; 75' tall andcylindrical bole is 30 - 50cm in diameter [Al-Dhabi, N. A., et al. (2012),Senaratne, LB

(2007-08-05)]. It is indigenous to the Amazon rainforest, is listed as a rare tree and flower in India.In India, it is considered as a sacred tree by Hindus which is the reason why it is generally grown in Lord Shiva temple because of its special featured flowers which look like hood of Naga (snake) protecting the Shivalinga, hence, it is called as "NagalingaPushpa".The tree was mistakenly believed to be *Shorearobusta* and has been planted around many Buddhist temples in Sri Lanka and Southeast Asia [Prance, G. T. & S. A. Mori2013,Brown.2013].

Kailashpatiis widely used for its medicinal properties and also extensively research for its pharmacological properties. *Couroupitaguianensis* is planted as an ornamental for its showy, scented flowers, and as a botanical specimen for its interesting fruit [Prance, G. T. & S. A. Mori2013].The fruit is edible, but is not usually eaten by people because, in contrast to its intensely fragrant flowers, it can have an unpleasant smell [Lim, T. K.2012] It is fed to livestock such as pigs and domestic fowl [Brown.2013]. The leaves of this plant have been used in the treatment of skin infections including protozoan disease in human.The plant has many medicinal uses. Native Amazonians use extracts of several parts of the tree to treat hypertension, tumors, pain, and inflammation. It has been used to treat the common cold, stomachache, skin conditions and wounds, malaria, and toothache [Prance, G. T. & S. A. Mori2013].

Material and methods:

List of chemicals: The solvents used for root extraction are Methanol and Ethyl Acetate. The reagents used for phytochemical screening and estimation were of laboratory reagent grade and were purchased for Merck chemicals private limited, Mumbai, Fisher scientific, Mumbai and SD fine chemicals Mumbai. Distilled water has been used for aqueous extraction. Alpha amylase enzyme was purchased from Sigma Aldrich chemicals.

List of apparatus: Denver electronic balance, TECHCOMP – UV 2301 Double Bean UV Visible Spectrophotometer with HITACHI 2.2 software, Tech-comp UV visible spectrophotometer, soxhlet extraction apperatus, heating mantle, incubator, autoclave.

Sample collection: Root material of *Couroupitaguianensis*Aublplant was collected from farm in Vijayawada, Kirshna district, Andhra Pradesh, India. The roots are separated and allowed to shade dry. The root sample was grounded and powdered for solvent extraction.

Solvent extraction: The phytochemicals present the roots of the collected plants were isolated using different solvents like hexane, ethyl acetate, methanol and water in a series of extraction method from low polarity to high polarity using soxhlet extraction method.

Preliminary phytochemical screening [Dey PM and Harborne J B.1987**,** Evans WC.1989**]**

1. **Test for steroids**

Salkowski Test

Few drops of concentrated sulphuric acid are added to the plant extract, shaken and on standing; lower layer turns red in colour.

Liebermann Burchard's Test

To the extract, few drops of acetic anhydride was added and mixed well. 1 ml of concentrated sulphuric acid is added from the sides of test tube, a reddish brown ring is formed at the junction of two layers.

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2. Tests for triterpenoids

Salkowski Test

Few drops of concentrated sulphuric acid are added to the extract, shaken and on standing, lower part turns golden yellow colour.

Lieberman Burchard's Test

To the extract, few drops of acetic anhydride was added and mixed well. 1 ml of concentrated sulphuric acid is added from the sides of test tube, a red ring indicates triterpenes.

Ischugajiu Test

Excess of acetyl chloride and pinch of zinc chloride are added to the extract solution, kept aside for reaction to subside and warmed on water bath, cosin red colour is produced.

Brickorn and Brinar Test

To the extract, few drops of chlorosulfonic acid in glacial acetic acid (7:3) are added, red colour is produced.

3. Test for Saponins

Foam Test

Small amount of extract is shaken with little quantity of water; the foam produced persists for 10 minutes. It confirms the presence of saponins.

Haemolysis Test

To 2ml of 1.8% Sodium chloride solution in two test tubes, 2ml distilled water is added to one and 2ml of 1% extract to the other, 5 drops of blood is added to each tube and gently mixed with the contents. Haemolysis observed under the microscope in the tube containing the extract indicates the presence of saponins.

4. Test for Steroidal Saponin

The extract is hydrolysed with sulphuric acid and extracted with chloroform. The chloroform layer is tested for steroids.

5. Tests for TriterpenoidalSaponin

The extract is hydrolysed with sulphuric acid and extracted with chloroform. The chloroform layer is tested for triterpenoids.

6. Tests for Alkaloids

Mayer's Test

The acid layer when mixed with Mayer's reagent (Potassium mercuric iodide solution) gives creamy white precipitate.

Dragendroff's Test

The acid layer with a few drops of Dragendroff's reagent (Potassium bismuth iodide) gives reddish brown precipitate.

Wagner's Test

The acid layer when mixed with few drops of Wagner's reagent (solution of iodide in potassium iodide) gives brown to red precipitate.

Hager's Test

The acid layer when mixed with few drops of Hager's reagent (Saturated solution of pricric acid) gives yellow coloured precipitate.

7. Tests for Carbohydrates

Fehlings's Test

The extract when heated with Fehling's A and B solutions gives an orange red precipitate showing the presence of reducing sugar.

Molischs's Test

The extract is treated with Molisch's reagent and concentrated sulphuric acid along the sides of the test tube, a reddish violet ring shows the presence of carbohydrate.

Benedict's test

The extract on heating with Benedict's reagent, brown precipitate indicates the presence of sugar.

Barfoed's Test

Barfoed's reagent is added and boiled on water bath for few minutes; reddish precipitate is observed for the presence of carbohydrate.

8. Test for Flavonoids

Shinoda Test

The extract solution with a few fragments of magnesium ribbon and concentrated hydrochloric acid produced magenta colour after few minutes.

Ferric chloride test

Alcoholic solution of extract reacts with freshly prepared ferric chloride solution and given blackfish green color.

Lead Acetate Test

Alcoholic solution of extract reacts with 10% lead acetate solution and given yellow precipitate.

9. Test for Glycosides

Anthraquinone test

Drug is powdered and extracted with either ammonia or caustic soda. The aqueous layer shows pink color

Keller-killiani test

This is for cardiac glycosides. The extract and 0.4 glacial acetic acid are mixed with ferrous chloride and 0.5 mi of concentrated sulphuric acid. The acetic acid layer shows blue color.

10. Test for Phenolic Compounds

Ferric chloride test

Treat the extract with ferric chloride solution then blue color appears if hydrolysable tannins are present and green color appears if condensed tannins are present.

Gelatin test

To the test solution add 1% gelatin solution containing 10% NaCl, and then precipitate is formed.

Test for chlorogenic acid

Treat the test solution with aqueous ammonia and expose to air gradually, green color is developed.

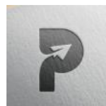
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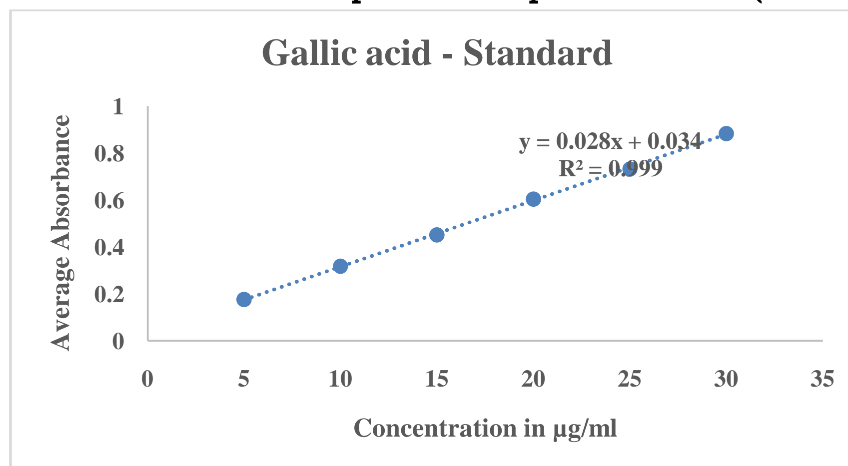
Table 1: Results of Phytochemical screening

S.No	Compound	Ethyl acetate extract	Methanol extract	Aqueous extract
1.	Steroids	Positive	Positive	Negative
2.	Triterpenoids	Negative	Negative	Negative
3.	Saponins	Negative	Positive	Positive
4.	Steroidal Saponin	Positive	Negative	Negative
5.	TriterpenoidalSaponin	Positive	Positive	Negative
6.	Alkaloids	Negative	Positive	Positive
7.	Carbohydrates	Positive	Positive	Negative
8.	Flavonoids	Negative	Positive	Positive
9.	Glycosides	Negative	Negative	Negative
10.	Phenolic Compounds	Positive	Positive	
11.	Tannins	Positive	Positive	

Quantitative analysis of Phenoilc Compounds: [Evans WC. Trease.1996, Parekh J and Chanda SV.2007**]**

The total phenolic content in different solvent extracts was determined with the Folin-Ciocalteu's reagent (FCR). In the procedure, 1ml of extract was mixed with 0.4 ml FCR (diluted 1:10 v/v). After 5 min 4 ml of sodium carbonate solution was added. The final volume of the tubes were made up to 10 ml with distilled water and allowed to stand for 90 min at room temperature. Absorbance of sample was measured against the blank at 765nm using a spectrophotometer. A calibration curve was constructed using gallic acid solutions as standard and total phenolic content of the extract was expressed in terms of milligrams of gallic acid per gram of dry weigh.

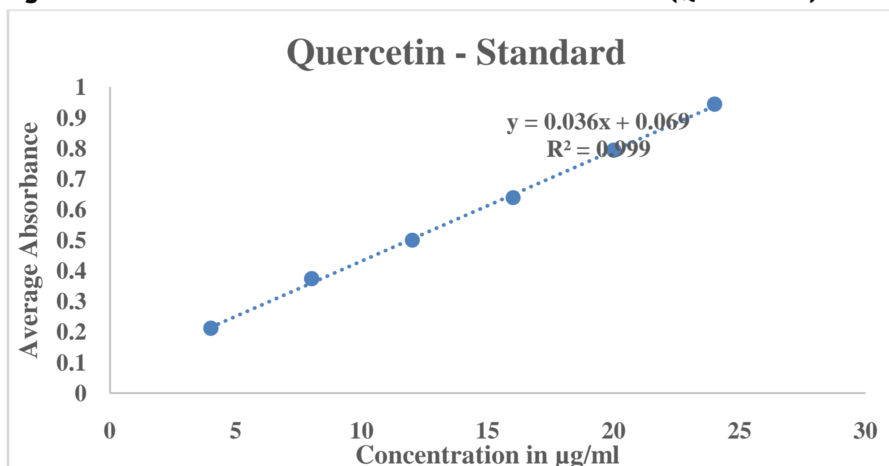
Figure A: Calibration curve of phenolic compounds standard (Gallic acid)



Determination of total flavonoid content: [Sastri BN.1962 ,Schanderl, S H (1970)]

Total flavonoid content was determined using aluminium chloride ($AlCl_3$) according to a known method using quercetin as a standard. The plant extract (1 ml) was added to 3 ml distilled water followed by 5% NaNO2 (0. 3ml). After 5 min at 25°C, AlCl3 (0.3 ml, 10%) was added. After further 5 min, the reaction mixture was treated with 2.0 ml of 1 mMNaOH. Finally, the reaction mixture was diluted to 10ml with water and the absorbance was measured at 510 nm. A calibration curve was constructed using quercetin solutions as standard and total phenolic content of the extract was expressed in terms of milligrams of quercetin per gram of dry weigh.

Figure B: Calibration curve of Flavanoids standard (Quercetin)



Determination of total Alkaloid content: [Trease GE,2002]

A part of extract residue was dissolved in 2N HCL and then filtered. 1 ml of this solution was transferred to separatory funnel and washed with 10 ml chloroform (3 times). The pH of this solution was adjusted to neutral with 0.1 N NaOH. Then 5 ml of BCG solution and 5 ml of phosphate buffer were added to this solution. The mixture was shaken and complex extracted with 1, 2, 3 and 4 ml chloroform by vigorous shaking, the extract was then collected in a 10 ml volumetric flask and diluted with chloroform.Atropine standard solutions were used for preparation of standard calibration curve. The absorbance of the complex in chloroform was measured at spectrum of 470 nm in UV-Spectrophotometer against the blank prepared as above but without Atropine.

Figure C: Calibration curve of Alkaloids standard (Atropine)

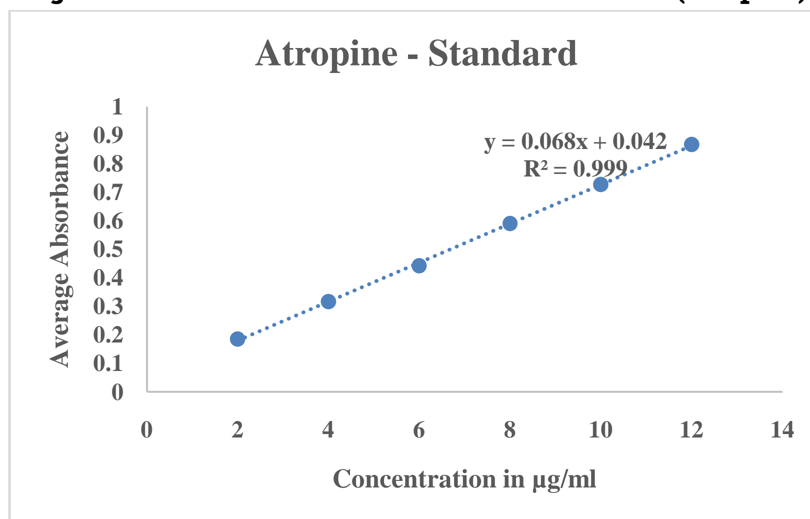


Table 2: Quantitative analysis of Phenolic compounds, Alkaloids and flavanoids in *Couroupitaguianensis*root extracts

Solvent extract	Phenolic compounds	Flavanoids	Alkaloids
Ethyl acetate	14.8mg/gram	------	------
Methanol	39.4mg/gram	58.91mg/gram	9.46mg/gram
Aqueous	-------	26.7mg/gram	18.73mg/gram

Measurement of Antioxidant Activity using DPPH method: [K.K. Teraos, N. Shinamoto.1988**]**
The antioxidant activity of the different root extracts was determined on the basis of their scavenging activity of the stable 1, 1- diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. lml of each solution of different concentrations (1-500g/ml) of the extracts was added to 3 ml of 0.004% ethanolic DPPH free radical solution. After 30 minutes the absorbance of the preparations were taken at 517 nm by a UV spectrophotometer which was compared with the corresponding absorbance of standard ascorbic acid concentrations (1-500µg/ml).The method described by **Hatano et al** was used to measure the absorbance with some modifications. Then the % inhibition was calculated by the following equation:

$$\%\ \text{Radical Scavenging Activity} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

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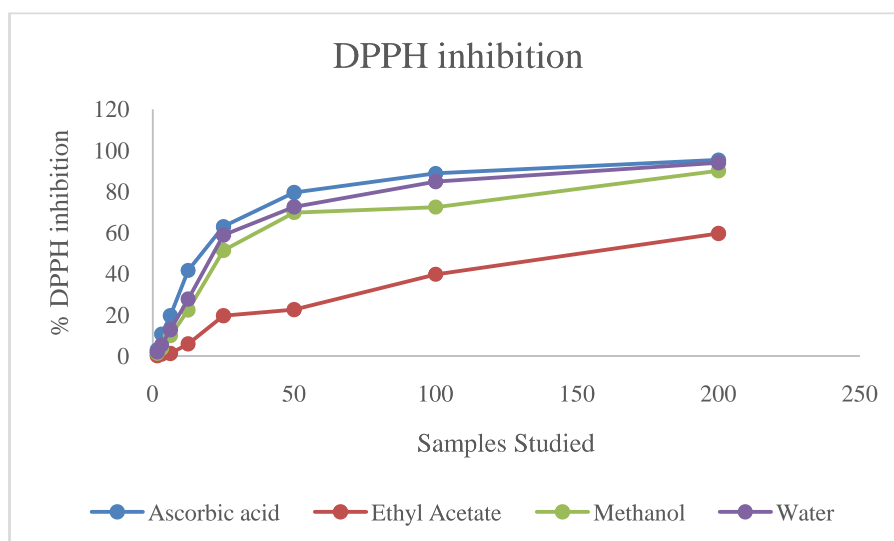
Table 3: Results of Antioxidant Activity using DPPH method

Concentration in µg	Ascorbic acid	Ethyl Acetate	Methanol	Water
1.5625	2.91	0.13	1.28	1.95
3.125	10.63	0.91	3.63	5.32
6.25	19.58	1.28	9.85	12.69
12.5	41.66	5.91	22.36	27.63
25	62.91	19.63	51.32	58.81
50	79.52	22.58	69.79	72.44
100	88.79	39.73	72.41	84.73
200	95.31	59.59	90.11	93.98

Figure D: Comparative graph of Antioxidant Activity using DPPH method



Antidiabetic activity:

In vitro study α- amylase inhibition activity by Spectrophotometric method: [Ali H, Houghton PJ, Soumyanath A.2006**]**

1ml of alpha amylase and 1 ml of plant extract in a test tube and incubated at 37 °C for 10 min. After pre-incubation, 1ml of 1% (v/v) starch solution was added to each tube and incubated at 37◦C for 15min. The reaction was terminated with 2 mL DNSA reagent, placed in boiling water bath for 5min, cool to room temperature, diluted, and the absorbance measured at 546 nm. The control reaction representing 100% enzyme activity did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract controls were also including.

% inhibition of alpha amylase by each plant extract can be calculated using the formula:

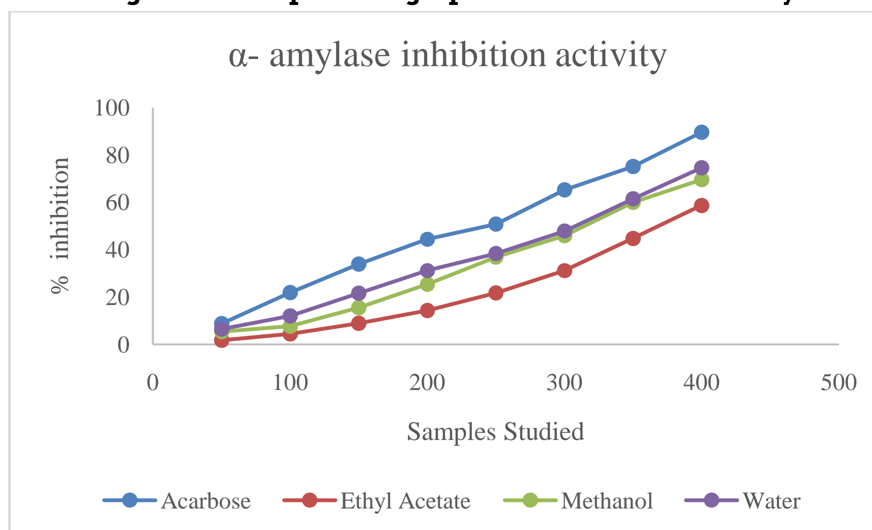
$$\% \text{ Indibition} = \frac{(\text{Enzyme activity of control} - \text{Enzyme activity of extract}}{\text{Enzyme activity of control}} \times 100$$

- Hansawasdi C, Kawabata J, Takanori K. α-amylase inhibitors from Roselle (*Hibiscus sabdariffa Linn.*) Tea. BiosciBiotechnolBiochem. 2000; 64:1041–3.

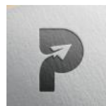
Table 4: Results of Anti-diabetic activity

Concentration in µg	Acarbose	Ethyl Acetate	Methanol	Water
50	8.69018	1.637279597	5.2896725	6.423173804
100	21.7884	4.282115869	7.5566751	11.96473552
150	33.8791	8.816120907	15.491184	21.53652393
200	44.3325	14.23173804	25.314861	31.10831234
250	50.7557	21.66246851	36.775819	38.28715365
300	65.2393	31.10831234	45.843829	47.85894207
350	75.063	44.71032746	59.949622	61.46095718

Figure E: Comparative graph of Anti-diabetic activity



Anti-inflammatory Activity by Albumin denaturation Assay: [Mizushima Y, Kobayashi M.,1968**]**
A solution of 0.2% W/V of BSA was prepared in Tris buffer saline and PH was adjusted to 6.8 using glacial acetic acid. Stock solutions plant extract were prepared by using methanol as a solvent. From these stock solutions 6 different concentrations of 10-500µg/ml were prepared by using methanol as a solvent. 50µl of each extract was transferred to Eppendorf tubes using 1ml micro pipette. 5ml of 0.2% W/V BSA was added to all the above Eppendorf tubes. The control consists of 5ml 0.2% W/V BSA solution with 50 µl methanol. The test tubes were heated at 72° C for 5 minutes and then cooled for 10 minutes. The absorbance of these solutions was determined by using UV/Vis Double beam spectrophotometer at a wavelength of 660nm. The % inhibition of precipitation (denaturation of the protein) was determined on a % basis relative to the control using the following formula.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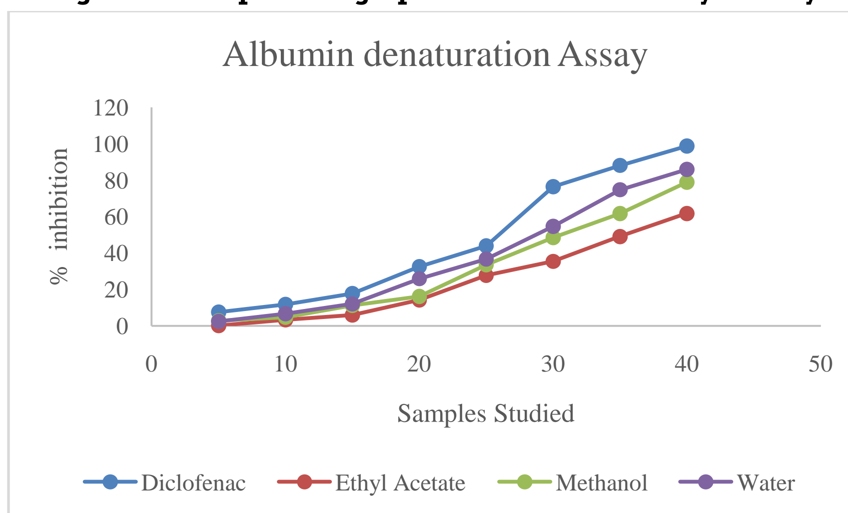
$$\% \text{ inhibition of denaturation} = \frac{(\text{Abs of control} - \text{Abs of extract})}{\text{Abs of control}} \times 100$$

Table 4: Results of Anti-inflammatory activity

Concentration in µg	Diclofenac	Ethyl Acetate	Methanol	Water
5	7.58197	0.204918033	2.8688525	2.459016393
10	11.7828	3.381147541	4.8155738	6.659836066
15	17.8279	5.942622951	11.270492	12.09016393
20	32.582	14.24180328	16.290984	25.92213115
25	43.9549	27.76639344	33.606557	36.78278689
30	76.332	35.45081967	48.463115	54.61065574
35	88.0123	49.07786885	61.680328	74.79508197
40	98.668	61.78278689	78.893443	85.96311475

Figure F: Comparative graph of Anti-inflammatory activity



Result and discussion:

Plants used for the treatment of diseases is as old as mankind, medicinal plants are an important source of potentially useful structures for the development of new drugs. Bioactive components are naturally found everywhere in most dietary higher plants available to humans and livestock. The natural products such as plant extracts provide unlimited opportunities for new drug discoveries, mostly because of plethora of varieties of phytochemicals.

The preliminary screening of phytochemicals with the root extracts was conducted to evaluate the phytochemicals present in the root part of the plant. Ethyle acetate, methanol and

aqueous extracts were studied for phytochemical compounds. Among three extracts more phytochemicals are reported in the methanolic extract where Steroids, Saponins, TriterpenoidalSaponin, Alkaloids, Carbohydrates, Flavonoids, Phenolic Compounds, Tannins have been identified. In ethanolicextract Steroids, Steroidal Saponin, TriterpenoidalSaponin, Carbohydrates, Phenolic Compounds, Tanninsave been identified. In aqueous extract only Saponins, Alkaloids, Flavonoids re reported. Results of qualitative analysis are presented in table 1.

Quantitativeanalysis of the extracts has been conducted inorder to estimate phenolic compounds,Alkaloids and flavanoids. Flavanoids have been found high in quantity (58.91mg/gram)than other to compounds analyzed. phenolic compounds were found14.8mg/gram extract in ethyle extract and 39.4mg/gram in methanolic extract. Whereas flavonoids are present in methanolic extract with high amount 58.91mg/gram and in aqueous extract it was found 26.7mg/gram. Amount of Alkaloids quantified was 9.46mg/gram extract and in aqueous extract it was found 18.73mg/gram extract. Hence the root of plant found rich in phytochemical compounds like flavanoids, phenolic compounds and Alkaloids which have high efficient pharmaceutical activity like antioxidant, anti diabetic and anti-inflammatory activities. Quantitative results are presented in table2 and figure A,B,C. The literature review proves that the *Couroupitaguianensis*plant is rich with many medicinal and bioactive compounds like flavanoids, phenolic compounds, glycosides, terpinoids etc. various studies are reported these compounds in various areal parts of the plant. There is no study reported with the root part of plant material. Hence the study was novel for phytochemical study in the *Couroupitaguianensis.*

The need for antioxidant agents, which can prevent oxidative stress, has become a major priority. Most plant-derived polyphenols exhibit strong antioxidant potentials. The antioxidant activities of these compoundshave been attributed to various mechanisms which is established by various assay procedures. The present study of antioxidant activity with three extracts has been performed and dose dependent antioxidant activity was reported. Among three extracts aqueous extract (93.98%) and methanol (90.11%) exhibit more activity than ethyl acetate extracts (59.59%).Results of antioxidant activity are presented in table 3 and figure D. Diabetes mellitus is a common metabolicdisorder which may eventually lead to multiple organ damage and syndromes [Rahimi R, Nikfar2005]. Management of the blood glucose level is a critical strategy in the control ofdiabetes and its complications.Among three extracts aqueous extract (74.56%) exhibit more activity than ethyl acetate (58.69%) and methanol extracts (69.52%) when In vitro study α- amylase inhibition activity analysis performed. Results of ant diabetic activity are presented in table 4 and figure E. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leucocytes from the body into the injured tissues. It is considered as a primary physiological defense mechanism that helps body to protect itself againstinfection, burn, toxic chemicals, allergens or other noxious stimuli. An uncontrolled and persistent inflammation may actas an etiologic factor for many of this chronic illness. Potent anti inflammation activity was found with aqueous extract (85.96%) when analysis by Albumin denaturation Assay. Similar activity was found with methanol extract (78.89%) and ethyl acetate found less activity (61.78%). Hence in the present study effort has been made to establish the scientificvalidity to the anti-inflammatory property of *Couroupitaguianensis*root extract using Albumin denaturation Assay.Results of anti

inflammation activityactivity are presented in table 5 and figure F. The present study of proving various biological activities are novel observations, where no reports have been presented with the root extracts of *Couroupitaguianensis.*

Literature review reveals that Regina et al (2012) , Y Reshma et al (2018) , Regina V et al (2012) , Shetesa et al (2013) , Naif Abdullah Al-Dhabi et al (2012) , reported various Preliminary Phytochemical analysis, antioxidant and antimicrobial studies of fruit of Couroupitaguianensis. C.stalin et al (2012) , Raghavendra et al (2017) ,Manimegalai S et al (2014) , Snehalimajumder et al (2014) studied the flower part of the Couroupitaguianensis and reported potent biological activities including antimicrobial activities. Praveen Kumar Uppala et al (2016) , Himanshu N et al (2018) , Mendhulkarv.d et al (2014) ,Reetikasingh et al (20145 were reported the biological activity and qualitative and quantitavie phytochemical results in leaf part of the plant. Similarly S. Manimegalai et al (2017) in stem and Shivashankar M et al (2013) in bark, D. Lavanya et al (2014) , Sirisha M et al (2018) ,C.Ramalakshmi et al (2013) ,MitalKaneria et al (2017) ere reported various screening results and biological activities in plant. Some of these observations have helped in identifying the active phytochemicals responsible for such therapeutic activities and in developing synthetic drugs for the treatment and management of a number of ailments in human beings. As of now there is no report has been established with root part of the plant the present study is novel approach for screening, estimation of phytochemicals and evaluation of their antioxidant, ant diabetic and anti inflammatory activity with root extract of*Couroupitaguianensis.*

Conclusion:

In the present investigation, primary and secondary metabolites of the root were qualitatively and quantitatively analyzed then the biological activity (anti oxidant, anti inflammatory, and anti diabetic) was studied. Methanol and aqueous extracts were found rich in bioactive compounds and the same was reported when biological activities were conducted. Further study will be carried out to isolate and characterize other anti-inflammatory chemical constituents present in the extract of this plant.

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