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A Research Article on-

"Phytochemical Analysis and Pharmacological Assessment of Medicinal Plant Extracts for Their Potential Antioxidant Activity"

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ABSTRACT:

The objective of the study was to investigate the Phytochemicals of Ethyl acetate and Methanol extracts of *Annona reticulata* L. stem bark.

The present study reports physicochemical characterization, antioxidant of stem bark extracts from *Annona reticulata* L. plant collected from local region of Nanded, Maharashtra, India, and authenticated by botanist. Different physical parameters like ash values, extractive value, Loss on drying, etc. were evaluated for powdered drug. The extracts were obtained from Soxhlet method by using Petroleum ether, Ethyl acetate and methanol as solvents for extraction and subjected for preliminary phytochemical evaluation and antioxidant studies.

Total phenolic and flavonoids content were also analyzed. The presence of primary and secondary metabolites such as carbohydrate, proteins, alkaloids, phenolic compounds, was confirmed through preliminary phytochemical analysis. DPPH free radical scavenging assays showed antioxidant activities with increase in concentration of Ethyl acetate and Methanol stem bark extracts.

Keywords: *Annona reticulata* L. Ethyl acetate and methanol extracts, Anti-oxidant activity.

I. INTRODUCTION:

Plants have been recognized as one of the sources of the medicinal products which are helpful to mankind. A different plant shows different pharmacological activities. Extracts obtained from various parts of plant posses' various medicinal properties. They are also used for cosmetic preparations as an herbal product shows lesser side effects as compared to the synthetic products. The repetitive or regular consumption of the synthetic drugs results in addiction or toxicity thus it is safer to use herbal medicines than the synthetic medicines.

Many developing countries use the plant-based products for their needs. Our country India has developed the traditional system known as Ayurvedic system which is based on herbal products. Ayurveda have developed uncountable herbal products which helps the people according to their needs with the safe environment. A medicinal plant typically contains mixture of different phytochemical which may improve quality of life.

Annona reticulata Linn. (Bullock's heart) is one of the traditionally important plants used for the treatment of various diseases. It belongs to family Annonaceae. Numerous phytoconstituents have been identified from different parts of *A. reticulata*. Stem bark contains tannins, alkaloid and phenolic compounds.

Leaves contain wide range of chemicals like alkaloids, amino acids, carbohydrates, steroids, flavonoids, proteins, tannins, glycosides and phenolic. The root has been identified for the content of acetogenin, alkaloid, carbohydrates, proteins, flavonoids, tannins.

Traditionally the plant has been employed for the treatment of epilepsy, dysentery, cardiac problem, parasite and worm infestations, constipation, hemorrhage, bacterial infection, dysuria, fever, ulcer and as insecticide. Bark is a powerful astringent and used as a tonic whereas leaves used for helminthiasis treatment. *Annona reticulata* Linn has been used to the treatment of pain and inflammation. Also, it reduces hyperthyroidism, and hyperglycaemia.

a. Morphology:

Rampal is a small tree with a rounded crown and trunk of 25-35 cm thick its height ranges from 5-10 meter the leaves are alternate oblong and lanceolate, 10-20 cm long and 2-5cm wide. Flowers are fragrant collateral with narrow petals of 2-3cm long the flowers never fully open. The fruits are nearly heart shaped 8-16cm wide. The skin of fruits may be yellow or brownish when ripe with brownish red blush there is thick, cream- white layer of custard like flesh under the skin with moderately juicy segments. Actual seed counts 55, 60 and 76. A pointed central core is attached to the thick stem of the fruit.

b. Geographical distribution:

It is specially found in tropical regions of India. It is also found in west indies and central and south America, Taiwan, Bangladesh, Australia and West Africa.

Image No.1: *Annona reticulata* L. Plant

Table 1 Scientific classification

Scientific classification	
Kingdom	Plantae
Order	Magnoliids
Family	Annonaceae
Genus	Annona
Species	*Annona reticulata*

Table 2 Botanical, Common Names

Botanical, common and vernacular names	
Botanical name	*Annona reticulata* Linn.
Common name	Netted Custard apple
English	Bullock's heart,
Corazon Portuguese	Frutoda-Condessa
Indonesian	Buah nona
India	Ramphal

c. Medicinal uses:

- The plant has been using to treatment of various diseases like epilepsy, parasite, cardiac problem, worm infection, hemorrhage, dysuria, fever, ulcer.
- The **barks** of this plant have astringent property that's why this plant used as a tonic.
- The **leaves** of *Annona reticulata* L. used for the helminthiases. also, the plant has been used as an anti-inflammatory in wound healing, antianxiety, anti-stress, spasmolytic effects.
- Leaf and stem extracts show positive inotropic, and spasmolytic effect.
- The **fruit** is used as an ant dysenteric and anthelmintic. *Annona reticulata* Linn has been used to the treatment of pain and inflammation. Also, it reduces hyperthyroidism, and hyperglycaemia.

II. MATERIALS AND METHODS:

A. Collection, identification and authentication of plant material:

The fresh stem barks of ***Annona reticulata* L**. were collected from local region and were authenticated by **Dr. Shrirang S. Bodke**, Associate professor and Head of Botany and Horticulture, Yashwant Mahavidyalaya, Nanded. Authentication of plant ***Annona reticulata* L**. was done by specimen No. **H-2**. Collection, authentication, identification, processing and storage had been done according to standard procedure for the plant material.

B. Pharmacognostic Evaluation of Plant Material:

Colour	Dark brown
Taste	Slightly aromatic
Shape	Cylindrical
Odour	Characteristic

a. Microscopic characteristics:

Transverse section of stem bark of ***Annona reticulata* L**. The thin section of stem was cut with the sharp blade and stained with phloroglucinol and dil. HCl (1:1) and presence or absence of following characteristic was observed.

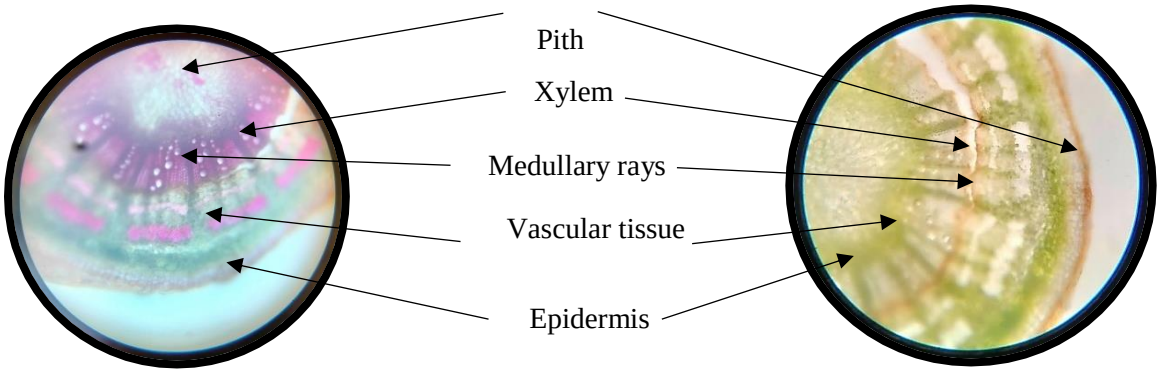


Image No.2: TS of plant Stain with Phloroglucinol + HCL

Image No.3: TS of plant Without stain

b. Powdered characteristics of stem bark

Some amount of powdered drug stained with phloroglucinol and HCL (1:1) it gives pink color to fibers which was observed under microscope with 40X lens.

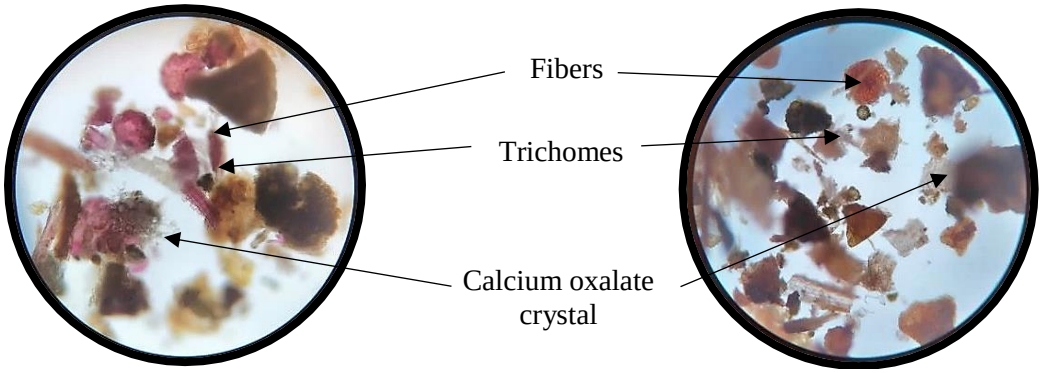


Image No.: Powdered characteristics Stain with Phloroglucinol + HCL

Image No.5: Powdered characteristics without stain

c. Processing of crude drug:

The fresh stem barks of plant ***Annona reticulata*** **L.** were subjected to shade drying and further crushed to coarse powder, and then the powder passed through mesh No. 14 and stored in air tight container for further use.

Image No.6: Processing of crude drug

B. Physicochemical Evaluation and Phytochemical Screening:

i. Total Ash value

Porcelain dish or a tarred silica crucible ignited and weighed. About 2 g of powder was weighed into dish/crucible. the dish Support on pipe-clay triangle placed on ring of stand. Heated using burner, with flame about 2 cm high and supporting the dish about 7 cm above the flame, heated till vapours almost crease was evolved, after that lowered the dish and heated more strongly until all the carbon is burnt off cooled in to desiccator. Ash was weighed and the total ash value was calculated the with reference to the air-dried sample of the crude drug **(Khandelwal k. 2002).**

ii. Acid Insoluble Ash

Proceeded as per the steps was mentioned in the procedure for determination of total ash value of a crude drug. After that using 25 ml of dilute hydrochloric acid, ash was washed from the dish used for total ash into 100 ml beaker. The mere gauze was placed was placed over a Bunsen burner and boiled for 5 min. It is filtered with an 'ash less' filter paper, and residue was washed twice. Crucible ignited on in the flame, cooled and weighed. The filter-paper and residue were put together into crucible, heated gently until vapours cease was evolved and then more strongly until all carbon has been removed. Cooled in to desiccator the residue was weighed and acid–insoluble ash calculated with the crude drug with reference to the air-dried sample of the crude drug. **(Khandelwal K. 2002)**

iii. Water Soluble Ash

Proceded as per the steps was mentioned in the procedure for determination of total ash value of a crude drug. After that using 25 ml of water, ash was washed from the dish used for total ash into 100 ml beaker. The mere gauze was placed over a Bunsen burner and boiled for 5 min. It is filtered with an 'ash less' filter paper, and residue was washed twice. Crucible ignited on in the flame, cooled and weighed. The filter-paper and residue were put together into crucible, heated gently until vapours cease was evolved and then more strongly until all carbon has been removed. Cooled in to desiccator. The residue was weighed and water-soluble ash calculated with the crude drug with reference to the air-dried sample of the crude drug. **(Khandelwal K. 2002)**

Image No.7: Burning of ash

iv. Determination of Loss on Drying (LOD)

Weighed about 2 g of the powdered drug into a flat and thin porcelain dish. Dried in the oven at 105◦C, until two consecutive weighing do not differ by more than 0.5 mg. Cooled in desiccator and weighed. The loss in weight is usually recorded as moisture. **(Khandelwal K. 2002)**

v. Extractive Value

4 gm of air-dried drug was macerated with 100 ml of solvents like pet ether, chloroform, acetone, methanol etc. and the specified strength in a closed flask for 24 hours. It was frequently Shaked during the first 6 hours and allowed to stand for 24 hours. Thereafter, it was filtered rapidly without loss of solvents, and dried in petri dish, and weighed. The percentage of extractive value was calculated **(Khandelwal K. 2002).**

Image No.08: Extractive value stand for 24 hr. & Evaporation

C. Extraction of Plant Material:

i. Selection of Solvent

On the basis extractive value and nature of phytochemical present in drug and literature review solvents were selected for the extraction of the Stem bark of *Annona reticulata* L. like Petroleum ether, Ethyl acetate & Methanol.

ii. Selection of Extraction Method:

Study of literature survey revealed that most of the chemical constituents of the plant extract are heat stable and most of the researchers selected continuous hot extraction method for plant extraction, **Soxhlet extractor** is very essential with less time usage and with high efficiency, solvent penetrates faster to the plant and it is most convenient method. on this basis the **Soxhlet extraction method** was selected for extraction of stem bark powder of *Annona reticulata* L.

Image No.9: Soxhlet Extraction Ethyl acetate and Methanol Extraction

Preparation of extracts

Three extracts of Stem bark of *Annona reticulata* L. was prepared

- **i.** Petroleum ether extracted by continuous hot extraction method
- **ii.** Ethyl acetate extracted by continuous hot extraction method
- **iii.** Methanol extracted by continuous hot extraction method

The extract obtained and the dried mass was weighed and recorded. The percentage of yield was calculated.

$$\text{(\%) yield} = \frac{\text{Wt. of extract}}{\text{Wt. of powdered drug}} \times 100$$

- **Preparation of Petroleum ether extract**

Dried powdered plant was successfully extracted with petroleum ether by Soxhlet extractor apparatus according to the standard method till colorless solution was observed in siphon tube. 270 gm of the powdered plant and 1500 ml petroleum ether was used for extraction. After completion of extraction extract was cooled & evaporated by using Superfit Rotary evaporator. The extract was stored in air tight container. % yield of extract was calculated.

- **Preparation of Ethyl acetate extract**

Dried powdered plant was successfully extracted with Ethyl acetate by Soxhlet extractor apparatus according to the standard method till colorless solution was observed in siphon tube. 260 gm of the powdered plant and 1500 ml Ethyl acetate was used for extraction. After completion of extraction extract was cooled & evaporated by using Superfit Rotary evaporator. The extract was stored in air tight container. % yield of extract was calculated.

- **Preparation of Methanol extract**

Dried powdered plant was successfully extracted with methanol by Soxhlet extractor apparatus according to the standard method till colorless solution was observed in siphon tube. 250 gm of the powdered plant and 1500 ml methanol was used for extraction. After completion of extraction extract was cooled & evaporated by using Superfit Rotary evaporator. The extract was stored in air tight container. % yield of extract was calculated.

D. Phytochemical Qualitative Analysis of Extract:

i. Chemical tests

The extracts obtained by successive extraction were subjected to qualitative tests for the identification of various secondary metabolites such as carbohydrates, proteins, tannins, steroids, flavonoids, alkaloids and glycosides. Phytochemical examinations were carried out for all the extracts as per standard methods.

- **Test for carbohydrate:**

a) Molisch test:

The aqueous extract treated with few drops of alpha-naphthol solution in alcohol, shake and add conc. H_2SO_4 from sides of the test tube. Violet ring is formed at the junction of two liquids.

b) Fehling's test:

1 ml Fehling's A and 1 ml Fehling's B solution was mixed, boiled for one minute. Equal volume of test solution added. Heated on boiling water bath for 5-10 min. First yellow then brick red ppt is observed.

c) Benedict's test:

Mix equal volume benedict's reagent and test solution in test tube. Heat in boiling water bath for 5min solution appears green, yellow or red depending on amount of reducing sugar present in the test solution.

- **Test for proteins:**

a) Biuret test:

Mix 3 ml T.S. Add 4 % NaOH and few drops of 1% $cuso_4$ solution. Violet or pink colour appears.

b) Million's test:

Mix 3 ml T.S with 5 ml Million's reagent. White ppt. turns brick red or the ppt. dissolves giving red coloured solution.

c) Test for Protein containing sulphur:

Mix 5 ml T.S. with 2ml 40% NaOH and 2 drops 10 % lead acetate solution. Boil mixture. Solution turns black or

brownish due to PbS formation.

- **Test for amino acids:**

a) Ninhydrin test (general test):
Heat 3 ml T.S. and 3 drops 5% Ninhydrin solution in boiling water bath for 10 min. purple or bluish colour appears.

- **Test for steroids:**

a) Salkowski reaction:
To 2ml of extract at 2 ml chloroform and 2 ml conc. H_2SO_4. Shake well chloroform layer appears red and acid layer shows greenish yellow fluorescence.
b) Liebermann-Burchard reaction:
Mix 2ml extract with chloroform add 1-2 ml acetic anhydride and 2 drops conc. H_2SO_4 from side of test tube. First red then blue finally green colour appears.

- **Test for glycosides:**

a) Legal's test:
To aqueous or alcoholic extract, add 1 ml pyridine and 1 ml sodium nitroprusside. Pink to red colour appears.
b) Keller-killiani:
To 2 ml extract, add glacial acetic acid, one drop 5% $FeCl_3$ and conc. H_2SO_4. Reddish brown colour appears at junction of the two liquid layers and upper layer appears bluish green.

- **Test for saponins:**

a) Foam test:
shake the drug extract or dry powder vigorously with water. Persistent stable foam observed.

- **Test for flavonoids:**

a) Shinoda test:
To dry powder or extract added 5 ml 95% methanol few drops conc. H_2SO_4 and 0.5 gm. magnesium turnings. Orange, pink, red to purple colour appears. Added t-butyl alcohol before adding the acid to avoid accidents from a Violent reaction and to dissolve coloured compounds in to the upper phase by using zinc instead of magnesium, only flavones give a deep red to magenta colour while flavanones and flavonols give weak pink to magnetic colour or no colour is observed.
b) Sulphuric acid test:
On addition of sulphuric acid (66 or 80%) flavones and flavonols dissolve into it and give a deep yellow solution. Chalcones and aurones give red, bluish solutions. Flavanes give orange to red colours.

- **Test for tannins and phenolic compounds**

To 2-3 ml of aqueous or alcoholic extract, add few drops of following reagents:
a) Lead acetate test:
bark extract was taken and 0.5 ml of 1% lead acetate solution was added and the formation of white ppt. observed.
b) 5% Ferric chloride solution:
Deep blue-black colour is observed**.**
c) Dilute iodine solution:
Transient red colour.
d) Bromine water:
Decolouration of bromine water**.**

- **Test for alkaloids:**

The small portion of solvent free extract/ fraction was stirred separately with a few drops of dilute HCL, filtrated and used to test the presence of alkaloids:
a) Mayer's test:
2-3 ml filtrate with few drops of Mayer's reagent gives ppt.
b) Hager's test:
2-3 ml filtrate with Hager's reagent gives yellow ppt. indicates presence of alkaloids.
c) Wagner's test: 2-3 ml filtrate with few drops of Wagner's reagent gives reddish brown ppt.

E. Thin Layer Chromatography:

- **Introduction**

Thin layer chromatography is a method of analysis in which the stationary phase, a finely divided solid, is spread as a thin layer on a rigid supporting plate and the mobile phase, a liquid, is allowed to migrate across the surface of the plate by capillary action by gravity or pressure. TLC separation takes place in the open layer with each component having the same total migration time but different migration distance. Numerous fixed sorbents have been used, including Silica gel, Cellulose, Polyamide, Alumina, Ion exchange and chemically bonded silica gel. Mobile phase consists of a single solvent or a mixture of solvents. The stationary phase of the TLC is prepared using various techniques such as pouring, dipping and spraying.
The prepared plates are allowed for setting (air drying). This is done to avoid cracks on the surface of adsorbent. After setting the plates are activated by keeping in an oven at 100 to 200°C for one hour. Activation of TLC plates is nothing but removing water/moisture and other substances from the surface of any absorbent, by heating at temperature around 1100°C so that adsorbent activity is retained. TLC studies were carried out using various extracts to confirm the presence of different Phytoconstituents in the extracts.

- **Analysis**

In TLC qualitative analysis of the unknown compound is done by comparing the Rf values. As solutes never travel the full length of the stationary phase in TLC all the Rf value depends on the amount of the stationary phase, the humidity, layer thickness, solvent quality, saturation of chamber, development distance, temperature, amount of substance added, and the presence of impurities.

$$Rf = \frac{\textbf{Distance Travelled by solute}}{\textbf{Distance travelled by Solvent}}$$

Rf = Retention factor

- **Development of TLC profile of the extracts**

All the extracts of selected plant material were subjected to TLC studies using various solvent systems to determine the presence of various Phytoconstituents. The Rf of observed compounds were noted for all extracts. The characteristic of the various chemical constituents in each extract under UV light and after derivatization with suitable reagents was recorded.
Preliminary phytochemical screening revealed the presence of flavonoids, Tannins, glycoside, amino acid. Compounds of varying polarity in the extracts well separated using various solvent systems on TLC. Rf value of the separated compounds were recorded. **(Cai, Li et.al 2014)**

F. Phytochemical Quantitative Analysis of Extracts:

i. Total Phenolic content

- **Principle:**

The Total phenolic content of the extract was determined by the Folin–Ciocalteu method. This test is based on oxidation of phenolic groups with phosphomolybdate and phosphotungstate. After oxidation a green-blue complex formed which measure at 666 nm.

- **Chemical required:**

Folin-Ciocalteu (1:10 diluted with water)
Sodium carbonate (7%)
Extract (50 µg/ml)

- **Procedure**

The Total phenolic content of the extract was determined by the Folin–Ciocalteu method. Extracts (50 µg/ml) were

made up to 3 ml with distilled water, mixed thoroughly with 0.5 ml of Folin-Ciocalteu reagent for 3 min, followed by the addition of 2 ml of 7% (w/v) Sodium Carbonate. Then final volume made up to 10 ml.
The mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 666 nm. The total phenolic content was calculated from the calibration curve of gallic acid (2, 4, 6, 8, 10 µg/ml) and by using equation obtained from standard calibration curve ($r^2 = 0.9919$) and the results were expressed as mg per gallic acid equivalent per gram dry weight. **(Subba, Bimala et.al 2016)**

ii. Total Flavonoid content

- **Principle:**

This method is based on the nitration of any aromatic ring bearing a catechol group with its 3-4 position unsubstituted or not sterically blocked. After addition of $AlCl_3$ a yellow solution of complex was formed which then turned immediately to red after addition of NaOH and the value of absorbance is measured at 510 nm.

- **Chemical required:**

Aluminum trichloride (10%)
Distilled water
Sodium hydroxide (1molar)
Sodium nitrate 5%
Extract solution (50 µg/mL)

- **Procedure**

The total flavonoid content of crude extract was determined by the aluminum chloride colorimetric method. 1ml extract solution (50 µg/mL) was mixed with 4ml distilled water and 0.3 ml of 5% $NaNO_2$ solution and 0.3 ml of 10% $AlCl_3$ solution was added after 5 min add 2 ml /1molar NaOH, and the mixture was allowed to stand for 6 min, and the final volume of the mixture was brought to 10 ml with distilled water.
The mixture was kept for 30 min, and absorbance was measured at 510 nm against blank (without standard). quercetin was used as standard. The total flavonoid content was calculated from a calibration curve, (quercetin 2,4,6,8,10 µg/ml) and by using equation obtained from standard calibration curve (r^2=0.9911) result was expressed as mg quercetin equivalent per gram dry weight. **(Subba, Bimala et.al 2016)**

G. Pharmacological Screening of Plant Extracts:

- ***In-vitro* studies**

In-vitro study of Stem bark of *Annona reticulata* L. is done in which, following study was carried out which includes,

i. *In-vitro* Anti-oxidant Activity
An antioxidant is a molecule capable to inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions.

A. DPPH (2, 2-dipheny-1-picrylhydrazyl) radical scavenging activity.

- **Principle**

DPPH assay method is based on the reduction of Methanolic solution of colored free radical DPPH by free radical scavenger. DPPH composed of stable free radical molecules. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 517 nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution. The activity is expressed as effective concentration EC_{50}.

- **Reagents Required**

DPPH (0.1 mM)
Methanol
Quercetin (standard)
Extract solution

- **Preparation of samples and standard solutions**

Accurately weighed 10 mg of Ethyl acetate and Ethanol extracts and the standard Quercetin dissolved in methanol. These solutions were serially diluted with methanol to obtain the lower dilutions.

- **Procedure:**

The ability of *Annona reticulata* L. stems bark to scavenge DPPH radicals was measured according to the method as previously described with some modifications. Initially, absorbance of DPPH solution (400mg in 10 ml methanol 0.1 mM) was read at decreasing wavelength 513 nm. Various concentrations of extracts 20,40,60,80,100 µg/ml) were mixed with 0.4 ml of 0.1 mM DPPH radical solution in methanol and made up to final volume of 10 ml with methanol. A similar solution without samples was applied as a control. The mixture was shaken vigorously and incubated in the dark for 1 hr. at room temperature. Quercetin was used as a standard. The absorbance was measured at decreasing wavelength 513 nm by using uv-visible spectrophotometer. The percent radical scavenging activity of tested samples was expressed by using following formula.

$$\% \text{ Scavenging Activity} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100$$

III OBSERVATIONS AND RESULTS:

A. Physicochemical Evaluation of Plant Material:

i. Total ash value:

Table 2 Total ash value of *Annona reticulata* L.

Parameters	(Z)Wt. of crucible+ ash(gm)	(X)Wt. of empty crucible(gm)	(Y)Wt. of drug (gm)	% of ash =Z-X÷Y×100
Total ash value	26.55	26.40	2	7.5%
Acid insoluble ash	44.06	44.05	2	0.5%
Water soluble ash	26.47	26.40	2	2%

ii. Determination of Loss on Drying (LOD):

Loss on drying can be calculated using following formula.

$$LOD = \frac{(W2 - W3)}{(W2 - W1)} \times 100$$

W1 = wt. of empty dish = 67.10
W2 = wt. of dish + drug = 68.69
W3 = wt. of drug after drying = 68.40

$$LOD = \frac{(68.69 - 68.40)}{(68.69 - 67.10)} \times 100$$

LOD = 18%

iii. Extractive value:

Table 3 Extractive value of *Annona reticulata* L.

Solvent	(a)Wt. of petri dish with lead(gm)	(b)Wt. of petri dish with extraction (gm)	Wt. of extract (gm)	Percent of extract(%)=b-a÷2×100
Pet ether	87.29	87.55	0.26	6.5
Ethyl acetate	**81.68**	**81.89**	**0.21**	**10.5**
Chloroform	92.28	92.37	0.09	2.25
Acetone	92.23	92.27	0.04	1
Methanol	**90.39**	**90.72**	**0.33**	**16.5**
Ethanol	88.11	88.37	0.26	13
Water	86.30	86.62	0.32	8

iv. Extraction of plant material:

Table 4 % of Yield of *Annona reticulata* L. Stem bark extracts

Sr. No.	Drug taken (g)	Solvent used (ml)	Consistency	Colour of extract	Yield (g)	(%) Yield
1.	**270**	**1500 (Pet Ether)**	**Sticky**	**Dark Green**	**1.26**	**0.46**
2.	**260**	**1500 (Ethyl Acetate)**	**Sticky**	**Dark brown**	**6.62**	**2.54**
3.	**240**	**1500 (Methanol)**	**Sticky**	**Dark brown**	**7.56**	**3.15**

Image No.10: AR-ME &AR-EAE

B. Phytochemical screening of extracts:

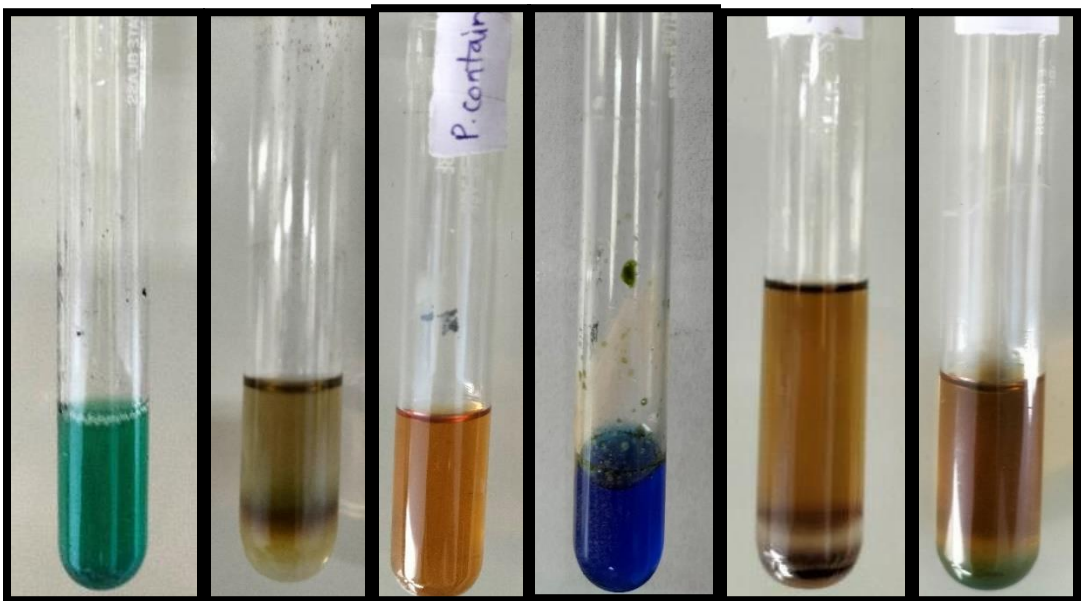
Table 5 Chemical tests of all three Stem bark extracts *Annona reticulata* L.

Chemical tests	Pet ether	Ethyl acetate	Methanol
Test for Carbohydrate			
Molisch test	-	+	+
Fehling's test	-	+	+
Benedict's test	-	+	-
Barford's test	-	-	-
Test for Proteins			
Biuret test	+	+	-
Millon's test	-	+	-
Test for protein S.	-	+	+
Test for Amino acid			
Ninhydrin test (general test)	-	-	-
Test Steroid			
Salkowski test	-	+	+
Liebermann Burchard reaction	-	-	-
Test for Glycosides			

Legal's test	-	+	-
Keller-killiani test	-	-	-
Test for Saponin Glycosides			
Foam test	-	+	+
Test for Flavonoids			
Shinoda test	-	+	+
Sulphuric acid test	-	+	+
Test for Tannins and phenolic compound			
Lead acetate test	-	+	+
5%Ferric chloride test	-	+	+
Dil. iodine solution	-	-	-
Bromine water	-	-	+
Test for Alkaloids			
Dragendroff's test	-	-	-
Wagner's test	-	+	+
Mayer's test	-	+	+
Hager's test	-	+	+

(+) Present, (-) Absent

Above observation table shows the presence of phytoconstituents in the *Annona reticulata* L. Stem bark extracts. It reveals that most of the chemical constituents present Ethyl acetate and Methanol extract. It contains carbohydrates, alkaloids, steroids, flavonoids, phenols, proteins, amino acids only contain in Ethyl acetate and Methanol extract not present in Petroleum ether extract.



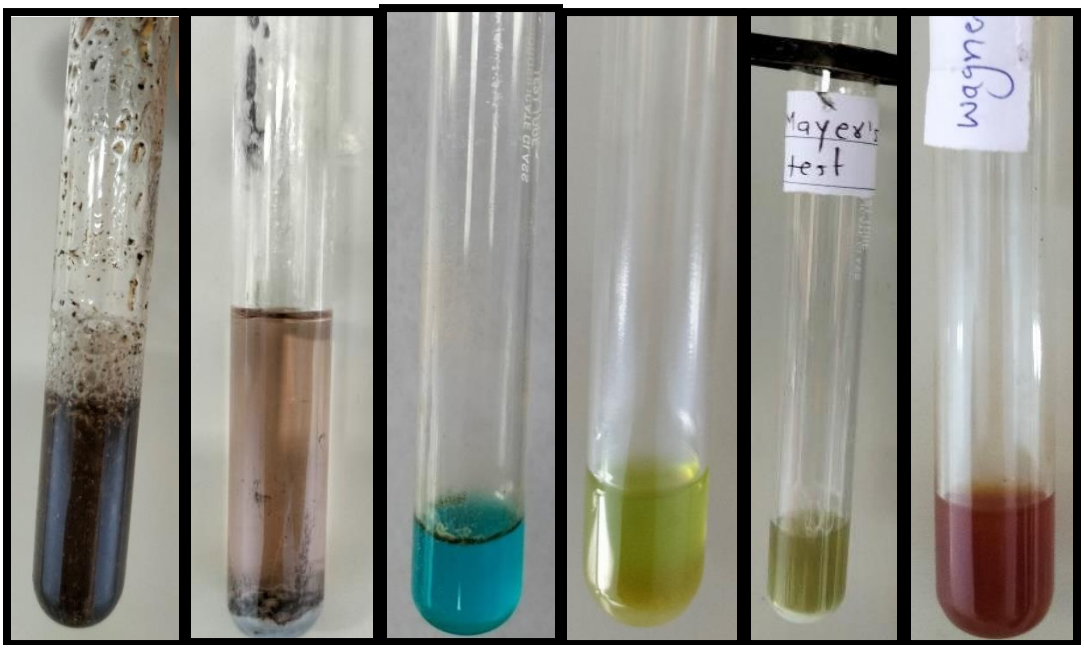


Image No.11: Chemical Tests of *Annona reticulata* L. stem bark extracts

Thin Layer Chromatography;

Thin Layer Chromatography of *Annona reticulata* L. stem bark extracts.

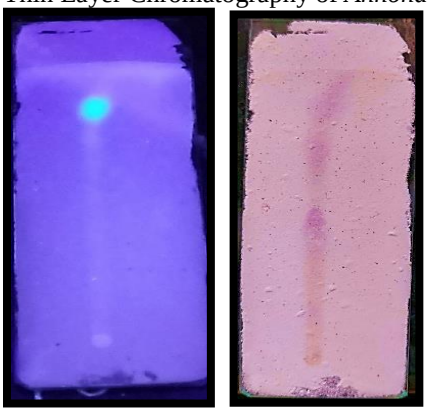


Image No.12: TLC of AR-PE

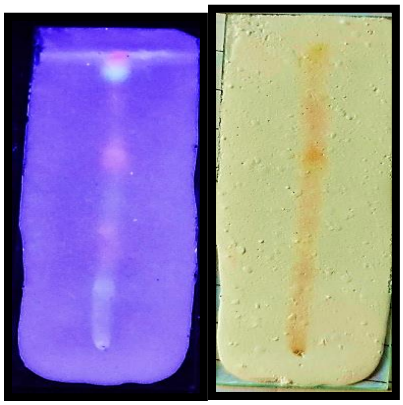


Image No.13: TLC of AR-ME

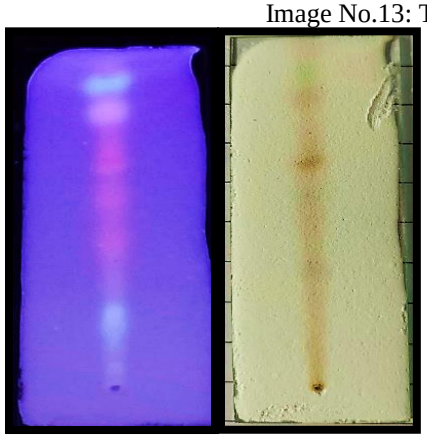


Image No.14: TLC of AR-EAE

Table 6 Rf values of All three extracts of *Annona reticulata* L.

Sr. No.	Extracts	Solvent System	Proportion	Spraying reagent	Rf Value	Colour	Chemical constituent
1.	AR-PE	Toluene: Ethyl Acetate: Methanol	8:1:1	10% H_2So_4	0.52	Purple	Triterpenoids
2.					0.77	Yellow	Flavonoids
3.					0.87	Purple	Triterpenoids
4.	AR-EAE	Chloroform: Ethyl acetate: Methanol	7:2:0.5		0.23	Light Brown	Flavonoids
5.					0.38	Brown	Alkaloid
6.					0.61	Pink	Diterpenes
7.					0.90	Faint Green	Chlorophyll
8.	AR-ME	n-hexane: Ethyl acetate: Methanol	5:4:0.5		0.29	Faint Brown	Alkaloid
9.					0.51	Brown	Flavonoids
10.					0.63	Faint pink	Diterpenes
11.					0.80	Brown	Alkaloid
12.					0.92	Green	Chlorophyll

C. Phytochemical Quantitative analysis of extracts:

i. Total Phenolic content:

Table 7 Absorbance of Gallic acid at different concentrations for determination of total phenolic content

Sr. No.	Conc. µg/ml	Absorbance of Gallic acid (at 666 nm)
1.	0	0.000
2.	2	0.101 ± 0.0008
3.	4	0.141 ± 0.0008
4.	6	0.218 ± 0.0008
5.	8	0.307 ± 0.0008
6.	10	0.372 ± 0.0008

Each value represents the mean ± SEM (n-3), Gallic acid as a standard.

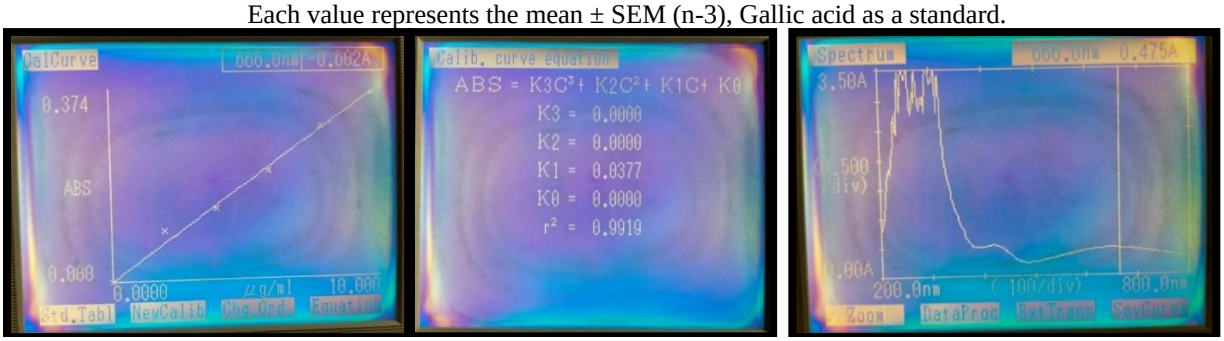


Image No.15: Calibration curve & calibration equation of Gallic acid

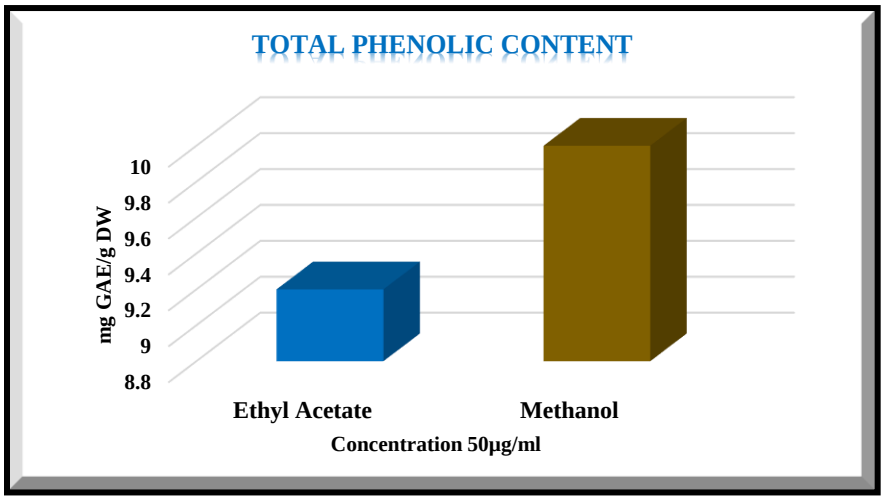
Table 8 Total phenolic content of AR stem bark extracts

Sr. No.	Extract	Conc. µg/ml	Absorbance	TPC (mg/GAE/g DW)
1.	Ethyl Acetate	50	0.175±0.002	**9.2**
2.	Methanol	50	0.203±0.003	**10**

Each value represents the mean ± SEM (n-3), Gallic acid as a standard.

Above observation table reveals that **Ethyl acetate** and **methanol** extract of *Annona reticulata* stem bark

have Phenolic content as 9.2(mg GAE/g DW), 10 (mg GAE/g DW) respectively. Methanol extract shows more phenolic content than Ethyl acetate as per comparative evaluation of phenolic content of extracts.
Note: mg/GAE/g DW denotes mg per Gallic Acid Equivalent per gram dry weight.



Graph No.1: TPC of *Annona reticulata* L.

ii. Total Flavonoid Content

Table 9: Absorbance of Quercetin at different concentrations for determination of total Flavonoid content

Sr. No.	Conc. µg/ml	Absorbance of Quercetin (at 510 nm)
1.	0	0.000
2.	2	0.021 ± 0.0008
3.	4	0.033 ± 0.0008
4.	6	0.048 ± 0.0008
5.	8	0.072 ± 0.0008
6.	10	0.090 ± 0.0005

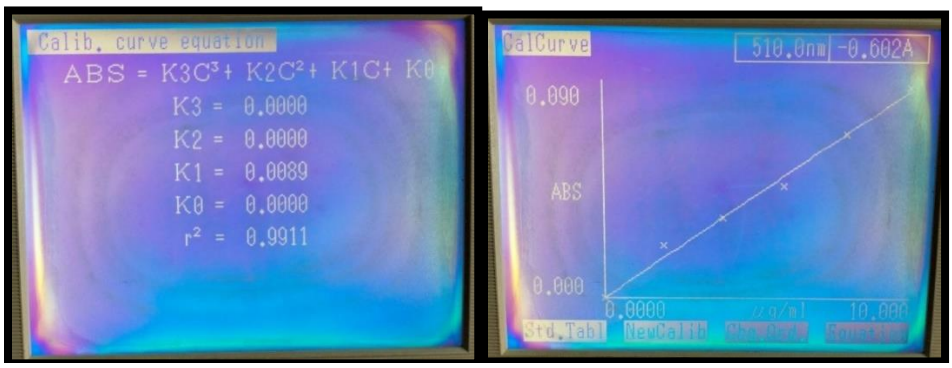
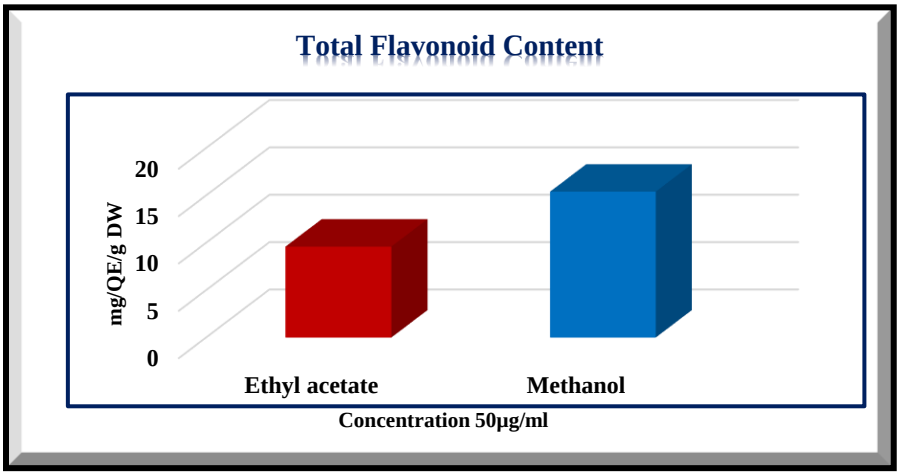


Image No.16: Calibration curve & calibration equation of Quercetin

Table 10 Total flavonoid content of AR Stem Bark extracts

Sr. No.	Extract	Conc. µg/ml	Absorbance	TFC (mg/QE/g DW)
1.	Ethyl acetate	50	0.031±0.0029	9.6
2.	Methanol	50	0.071±0.0017	15.4

Above observation table reveals that **Ethyl acetate** and **methanol** have Flavonoid content as 9.6 (mg/QE/g DW), 15.4 (mg/QE/g DW) respectively. Ethyl acetate extract shows more Flavonoid content than methanol as per comparative evaluation of flavonoid content of extracts Note: mg/QE/g DW denotes mg per Quercetin Equivalent per gram dry weight.



Graph No.2: TFC of *Annona reticulata* L.

D. Pharmacological screening of Plant extracts for:

➢ ***In-vitro* Antioxidant activity:**

Table 11 Absorbance of Gallic acid, Rutin, Ascorbic acid

Sr.no.	Conc (µg/ml)	Absorbance		
		Gallic acid	**Rutin**	Ascorbic acid
1	20	0.423 ± 0.002	0.251 ± 0.002	**0.484 ± 0.02**
2	40	0.380 ± 0.001	0.218 ± 0.001	**0.375 ± 0.001**
3	60	0.316 ± 0.002	0.193 ± 0.001	**0.282 ± 0.001**
4	80	0.287 ± 0.001	0.163 ± 0.002	**0.194 ± 0.001**
5	**100**	**0.195 ± 0.002**	**0.150 ± 0.001.**	**0.155 ± 0.002**

The Antioxidant activity of *Annona reticulata* L. was determined by *In-vitro* method DPPH free radical scavenging assay. The result was compared with Gallic acid, Ascorbic acid and rutin as a reference standard.

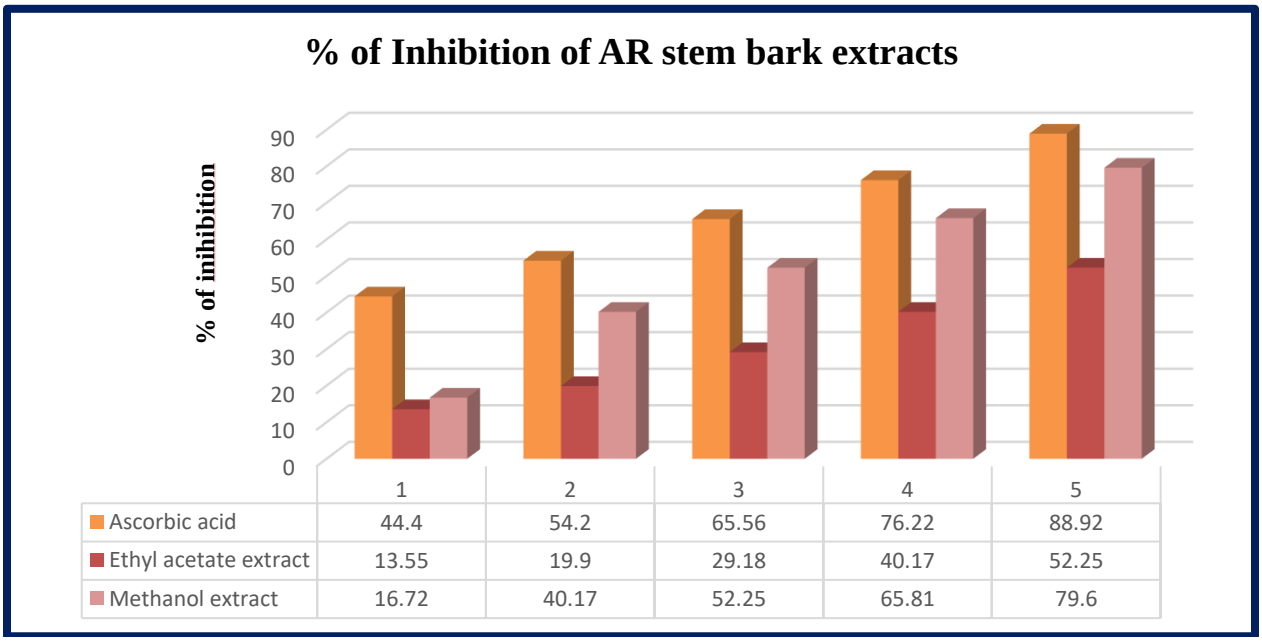
Table 12: % of inhibition Gallic acid, Rutin, Ascorbic acid

Sr.no.	Conc.µg/ml	% inhibition		
		Gallic acid	**rutin**	Ascorbic acid
1	20	48.30 ± 0.36	69.26 ± 0.28	**44.4 ± 0.10**
2	40	53.59 ± 0.21	73.33 ± 0.17	**54.20 ± 0.18**
3	60	61.37 ± 0.28	76.43 ± 0.18	**65.56 ± 0.18**
4	80	64.95 ± 0.18	80.09 ± 0.25	**76.22 ± 0.17**
5	**100**	**76.14 ± 0.24**	**81.68 ± 0.13**	**88.92 ± 0.10**

The standard gallic acid, rutin and Ascorbic acid shows 48.30 %, 69.26% and 44.4 % inhibition (per cent scavenging activity) at 20µg/ml conc. At 100 µg/ml concentration shows 76.14%, 81.68 % and 88.92% inhibition, respectively. The observation reveals that Ascorbic acid shows better antioxidant activity from concentration 20µg/ml to 100µg/ml in concentration dependent manner i.e., as conc. increases % inhibition of free radicals also increases.

Table 13 Absorbance of AR stem bark extracts for determination of Antioxidant activity

Sr. No.	Conc. (µg/ml)	Absorbance		%inhibition	
		Ethyl acetate extract	Methanol extract	Ethyl acetate extract	Methanol extract
1.	20	0.708±0.022	0.682±0.001	13.55	16.72
2.	40	0.656±0.0031	0.491±0.003	19.90	40.17
3.	60	0.580±0.004	0.391±0.003	29.18	52.25
4.	80	0.490±0.006	0.280±0.005	40.17	65.81
5.	100	0.391±0.007	0.167±0.008	52.25	79.60



Graph No.3: % of inhibition of AR stem bark extracts

In DPPH Scavenging assay method, the % inhibition of Ethyl acetate and methanolic stem bark extracts of *Annona reticulata* L. at 513 nm has been recorded at different concentration of 20, 40, 60, 80 and 100 µg/ml respectively. The results were compared with Ascorbic acid as a reference standard and both extracts showed very significant % inhibition close to reference standard. Among both extracts, Methanolic extract of *Annona reticulata* L. shows highest % inhibition activity (79.60% at 100µg/ml concentration).

IV. SUMMARY AND CONCLUSION:

The present study successfully demonstrated that the ethyl acetate and methanol extracts of *Annona reticulata* L. stem bark contain significant phytochemicals, including phenolics, flavonoids, alkaloids, carbohydrates, and proteins. Physicochemical parameters such as ash value, extractive value, and loss on drying confirmed the quality and purity of the plant material. The Soxhlet extraction method yielded extracts rich in bioactive compounds, with preliminary phytochemical screening confirming the presence of primary and secondary metabolites. Notably, both extracts exhibited concentration-dependent antioxidant activity in the DPPH assay, with methanol extract showing slightly higher potency. These findings suggest that *Annona reticulata* stem bark, particularly its methanol and ethyl acetate extracts, can serve as a potential natural source of antioxidants and bioactive phytochemicals for future pharmacological applications.

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