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# In vitro anti-urolithiaticactivity and in vitro antioxidant activity of ethanolic leaf extract of *Justicia adhatoda* (L.)

Sayoojya R [1], Jayachandran T P [2], Dr. Jyoti Harindran [3], Shruthy P [4], Aparna P V [5], Arathy Sreenivasan [6], Binny Binoy [7], Deepthi Krishna M [8], Sanusha T V [9].

*[1] Final year M. Pharm student, [2] Associate professor, [3] Principal Department of Pharmaceutical Sciences, [4] Final year M. Pharm student, [5] Final year M. Pharm student, [6] Final year M. Pharm student, [7] Final year M. Pharm student, [8] Final year M. Pharm student, [9] Final year M. Pharm student.*
*Department of Pharmaceutical Sciences, Department of Pharmaceutical Sciences Rd, Cheruvandoor, Ettumanoor, Kerala -686631*

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## ABSTRACT

*Justicia adhatoda* (L.) is a medicinal plant traditionally valued for its bioactive properties. This study aimed to evaluate the phytochemical composition and in-vitro antioxidant and in-vitro anti-urolithiatic potential of its ethanolic leaf extract. Dried leaves (225 g) were subjected to Soxhlet extraction using 95% ethanol for 72 hours, and the extract was concentrated under reduced pressure. Preliminary phytochemical screening revealed the presence of alkaloids, carbohydrates, reducing sugars, amino acids, cardiac glycosides, phenol, flavonoids, tannins, saponins, proteins and steroids. The antioxidant potential was assessed using the DPPH (2,2-diphenyl-1 picrylhydrazyl) radical scavenging assay and lipid peroxidation assay. These findings indicate that the leaf extract possesses significant free radical scavenging activity, likely due to its phenolic and flavonoid content. The in-vitro anti-urolithiatic potential was assessed using Nucleation assay and Aggregation assay, which indicate that the plant has the potential anti-urolithiatic effect. While the study provides preliminary insights into its bioactive profile, antioxidant and anti-urolithiatic efficacy, and further anti-urolithiatic investigations, including in vivo models, are warranted to explore its therapeutic potential. The findings suggest the leaf extract has strong scavenging properties against free radicals and strong efficacy to inhibit stone formation.

**KEY WORDS:** *Justicia adhatoda* (L.), ethanolic extract, DPPH assay, free radical scavenging, lipid peroxidation assay, nucleation assay, aggregation assay.

## I. INTRODUCTION

Stone formation is one of the painful urological disorders that occurs in approximately 12% of the global population, and the recurrence rate in males is 70-81% and 47-60% in females. The prevalent urological disorder of the urinary system accounts for a significant degree of morbidity and mortality in the global population. The rate of occurrence is three times higher in men than in women. [1,2]

Urolithiasis, also known as stone formation or calculi in the urinary tract, is the third most common urinary disease. The occurrence of this disease in any part of the urinary tract is through events like supersaturation, nucleation, aggregation, and retention. [3]

*Justicia Adhatoda* (L.) is also known as the Malabar nut tree, Adhatoda, Vasa, and Vasaka. It is a notable member of the family Acanthaceae with a broad geographic distribution across varied climatic conditions. It is known for its extensive medicinal properties for treating respiratory disorders, tuberculosis, malaria, diabetes, etc. The leaves produce a diuretic action, reducing the swelling of the kidneys and leading to clear urination. India's rich and diverse medicinal plant resources have played a vital role in developing traditional medicinal systems such as Ayurveda and Siddha. [4,5,6]

The phytochemical analysis of the leaf extract of the Malabar nut tree (*Justicia adhatoda*(L.)) showed the presence of various components such as phenols, tannins, alkaloids, saponins, flavonoids, amino acids, and reducing sugars. It possesses various therapeutic activities.

*Justicia adhatoda* (L.) in an ethylene glycol-induced renal calculus in male Wistar rats. The ethylene glycol-induced model is well-known for imitating the pathophysiology of urolithiasis and

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is an effective approach for evaluating anti-urolithiatic drugs. Then the ethylene glycol produces urinary stones like CaOx stones. The current study is to assess the anti-urolithiatic activity of *Justicia adhatoda* (L.) ethanolic leaf extract in ethylene glycol-induced calculi in a male Wistar rat model.

**PLANT DESCRIPTION**

*Justicia adhatoda*(L.) belongs to the Acanthaceaefamily.

Vernacular name:Malabar nut, Adhatoda,Vasa, Vasaka, Adulsa, Adalodagam, etc. [6]

**Table 1:** Plant Profile

| **Taxonomical representation** | |
|---|---|
| Kingdom | Plantae |
| Subkingdom | Angiospermae |
| Phylum | Tracheophyta |
| Class | Magnoliopsida |
| Order | Scrophulariales |
| Family | Acanthaceae |
| Genus | *Justicia* |
| Species | *J. adhatoda* |

## II. MATERIALS AND METHODS

### 1. PLANT MATERIAL AND AUTHENTICATION

*Justicia adhatoda*(L.) leaves were collected from Puthuchira, Kollam, Kerala, India, during February 2026, and it was identified by Dr Krishnaraj MV, Assistant Professor, Department of Botany, Baselius College, Kottayam (Autonomous), Kerala, India, and they were dried under shade (1kg). After grinding, 225g of powder was obtained.

### 2. PREPARATION OF ETHANOLIC LEAF EXTRACT

The collected *Justicia adhatoda* (L.) leaves were carefully washed and left to air dry. A 95% ethanol solvent was used to extract 225g of the dried and powdered *Justicia adhatoda* (L.) leaves in a Soxhlet extractor. The extraction was carried out continuously for 72 hours. After extraction, the solvent was removed under reduced pressure at 40°C using a rotary evaporator to obtain the crude ethanolic extract. The concentrated extract was stored at 4°C until further analysis. [7]

### 3. PHYTOCHEMICAL SCREENING OF THE PLANT

The leaf extracts obtained from sequential ethanol solvent extraction were then analysed using various qualitative chemical tests. These tests aimed to identify several phytoconstituents, such asalkaloids, carbohydrates, reducing sugars, amino acids, cardiac glycosides, phenol, flavonoids, tannins, saponins, proteins and steroids, by applying standard methods.

**Detection of alkaloids:**

i. Dragendroff's/ Kraut's test: Few ml filtrate and 1-2 mL of Dragendroff's reagents. An orange-brown precipitate.

ii. Hager's test: A few mL of filtrate and 1-2 ml Hager's reagents. A creamy white precipitate.

iii. Mayer's/ Bertrand's/ Valser's test: A few mL of filtrate and 1-2 drops of Mayer's reagent (Along the sides of the test tube). A creamy white/yellow precipitate.

iv. Wagner's test: A few mL of filtrate and 1-2 drops of Wagner's reagent (Along sides of test tube). A brown/reddish precipitate.

**Detection of carbohydrates:**

i. Molish's test: To 2-3 ml filtrate, add a few drops of α-naphthol solution in alcohol, shake, and add concentrated $H_2SO_4$ from the sides of the test tube. At the junction of two liquids, a violet colour ring appeared.

ii. Barfoed's test: Equal volumes of Barfoed's reagent and test solution were added. Heated for 1-2 min in a boiling water bath and cooled. A red precipitate is formed.

**Detection of reducing sugar:**

i. Fehling's test: 1 ml Fehling's A and 1 ml Fehling's B solution were mixed and boiled for 1 min. A yellow, then brick red precipitate is formed.

ii. Benedict's test: An equal volume of Benedict's reagent and test solution was mixed in a test tube. The solution may appear green, yellow or red.

**Detection of proteins:**

i. Biuret test: To 3 ml test solution, add 4% NaOH and a few drops 1% CuSO4 solution. A violet or pink colour is formed.

ii. Million's test: Mix 3 ml T.S. with 5 ml Million's reagent. A white precipitate is formed.

iii. Xanthoprotein test: Mixed 3 ml T.S. with 1 ml concentrated $H_2SO_4$. A white precipitate is formed.

**Detection of amino acids:**

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i. Ninhydrin test: Heat 3 ml T.S. and 3 drops 5% Ninhydrin solution in a boiling water bath for 10 min. A purple or bluish colour is formed.
ii. Heated 3 ml T.S. and 3 drops Million's reagent. A dark red colour is formed.

**Detection of fixed oils and fats:**

i. Spot test/Stain test: A little quantity of plant extract is pressed between two filter papers. Oil stain on paper.
ii. Saponification test: To the extract, add a few drops of 0.5 N alcoholic KOH and a drop of phenolphthalein. Soap formation.

**Detection of anthraquinone glycosides:**

i. Borntrager's test: To 3ml plant extract, add dil.$H_2SO_4$, boil on water bath for 10 min and filter. Add an equal volume of benzene, the benzene layer is separated, and add ammonia solution to the filtrate and shake. Formation of pink or red colour in the ammoniacal layer.
ii. Modified Borntrager's test: To 5ml plant extract, add 5% ferric chloride solution and add dil. HCl is boiled for 5 minutes, and the solution is cooled. Add an equal volume of benzene, and the benzene layer is separated, and add ammonia solution. Formation of pink or red colour in the ammoniacal layer.

**Detection of cardiac glycosides:**

i. Legal's test: Dissolve 50g of plant extract in pyridine, add sodium nitroprusside and 10% sodium hydroxide. A pink-coloured solution is formed.
ii. Keller-Killiani test: To 1ml filtrate, add 1.5ml of glacial acetic acid, add 1 drop of 5% ferric chloride and add conc.$H_2SO_4$ (along the side of the test tube). A blue-coloured solution in the acetic acid layer is formed.

**Detection of saponin glycosides:**

i. Foam test: The drug extract or dry powder was shaken vigorously with water. A persistent foam was observed.

**Detection of steroids:**

i. Salkowski test: To 2ml extract, add 2ml chloroform and 2ml conc.$H_2SO_4$, shake well. The chloroform layer appears red, and the acid layer shows greenish-yellow fluorescence.

**Detection of flavonoids:**

i. Alkaline reagent test: To 2-3 drops of NaOH were added 2ml of extract. Initially, a deep colour appeared, but it gradually became colourless by adding a few drops of dil. HCl.
ii. Shinoda test: To 10 drops of dil. HCl and a piece of magnesium were added to 1ml of extract. A pink colour is formed.
iii. Lead acetate test: A few drops of 10% lead acetate solution were added to a few mL of extract. A yellow precipitate is formed.

**Detection of phenolic compounds:**

i. Iodine test: To 1ml of extract, add a few drops of dil. Iodine solution. A transient red colour is formed.
ii. Ferric chloride test: To the extract, add a few drops of 5% $FeCl_3$ solution. A dark green/bluish black colour.

**Detection of tannins:**

i. Gelatin test: Plant extract is dissolved in 5ml distilled water, add 1% gelatin solution and 10% NaCl are added. A white precipitate is formed.
ii. Braymer's test: To 1ml filtrate, add 3ml distilled water and 3 drops of 10% $FeCl_3$ solution. A blue-green colour is formed.
iii. Bromine water test: To 0.5g of plant extract, add 10ml of bromine water. Decolouration of bromine is observed.[8, 9]

## 4. ESTIMATION OF TOTAL PHENOLIC CONTENT

**Method: Folin-Ciocalteu method**

The total phenolic content was determined using a Folin–Ciocalteu reagent according to the procedure reported by Singleton and Rossi in 1965. Many available methods of quantification of total phenolic content in food products or biological samples are based on the reaction of phenolic compounds with a colorimetric reagent, which allows measurement in the visible portion of the spectrum. The Folin–Ciocalteu (F–C) assay is such a method and has been proposed as a standardised method for use in the routine quality control and measurement of the antioxidant capacity of food products and dietary supplements. The F–C assay relies on the transfer of electrons in an alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at approximately 765 nm. The exact chemical nature of the F–C reaction is unknown; it is believed that sequences of reversible one- or two-electron reduction reactions lead to blue species. The equation estimated the phenolic content.

Total phenol content, C = x(V/M)

x-obtained from the equation for a straight line of the standard graph of gallic acid,

y = mx+c

y - mean absorbance of test at 765 nm

m – slope

c - y-intercept

V - volume of extract taken

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M - amount of extract in the taken volume

**5. ESTIMATION OF TOTAL FLAVONOIDS**

**Method: Aluminium Chloride Colorimetric Method**

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants. The phenylpropanoid pathway synthesises them. Total flavonoid contents can be determined in the sample extracts by reaction with sodium nitrite, followed by the development of pink-coloured flavonoid-aluminium complex formation using Aluminium Chloride in alkaline conditions, which can be monitored spectrophotometrically at a wavelength of 510 nm. The equation estimates the flavonoid content.

Total flavonoid content, C = x(V/M)

x-obtained from the equation for a straight line of the standard graph of quercetin

y = mx + c

y - mean absorbance of test at 510 nm

m – slope

c - y-intercept

V - volume of extract taken

M - amount of extract in the taken volume. [8, 9, 10]

**6. ESTIMATION OF PROTEIN**

**Method: Bradford Method**

The protein in a solution can be measured quantitatively by different methods. The method described by Bradford uses the protein's ability to bind a dye quantitatively. The method is simple, rapid and inexpensive. The assay is based on the ability of proteins to bind Coomassie brilliant blue G250, forming a complex with an extinction coefficient much greater than that of the free dye. 100 µl of each sample was mixed with 5ml of diluted dye. It was mixed well and allowed the colour to develop for at least 5 minutes, but no longer than 30 minutes. Absorbance was read at 595nm (UV-VIS Spectrophotometer- SHIMADZU-UV-1900i) after incubation. A standard graph was plotted, and the protein concentration was calculated using the standard curve. Bovine serum albumin was used as the standard.The equation estimates the protein content.

Total protein content, C = x(V/M)

x-obtained from the equation for a straight line of the standard graph of bovine serum albumin

y = mx + c

y - mean absorbance of test at 595 nm

m – slope

c - y-intercept

V - volume of extract taken

M - amount of extract in the taken volume. [11]

**7. IN-VITRO STUDY**

**i. DPPH Radical Scavenging Assay**

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable, organic nitrogen-centred free radical that is widely utilised to evaluate the antioxidant capacity of natural compounds and plant extracts. In its radical form, DPPH exhibits a deep violet colour with a maximum absorption spectrum at 517 nm, which decolourises to pale yellow upon accepting an electron or hydrogen atom from an antioxidant. This simple, rapid, and highly sensitive spectrophotometric assay serves as a standard benchmark in phytochemistry to quantify free radical scavenging activity. Different concentrations of the leaf extract and standard antioxidant (ascorbic acid) were prepared. 1 mL of 0.1 mM DPPH solution was mixed with 1 mL of the test sample and incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer. [8, 12]

And it is calculated by,

**ii. LIPID PEROXIDATION ASSAY**

Lipid peroxidation is referred to as the oxidative degradation of lipids. It was the process whereby free radicals “steal” electrons from the lipids in the cell membrane, resulting in cell damage. This process, preceded by a free radical, often affects polyunsaturated fatty acids because they contain multiple double bonds in between which lie methylene- $CH_2$ groups that possess especially reactive hydrolysis. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) upon decomposition.

50µlof cell lysate were added with 500µl of 70% alcohol and 1ml of 1% TBA. Then all the tubes were kept in a boiling water bath for 20 minutes. After cooling to room temperature, 50 µl of acetone was added to all the test tubes and the absorbance was read at 535 nm in a spectrophotometer. [13]

**iii. NUCLEATION ASSAY**

Solutions of calcium chloride and sodium oxalate were prepared at the final concentrations of 3 mM and 7.5 mM, respectively, in a buffer containing Tris-HCl 0.05 M and NaCl 0.15 M at pH 6.5. Different concentrations of sample 40, 80 µl from the stock solution 100 mg/ml, were mixed with 3 ml calcium chloride and sodium oxalate solution each

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and were kept in incubation at 37 °C for 30 min. The absorbance of the solution was monitored at 620 nm. [14].The percentage inhibition of nucleation was calculated as:

**iv. AGGREGATION ASSAY**

Solutions of each of 50 mmol/L of CaCl2 as well as NaC2O4 were added together, equilibrated in water bath at 60°C for 1 h, incubated at 37°C all night, followed by evaporation to yield CaOx crystals. Then, 1 mg of CaOX crystals was mixed with 1 mL of buffer consisting of TrisHCl 0.05 mol/L and NaCl 0.15 mol/L, having a pH of 6.5. Aliquots of 40 and 80µg/ml were taken from a 100 mg/ml stock solution of the sample, then mixed with 3 mL of CaOx solution and incubated at 37°C for 30 min. The OD of the sample was measured at 620 nm. The percentage inhibition of aggregation of CaOx crystals was anticipated through the formula represented in the nucleation assay. [15]

The percentage inhibition of aggregation was calculated as:

## III. RESULTS

### 1. EXTRACTION

The phytochemical process that identifies bioactive components in plant materials includes a crucial step called extraction. Choosing an appropriate extraction method is crucial for the standardisation of herbal products. The quality and purity of the medicine are determined by the crude drug's extraction yield. Many factors influence extraction yields from biomass, and some of these factors can even influence one another in interdependent ways. 225g of dried and coarsely powdered*Justicia adhatoda* (L.). Leaves were extracted using the Soxhlet extraction methodwith ethanol (95%) as a solvent. Evaporation is performed usinga rotary evaporator. Extract yield of*Justicia adhatoda* (L.). Leaves prepared bythe Soxhlet extraction method with ethanol are determined by = [ weight of the dried extract/weight of the leaves] *100

= [21.04/225] * 100

= 9.35 % w/w

The extraction yield was used as an indicator of the effects of the extraction conditions. The percentage yield obtained after ethanolic extraction of*Justicia adhatoda* (L.). leaves by the Soxhlet extraction method is 9.35 % w/w.

### 2. PHYTOCHEMICAL STUDIES

**QUALITATIVE PHYTOCHEMICAL ANALYSIS**

Ethanol extract of*Justicia adhatoda* (L.)leaves was subjected to qualitative chemical analysis for the identification of various phytoconstituents like alkaloids, carbohydrates, reducing sugars, amino acids, cardiac glycosides, phenol, flavonoids, tannins, saponins, proteins and steroids.The results are summarised in **Table 2**.

| Phytochemical | Result |
|---|---|
| Alkaloids | Present |
| Carbohydrates | Present |
| Reducing sugars | Present |
| Proteins: | Present |
| Amino acids | Present |
| Cardiac glycosides | Present |
| Anthraquinone glycosides | Absent |
| Saponins | Present |
| Fixed oils and fats | Absent |
| Steroids | Present |
| Flavonoids | Present |
| Phenolic compounds | Present |
| Tannins | present |

Table 2:Preliminary Phytochemical Screening ofJusticia adhatoda (L.) Leaf Extract

### 3. QUANTITATIVE PHYTOCHEMICAL ANALYSIS

**ESTIMATION OF TOTAL PHENOLIC CONTENT**

**Table 3:** Standard graph values of gallic acid

| Concentration (µg/ml) | Mean absorbance(765nm) |
|---|---|
| 25 | 0.372±0.001 |
| 50 | 0.556±0.003 |
| 75 | 0.744±0.02 |
| 100 | 0.951±0.01 |
| 125 | 1.133±0.001 |
| 150 | 1.328±0.002 |

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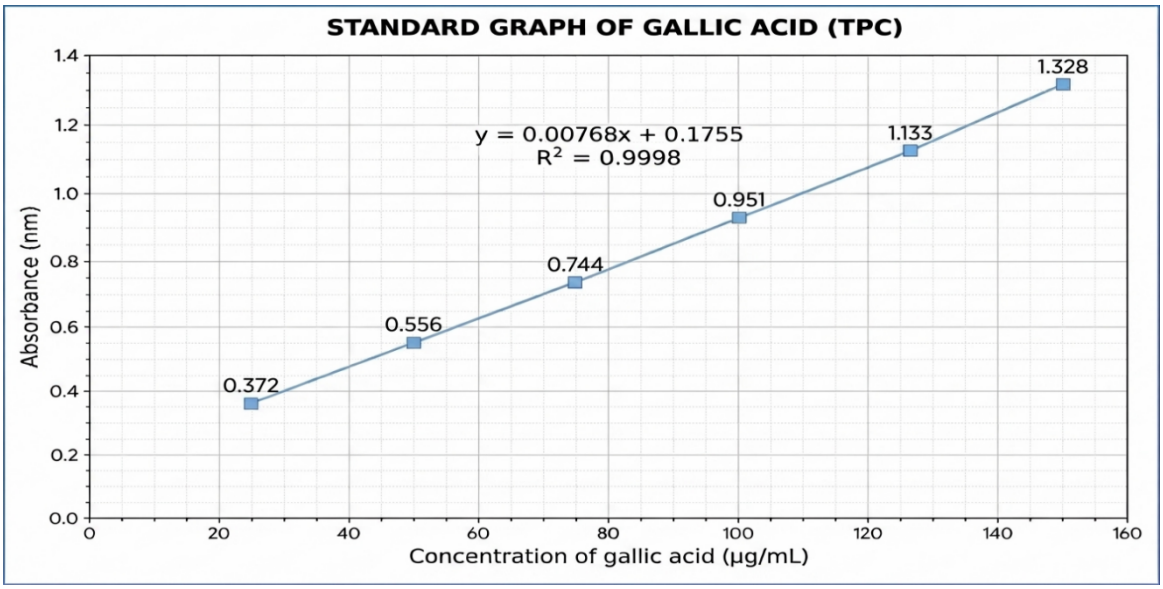


**Figure 1**: Standard graph of gallic acid

**Calculation**

Mean absorbance of*Justicia adhatoda* (L.) leaf extract at 765nm = 0.873.
Concentration from graph (µg/ml) = 90.82 (µg/ml).
From the calibration curve, thegallic acid phenolic content of the ethanolic extract was found to be90.82(µg/m

## 4. QUANTITATIVE PHYTOCHEMICAL ANALYSIS

### ESTIMATION OF TOTAL PHENOLIC CONTENT

**Table 4:** Standard graph values of quercetin

| Concentration (µg/ml) | Mean absorbance (510nm) |
|---|---|
| 25 | 0.245±0.001 |
| 50 | 0.342±0.002 |
| 75 | 0.436±0.01 |
| 100 | 0.532±0.003 |
| 125 | 0.632±0.05 |
| 150 | 0.748±0.004 |

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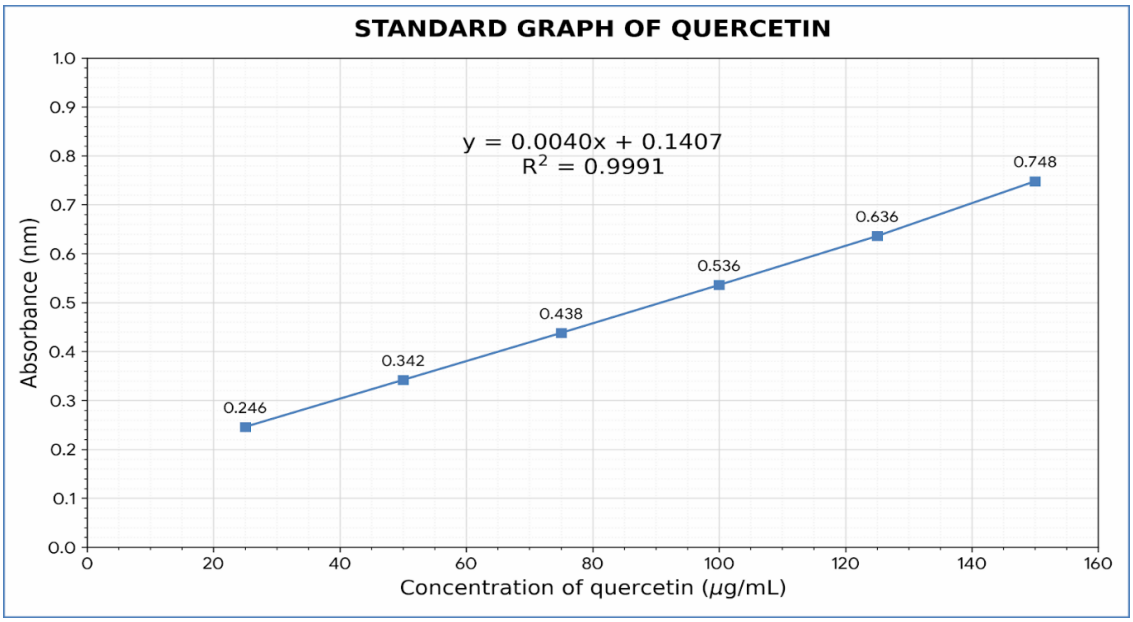


**Figure 2:** Standard graph of quercetin

**Calculation**

Mean absorbance of *Justicia adhatoda* (L.) leaf extract at 510nm = 0.271.

Concentration from graph (µg/ml) = 38.58 (µg/ml).

From the calibration curve, the quercetinflavonoid content of the ethanolic extract was found to be 32.58 (µg/ml)

## 5. QUANTITATIVE PHYTOCHEMICAL ANALYSIS

### ESTIMATION OF PROTEIN

**Table 5:** Standard graph values of bovine serum albumin

| Concentration of standard (mg/ml) | Absorbance (595nm) |
|---|---|
| 0.1 | 0.277 |
| 0.2 | 0.32 |
| 0.4 | 0.413 |
| 0.8 | 0.569 |
| 1 | 0.613 |

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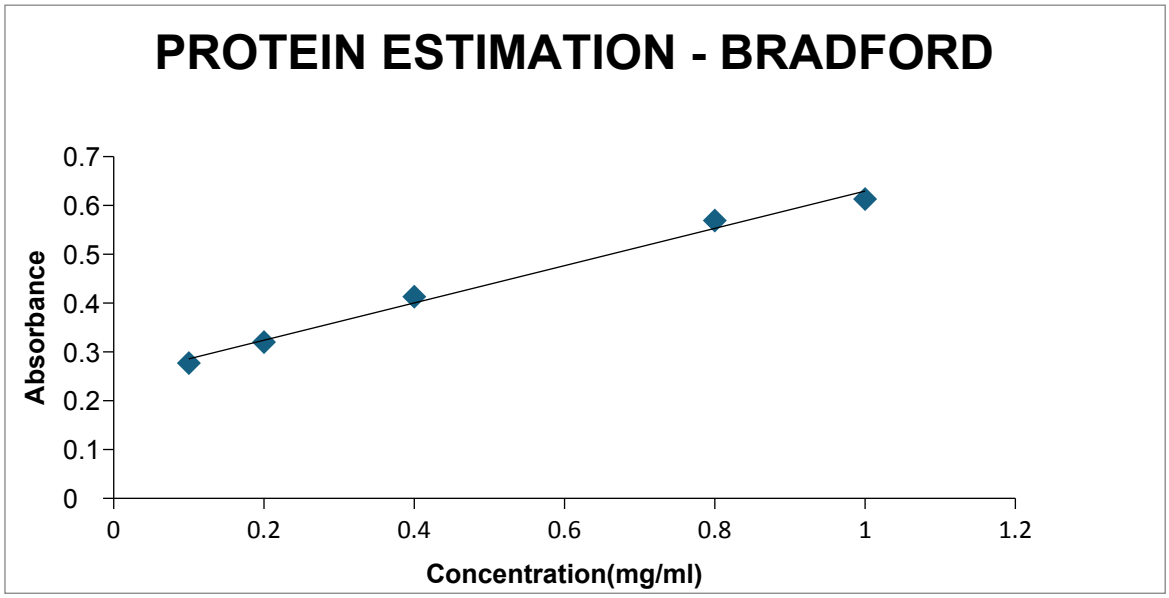


**Figure 3**: Standard graph of bovine serum albumin

**Table 6:** Standard values of control and *Justicia adhatoda*(L.) ethanolic extract

| SAMPLE CODE | ABSORBANCE (595nm) | CONCENTRATION OF PROTEIN (mg/mL) |
|---|---|---|
| Control | 0.575 | 0.8593 |
| *Justicia adhatoda* ethanolic extract | 0.566 | 0.8347 |

**Calculation**

Mean absorbance of *Justicia adhatoda* (L.) leaf extract at 595nm = 0.575.
Concentration from graph (mg/ml) = 0.8593 (mg/ml).
Mean absorbance of control at 595nm = 0.566.
Concentration from graph (mg/ml) = 0.8347 (mg/ml).
From the calibration curve, thebovine serum albumin protein content of the ethanolic extract was found to be 0.8593(mg/ml), and the bovine serum albumin protein content of the control was found to be 0.8347(mg/ml)

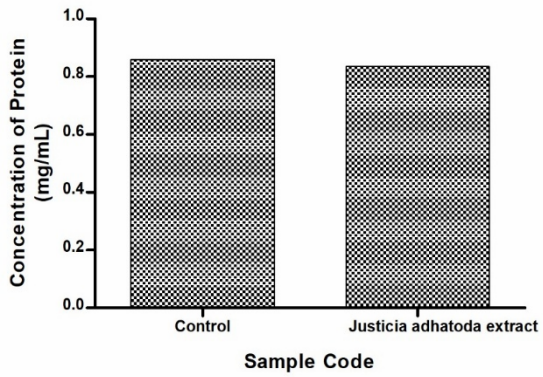


**Figure 4**: Graphical representation depicting Protein estimation by the Bradford method. Along the X axis, Sample code and along the Y axis, Concentration of protein.

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## 6. IN-VITRO STUDY

### i. DPPH RADICAL SCAVENGING ASSAY

The antioxidant potential of the extract was evaluated using the DPPH assay. The hydro alcoholic leaf extract demonstrated dose-dependent free radical scavenging activity, with an IC value of35.31µg/mL, which was comparable to the standard antioxidant, ascorbic acid (IC = 30.30 µg/mL). This indicates the extract has significant radical scavenging ability.

| SL.NO | SAMPLE | CONCENTRATION (µg/ml) | ABSORBANCE (517 nm) | PERCENTAGE INHIBITION (%) |
|---|---|---|---|---|
| | Control | | 0.529 | |
| 1 | Standard [ascorbic acid] | 10 | 0.429 ± 0.007 | 19.47 |
| 2 | | 20 | 0.321 ± 0.005 | 39.31 |
| 3 | | 30 | 0.268 ± 0.001 | 49.33 |
| 4 | | 40 | 0.183 ± 0.002 | 65.40 |
| 5 | | 50 | 0.110 ± 0.001 | 79.20 |
| 1 | Test [*Justicia adhatoda*(L.)] | 10 | 0.469 ± 0.005 | 11.34 |
| 2 | | 20 | 0.373 ± 0.003 | 29.48 |
| 3 | | 30 | 0.294 ± 0.002 | 44.42 |
| 4 | | 40 | 0.214 ± 0.001 | 59.54 |
| 5 | | 50 | 0.156 ± 0.001 | 70.51 |

**Table 7:** Standard graph values of standard (ascorbic acid) and *Justicia adhatoda*(L.) ethanolic extract

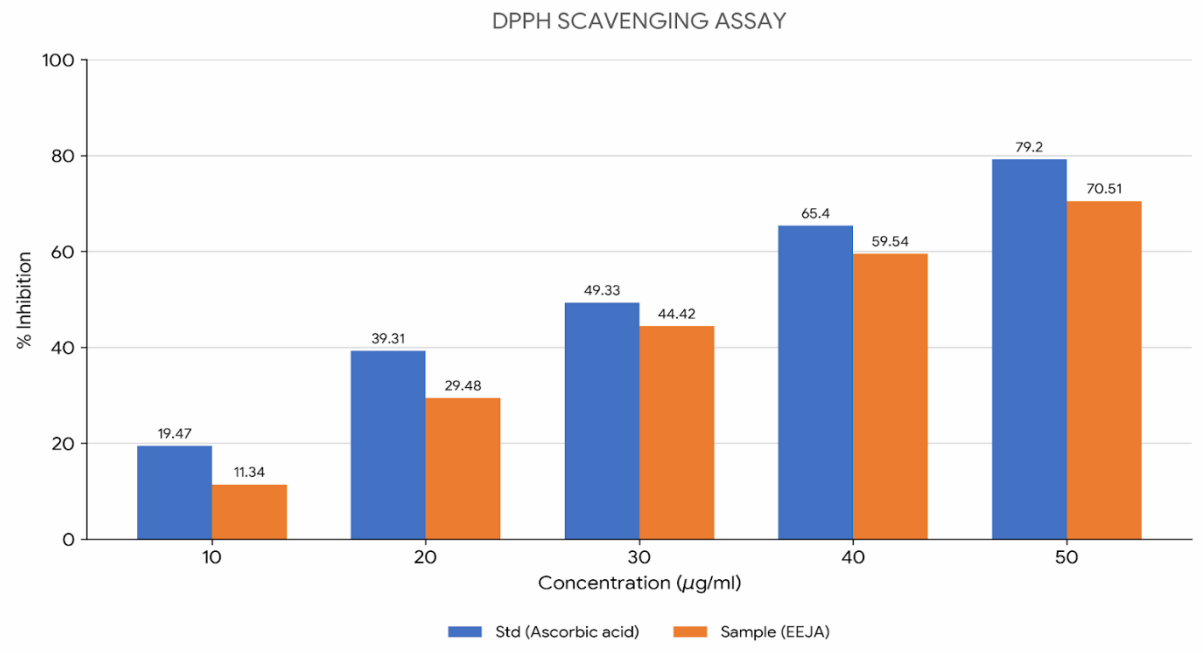


**Figure 5**: Graph of DPPH scavenging assay in standard (Ascorbic acid) and ethanolic leaf extract *Justicia adhatoda*(L.)

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**ii. LIPID PEROXIDAT**

**iii. ION ASSAY**

The anti-lipid peroxidation potential of the extract was evaluated using the MDA assay. The ethanolic leaf extract demonstrated dose-dependent protective activity, with an MDA value of 0.3699 µg/mg protein, which was significantly lower than the untreated control (0.5082 µg/mg protein). This indicates the extract has significant lipid peroxidation-inhibiting ability.

| Sample code | Absorbance (535nm) | µg MDA units | µg MDA units /mg protein |
|---|---|---|---|
| Control | 0.058 | 0.43674 | 0.5082 |
| Justicia adhatoda extract | 0.041 | 0.30873 | 0.3699 |

**Table 8:** Standard values of control and *Justicia adhatoda*(L.) ethanolic extract

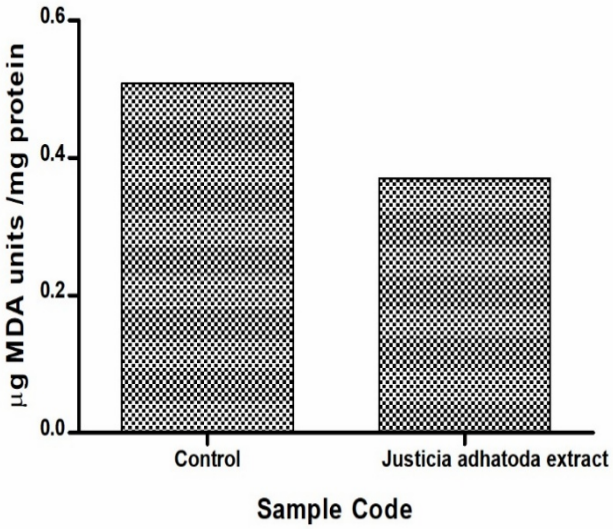


**Figure 6**: Graphical representation depicting the Lipid peroxidation of sample. Along X axis, Sample code and Along Y axis, MDA units/mg protein.

**iv. NUCLEATION ASSAY**

The anti-urolithiatic potential of the extract was evaluated using the nucleation assay. The ethanolic leaf extract demonstrated dose-dependent crystallization inhibition activity, with an $IC_{50}$ value of 943.78 µg/mL. The percentage of inhibition increased from 2.868% at 40 µg/mL to 4.954% at 80 µg/mL, corresponding to a parallel decrease in absorbance. This indicates the extract has significant nucleation inhibiting ability.

**Table 9:** Standard values of control and *Justicia adhatoda*(L.) ethanolic extract

| Sample concentration (µg/ml) | Absorbance (620nm) | Percentage of inhibition(%) |
|---|---|---|
| CONTROL | 0.767 | 0.000 |
| 40 | 0.745 | 2.868 |
| 80 | 0.729 | 4.954 |

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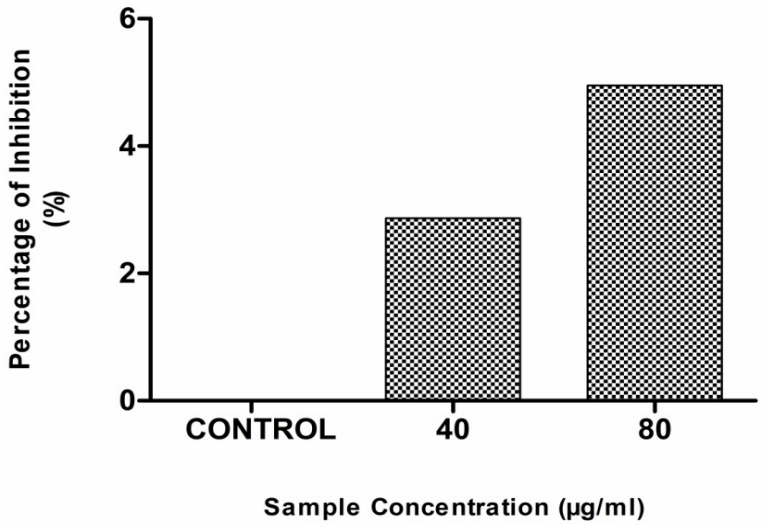


**Figure 7**: Graphical representation depicting the nucleation assay of*Justicia adhatota*(L.). Along Y axis Percentage inhibition, Along X axis varied concentration of *Justicia adhatota* (L.).

### v. AGGREGATION ASSAY

The anti-urolithiatic efficacy of the extract was evaluated using the crystal aggregation assay. The ethanolic leaf extract demonstrated dose-dependent aggregation inhibitory activity, showing an $IC_{50}$ value of 858.99 µg/mL. At 40 µg/mL, the extract showed 1.190% inhibition, which increased to 3.571% at 80 µg/mL as turbidity/absorbance decreased. This indicates the extract has significant crystal aggregation reducing ability.

**Table 9:** Standard values of control and *Justicia adhatoda*(L.) ethanolic extract

| Sample concentration (µg/ml) | Absorbance (620nm) | Percentage of inhibition |
|---|---|---|
| CONTROL | 0.767 | 0.000 |
| 40 | 0.745 | 2.868 |
| 80 | 0.729 | 4.954 |

**Table 10:** Standard values of control and *Justicia adhatoda*(L.) ethanolic extract

| Sample concentration (µg/ml) | Absorbance (620nm) | Percentage of inhibition(%) |
|---|---|---|
| Control | 0.252 | 0.000 |
| 40 | 0.249 | 1.190 |
| 80 | 0.243 | 3.571 |

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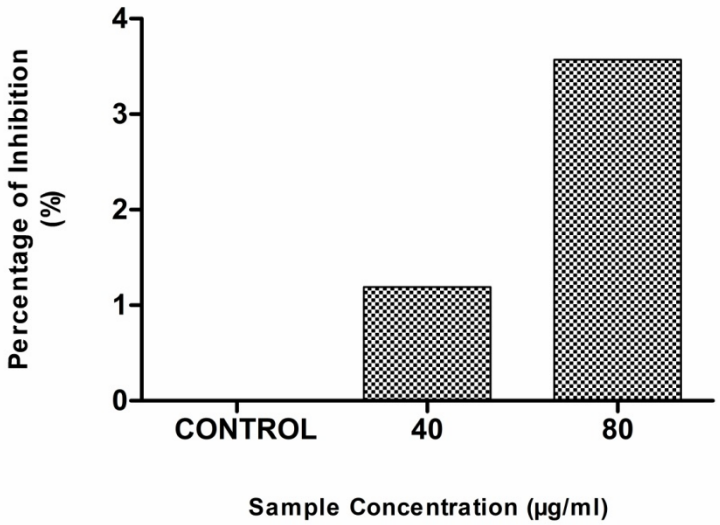


**Figure 8**: Graphical representation depicting the aggregation assay of*Justicia adhatota* (L.). Along Y axis Percentage inhibition, Along X axis varied concentration of *Justicia adhatota* (L.).

## IV.

## V. DISCUSSION

The primary aim of this research work was to evaluate the extraction yield, qualitative and quantitative phytochemical profile, antioxidant capacity, and *in vitro* anti-urolithiatic efficacy of the ethanolic leaf extract of *Justicia adhatoda* (L.). The phytochemical isolation process remains a foundational step in identifying bioactive molecules from plant matrices, and selecting an optimal extraction paradigm is essential for the standardisation of herbal therapeutics. In this study, optimised Soxhlet extraction of dried, coarsely powdered *Justicia adhatoda* (L.) leaves utilizing 95% ethanol as a solvent yielded a substantial extract recovery of 9.35% w/w. This extraction yield serves as an essential benchmark for processing conditions and crude drug purity. Following extraction, preliminary qualitative phytochemical screenings were conducted to categorise the secondary metabolites present in the leaf biomass. The analysis confirmed a robust phytochemical matrix, indicating the prominent presence of alkaloids, carbohydrates, reducing sugars, proteins, amino acids, cardiac glycosides, saponins, steroids, flavonoids, phenolic compounds, and tannins, while fixed oils, fats, and anthraquinone glycosides were absent. This diverse chemical profile directly accounts for the plant's multi-targeted pharmacological behaviour.

To precisely map the primary chemical drivers behind these activities, quantitative evaluations were performed for total phenolic and total flavonoid content using standard colorimetric methodologies. The total phenolic content was determined using the Aluminium Chloride Colorimetric Method (referenced to a standard gallic acid calibration curve), yielding a significant value of 90.82 µg/mL from a mean extract absorbance of 0.873. Similarly, the total flavonoid content was quantified against a standard quercetin calibration curve, revealing a concentration of 32.58µg/mL derived from a mean absorbance of 0.271. Phenolic compounds and flavonoids are crucial natural secondary metabolites that possess extensive electron-donating properties. These compounds are well-documented to alter physiological, biochemical, and pathological pathways in biological systems, acting as robust protective shields against cellular deterioration.Concurrently, quantitative protein estimation was executed via the Bradford method using bovine serum albumin (BSA) as the standard calibration marker. The total protein concentration of the *Justicia adhatoda* ethanolic extract was determined to be 0.8593 (absorbance: 0.575), compared to the control baseline of 0.8347 (absorbance: 0.566). Establishing this protein profile provides a crucial biochemical framework, as it serves as the descriptive baseline denominator required to normalise and calculate cellular biomarkers under oxidative duress.

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The therapeutic implications of these quantitative markers were initially evaluated through their *in vitro* antioxidant profiles. Oxidative stress is known to trigger cell injury and membrane degradation, which directly accelerates crystal retention in the renal pathways. The direct radical scavenging potential of the extract was confirmed via the DPPH assay, where it demonstrated an excellent dose-dependent free radical quenching capability. The extract achieved a potent $IC_{50}$ value of 35.31µg/mL, a performance highly comparable to the well-established reference antioxidant, ascorbic acid $IC_{50}$= 30.30 µg/mL).To understand how this radical scavenging ability protects cellular structures, an *in vitro* lipid peroxidation (TBARS/MDA) assay was utilised. Lipid peroxidation causes significant oxidative damage to membrane lipids, generating toxic aldehydes like malondialdehyde (MDA). In this assay, the *Justicia adhatoda*(L.)extract demonstrated substantial tissue-protective efficacy. By utilising the pre-established Bradford protein values, the raw MDA accumulation was normalised to demonstrate that the extract notably kept lipid damage down to 0.3699 (absorbance: 0.041) compared to the untreated control group, which exhibited severe lipid damage at 0.5082 (absorbance: 0.058). This synchronised antioxidant performance demonstrates that the extract suppresses oxidative degradative pathways, thereby preserving cellular membrane integrity.

Crucially, this cell-protective mechanism provides a strong scientific rationale for the extract's ability to prevent urolithiasis. Chronic urolithiasis is a complex pathological process involving the nucleation, growth, and aggregation of calcium oxalate (CaOx) crystals within the urinary tract. The anti-urolithiatic potential of *Justicia adhatoda* (L.) was evaluated across these key stages using standard nucleation and aggregation assays.During the primary crystallisation phase, as evaluated by the nucleation assay, the ethanolic extract inhibited crystal formation in a clear concentration-dependent manner, yielding an $IC_{50}$of 943.78 µg/mL. While the control group experienced uninhibited crystal precipitation, the extract-treated groups restricted crystal nucleation, showing an increase in inhibition from 2.868% at 40 µg/mL to 4.954% at 80 µg/mL, which correlated with a parallel reduction in turbidity and absorbance.Furthermore, the extract effectively disrupted the subsequent, highly critical step of stone growth in the aggregation assay. Crystal aggregation causes smaller particles to clump into larger stone masses that are easily retained in the kidneys. The extract successfully minimised this risk, showing a dose-dependent aggregation inhibitory trend with an value of $IC_{50}$858.99 µg/mL. It achieved an inhibition rate of 1.190% at 40 µg/mL, which rose significantly to 3.571% at 80 µg/mL as a result of a steady reduction in absorbance.This multi-stage inhibition highlights a compelling, direct link between the rich phenolic/flavonoid matrix of *Justicia adhatoda* (L.) and its anti-urolithiatic profile. By actively lowering systemic oxidative stress, preventing lipid membrane peroxidation, and directly blocking the nucleation and aggregation of mineral crystals, the ethanolic extract demonstrates an effective, multi-targeted therapeutic approach. This study provides valuable *in vitro* validation supporting the traditional use of *Justicia adhatoda* (L.) and establishes it as a promising natural candidate for the preventative management of urolithiasis.

## VI. CONCLUSION

The present study evaluated the ethanolic leaf extract of *Justicia adhatoda* (L.) for its phytochemical composition, quantitative biomarkers, and *in vitro* antioxidant and anti-urolithiatic potential. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, phenolics, saponins, tannins, and glycosides, indicating a rich profile of bioactive compounds. Quantitative assessments showed substantial levels of total phenolics (90.82 µg/mL) and total flavonoids (32.58 µg/mL), while Bradford protein estimation established an extract concentration of 0.8593 mg/mL (0.8347 mg/mL for control). The extract demonstrated notable antioxidant activity in the DPPH assay, with an value of 35.31 µg/mL, comparable to the standard antioxidant, ascorbic acid ( = 30.30 µg/mL), alongside significant cellular protection in the lipid peroxidation assay by limiting malondialdehyde accumulation to 0.3699 µg MDA units/mg protein. Furthermore, the extract displayed promising *in vitro* anti-urolithiatic efficacy, concentration-dependently inhibiting calcium oxalate crystallisation with values of 943.78 µg/mL and 858.99 µg/mL in the nucleation and aggregation assays, respectively.These findings suggest that the extract possesses compounds capable of scavenging free radicals, preventing lipid membrane peroxidation, and potentially mitigating stone-related crystal retention. The ultimate relevance of this research lies in validating the traditional ethnomedical claims of *J. adhatoda* through structured biochemical evidence, demonstrating that

its multi-targeted mechanism can actively disrupt critical stages of renal stone development.

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