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IN VITRO INVESTIGATION OF ANTIOXIDANT AND ANTI-UROLITHIATIC ACTIVITY OF *HOMONOIA RETUSA* ROOTS

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ABSTRACT

Ayurvedic practitioners utilise Pashanabheda as an anti-urolithiatic. The root of *Homonoia retusa* (Euphorbiaceae), which grows in the Indian town of Tirupathi, was chosen for this study's screening for its antioxidant and anti-urolithiatic properties. The hydrogen peroxide assay (H2O2), ferric reducing antioxidant power (FRAP), and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) techniques were utilised to assess antioxidant activity. The anti-urolithiatic activity was evaluated by nucleation and aggregation assay by adding 0.01 M sodium oxalate solutions that caused the crystallisation of Calcium oxalate. By measuring turbidity in the presence or absence of extract at 620 nm using a spectrophotometer, the effect of extract (200, 400, 600, 800, and 1000 µg/ml) was investigated. According to the result, aqueous root extract can prevent crystal growth and aggregation more than methanol-based root extract.

Keywords: *Homonoia retusa*, antioxidant, anti-urolithiatic, root, pharmacological significance.

INTRODUCTION

Ayurvedic practitioners in India use a class of medicinal herbs known as Pashanabheda (stone-breaking) plants as anti-urolithiatic medications.[1] *Homonoia retusa* are rheophytes and are usually found in groups at riverbanks of India.[2] It is commonly known as Pashanabheda, which is a plant of the Euphorbiaceae family.[2] It is a dioecious shrub that may grow up to 3 metres tall, having sturdy, woody branchlets, obovate to oblanceolate leaves that are 2-4 by 1-2 cm, coriaceous, edge serrate to dentate, apex obtuse to retuse, acute-cuneate at base, flowers in axillary spikes, globose capsules that are 3.5 mm across, and smooth, yellow seeds that are rounded on the back. [3] The herb is used as a diuretic, cough, sore throat, diabetes, and lithiasis remedy in traditional medicine.[4]

Persons who have urolithiasis develop kidney or urinary tract stones. [5] The most typical kind of stone comprises calcium together with either oxalate or phosphate.[6] In the current medical system, kidney stones are typically treated with surgical and interventional techniques such as extracorporeal shock wave lithotripsy, percutaneous nephrolithotomy, and ureteroscopy.[7] These surgical techniques are expensive; recurrence is rather typical. Due to the current medical system's lack of clinically effective medications that can be used to dissolve kidney stones or stop their formation and recurrence, doctors must rely on complementary and alternative therapies.[8]

Therefore, the present study was undertaken for anti-urolithiatic study and to elucidate the biological activities of *Homonoia retusa*; we analysed the total phenolic content (TPC) and the total flavonoid content (TFC) and determined petroleum ether, chloroform, alcohol and water extracts for antioxidant activity.

MATERIALS AND METHODS

Collection and identification of the plant

The roots of the *Homonoia retusa* plant were collected and dried under shade and made into coarse powder. The plant material was collected, identified, and authenticated by Scientist (Dr) K Madhava Chetty, plant taxonomist and Assistant Professor of the Department of Botany, Tirupathi, India.

Preparation of extracts

The extract was made from the previously powdered plant drug. Various extracts are made by extracting plant material using progressively more polar solvents, such as pet ether, chloroform, ethanol, and water.

Phytochemical screening

The procedures outlined in standard pharmacopoeias were followed in the phytochemical screening process. Their secondary chemical elements primarily cause the biological activity of plants or medications. Several chemical tests were carried out to identify distinct chemical components.[9,10]

Total phenolic content

Total phenol content was determined using Folin-Ciocalteu's assay, which used gallic acid as the standard. In the procedure, 0.5 ml of plant extracts were mixed with 1.5 ml Folin-Ciocalteu's reagent (FCR) diluted 1:10 v/v. Then, after 5 minutes, 1.5 ml of 7% sodium carbonate solution was added. The final volume of the tubes was made up to 10 ml with distilled water and allowed to stand for 90 minutes at room temperature. The absorbance of the sample was measured against the blank at 750 nm using a spectrophotometer. All the experiment was repeated three times for precision, and values were expressed in mean ± standard

deviation in terms of phenol content (Gallic acid equivalent, GAE) per g of dry weight.[11]

Total flavonoid content

Total flavonoid content was determined using the aluminium chloride method, which used quercetin as a standard. 1 ml of the test sample and 4 ml of water were added to a volumetric flask (10 ml volume). Add 0.3 ml of 5 % Sodium nitrite and 0.3 ml of 10% aluminium chloride was added after 5 minutes. After 6 minutes incubation at room temperature, 1 ml of 1 M sodium hydroxide was added to the reaction mixture. The final volume was made up of 10 ml of distilled water. The absorbance of the sample was measured against the blank at 510 nm using a spectrophotometer. All the experiment was repeated three times for precision, and values were expressed in mean ± standard deviation in terms of flavonoid content (Quercetin equivalent, QE) per g of dry weight.[12,13]

***In-vitro* antioxidant activity**

DPPH reducing assay

The ability of *Homonoia retusa* root extracts to scavenge ferric ions was determined according to the method described by Patel *et al.* (2010) and Patel *et al.* (2012), with some modifications. The 2.5 ml extract added 1 ml of 0.2 M phosphate buffer pH 6.6 and 1 ml of 1 % potassium ferricyanide. The reaction mixture was incubated in a water bath at 50 °C for 20 minutes. Afterwards, the reaction mixture was rapidly cooled, and 2.5 ml of 10 % trichloroacetic acid was added to stop the reaction. Then, it was centrifuged for 10 minutes. 2.5 ml of aliquots was pipetted, 2.5 ml of distilled water was added, and 0.5 ml of 0.1 % ferric chloride solution was added. The colour changes to green. The mixture was allowed to stand for 10 minutes, and absorbance was measured at 593 nm in a spectrophotometer. The blank was performed using a reagent blank and solvent. Ascorbic acid was used as standard. The extracts were performed in triplicate.[14]

The percentage scavenging activity of both extracts and standard sample were calculated according to the following formula

$$\text{Inhibitory activity}(\%) = \frac{\text{Abs Sample (AS)} - \text{Abs Control (AC)}}{\text{Abs Sample (AS)}} \times 10$$

Where AC is the absorbance of the control, AS is the absorbance in the presence of the *Homonoia retusa* root extracts or standards sample.

Ferric-reducing power assay (FRAP)

The ability of *Homonoia retusa* root extracts to scavenge ferric ions was determined according to the method described by Patel *et al*. (2010) and Patel *et al.* (2012), with some modifications. The 2.5 ml extract added 1 ml of 0.2 M phosphate buffer pH 6.6 and 1 ml of 1 % potassium ferric cyanide. The reaction mixture was incubated in a water bath at 50 °C for 20 minutes. Afterwards, the reaction mixture was rapidly cooled, and 2.5 ml of 10 % trichloroacetic acid was added to stop the reaction. Then, it was centrifuged for 10 minutes. 2.5 ml of aliquots was pipetted, 2.5 ml of distilled water was added, and 0.5 ml of 0.1 % ferric chloride solution was added. The colour changes to green. The mixture was allowed to stand for 10 minutes, and absorbance was measured at 593 nm in a spectrophotometer. The blank was performed using a reagent blank and solvent. Ascorbic acid was used as standard. The extracts were performed in triplicate.[14]

The percentage scavenging activity of both extracts and standard sample were calculated according to the following formula

Inhibitory activity (%) = Abs Control (AC) − Abs Sample (AS) Abs Control (AC) × 100

Where AC is the absorbance of the control, AS is the absorbance in the presence of the *Homonoia retusa* extracts or standards sample.

H_2O_2 reducing assay

The ability of *Homonoia retusa* extracts to scavenge hydrogen peroxide was determined according to the method described by Ruch *et al.* (1989) with some modifications. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). To 1 ml of extracts in distilled water, hydrogen peroxide solution (0.6 mL, 40 mM) was added. The mixture was allowed to stand for 10 minutes, and absorbance was measured at 230 nm in a spectrophotometer. The blank was performed using a reagent blank and solvent. Ascorbic acid was used as standard. The extracts were performed in triplicate.[15]

The percentage scavenging activity of both extracts and standard sample were calculated according to the following formula

Inhibitory activity (%) = Abs Control (AC) − Abs Sample (AS) Abs Control (AC) × 100

Where AC is the absorbance of the control, AS is the absorbance in the presence of the *Homonoia retusa* extracts or standards sample.

***In-Vitro* Anti-Urolithiatic Activity**

Nucleation assay

Solution of calcium chloride and sodium oxalate will be prepared at the final concentrations of 5 mmol/L and 7.5 mmol/L, respectively, in a buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5. 950 µL of calcium chloride solution will be mixed with 100 µL of herb extracts at different concentrations (100 µg/ml-1000 µg/ml). Crystallisation will be started by adding 950 µL of sodium oxalate solution. The temperature will be maintained at 37 °C. The OD of the solution will be monitored at 620 nm. The nucleation rate will be estimated by comparing the induction time in the presence of the extract with that of the control.

Aggregation Assay

Calcium Oxalate monohydrate (COM) crystals will be prepared by mixing calcium chloride and sodium oxalate at 50 mmol/L. Both solutions will be equilibrated to 60 °C in a water bath for 1 hour and then cooled to 37 °C overnight. The crystals will be harvested by centrifugation and then evaporated at 37 °C. CaOX crystals will be used at a final concentration of 0.8 mg/ml, buffered with Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.55. After stopping the stirring, experiments will be conducted at 37 °C in the absence or presence of the plant extract. The Cystone tablets will be used as a standard drug solution. The percentage aggregation inhibition rate (Ir) will be then calculated by comparing the turbidity in the presence of the extract with that obtained in the control using the following formula.[16]

$$Ir = (1 - \text{Turbidity}_{\text{sample}}/\text{Turbidity control}) \times 100$$

Table 1: Total phenolic content and total flavonoid content of various extracts of *Homonoia retusa*

***Homonoia retusa* plant extract**	**Concentration of phenolic content (GAE)** (µg/ml)	**Concentration of Total flavonoid content (QE)** (µg/ml)
Pet ether	1.926±0.0268 µg/ml	1.257±0.0325µg/ml
Chloroform	6.645±0.029 µg/ml	3.563±0.0478 µg/ml
Ethanol	19.518±0.021 µg/ml	28.5601±0.0356 µg/ml
Water	26.86±0.0233 µg/ml	35.2305±0.0469 µg/ml

Table 2: Antioxidant effects of root extract of *Homonoia retusa* using DPPH inhibition assay

Extract concentration (µg/ml)	**Percentage inhibition**			
	Pet ether	**Chloroform**	**Ethanol**	**Water**
10	3.72±0.01	11.38±0.01154	13.18±0.0115	30.75±0.01154
20	9.13±0.01732	17.24±0.01	18.59±0.00577	34.81±0.00577
40	14.54±0.00577	22.64±0.00577	26.70±0.01	38.86±0.01
60	21.29±0.01	32.10±0.01	36.16±0.00577	45.62±0.01
80	26.7±0.0057	40.21±0.0115	44.2±0.005774	49.67±0.01
100	37.51±0.005774	45.62±0.005774	48.32±0.01	55.08±0.01
IC 50	140.58	108.92	98.55	80.07

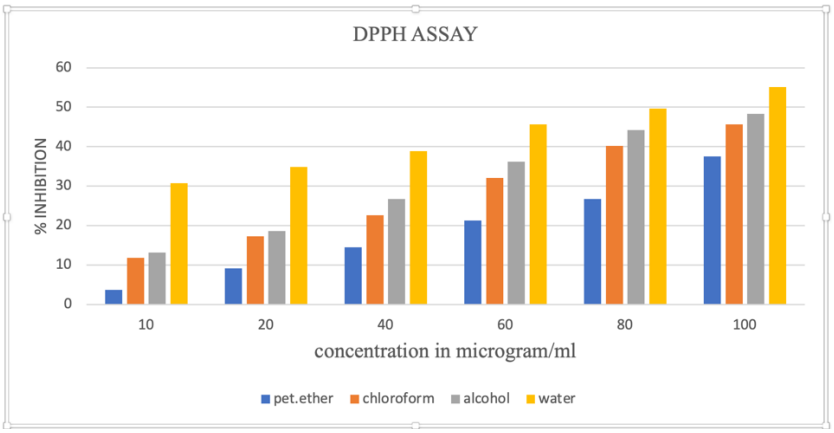

Figure 1: Antioxidant effects of root extract of *Homonoia retusa* using DPPH inhibition assay

Table 3: Antioxidant effects of various extracts of *Homonoia retusa* using ferric chloride inhibition assay

Extract concentration (µg/ml)	**Percentage inhibition**			
	Pet ether	**Chloroform**	**Ethanol**	**Water**
10	3.33±0.02	13.33±0.03	18.33±0.05	31.66±0.0321
20	6.66±0.02516	20±0.036	26.66±0.02	33.33±0.0416
40	15±0.01527	23.36±0.015	31.56±0.017	41.65±0.0360
60	20±0.01527	30.02±0.026	36.65±0.0173	48.32±0.02309
80	28.33±0.02645	35.01±0.026	48.33±0.0152	53.33±0.03511
100	38.32±0.026	41.66±0.0360	50±0.02	63.03±0.01
IC 50	150.06	128.84	94.075	65.523

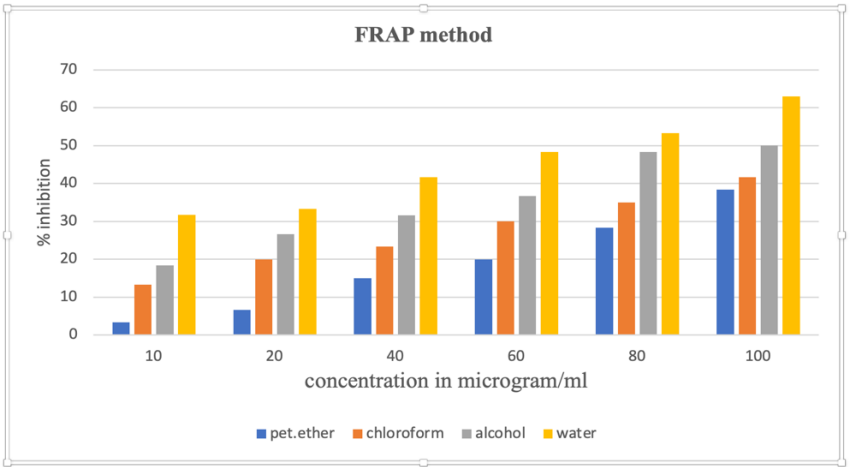

Figure 2: Antioxidant effects of root extract of *Homonoia retusa* using FRAP method

Table 4: Antioxidant effects of various extracts of *Homonoia retusa* using H_2O_2 inhibition assay

Extract concentration (µg/ml)	Percentage inhibition			
	Pet ether	Chloroform	Ethanol	Water
10	2.85±0.01	10±0.01	22.85±0.0115	27.14±0.01
20	7.14±0.01	17.1±40.0115	27.14±0.0115	34.28±0.01
40	15.71±0.0152	21.42±0.01	34.28±0.01	48.04±0.017
60	21.42±0.01	25.71±0.0208	40±0.01	58.57±0.01
80	25.71±0.01	30±0.01	44.28±0.0115	65.71±0.01
100	30±0.01	34.28±0.01	47.14±0.0057	71.05±0.01
IC_{50}	161.99	159.95	103.31	50.054

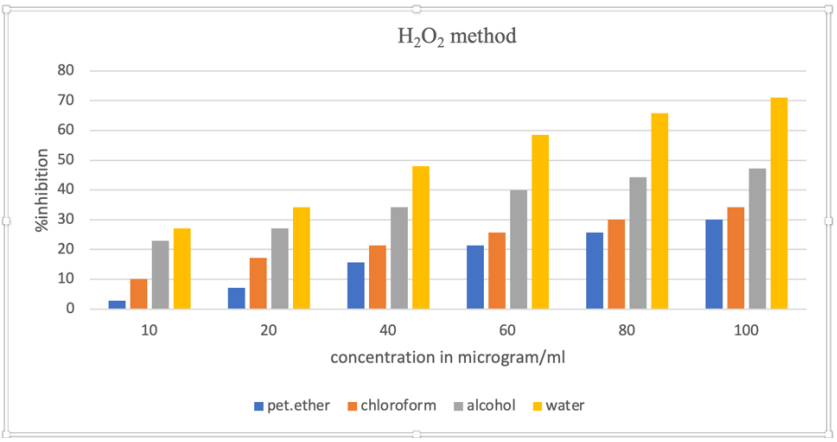

Figure 3: Antioxidant effects of root extract of *Homonoia retusa* using H_2O_2 inhibition assay.

Table 5: Anti-urolithiatic effect of different concentrations of aqueous and ethanol extracts of the root of *Homonoia retusa* by Nucleation assay

Concentration in (µg/ml)	% inhibition		
	Aqueous root extract	Ethanol root extract	Standard Cystone
200	49.2±0.0014	36.42±0.0012	72.3±0.0013
400	52.04±0.002	39.03±0.001	78.6±0.0023
600	56.56±0.0065	43.4±0.044	81.5±0.0032
800	61.7±0.0023	49.09±0.005	88.1±0.056
1000	68.69±0.005	52±0.0032	94.4±0.005

The values are expressed as Mean ± SEM, n=3

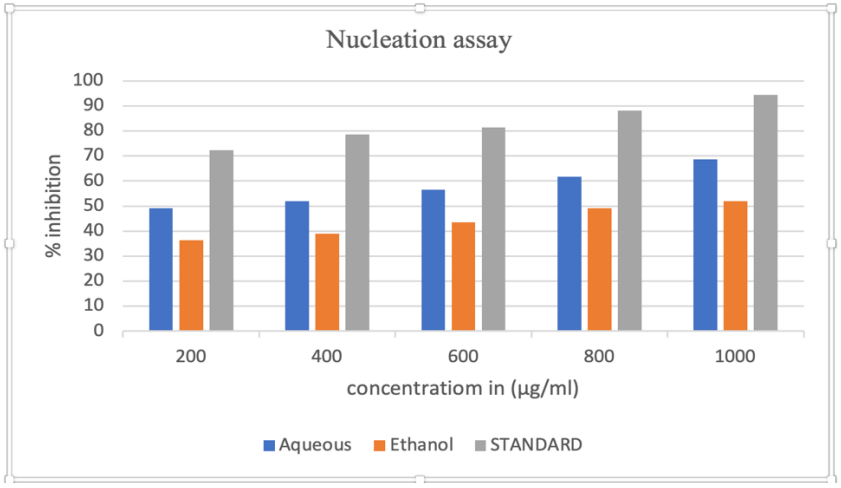

Figure 4: Anti-urolithiatic activity of root extract of *Homonoia retusa* by Nucleation assay

Table 6: Anti-urolithiatic effect of different concentrations of aqueous and ethanol extracts of the root of *Homonoia retusa* by Aggregation assay

Concentration in (µg/ml)	% inhibition		
	Aqueous root extract	Ethanol root extract	Standard Cystone
200	34.03±0.003	32.22±0.026	71.3±0.0023
400	41.00±0.0032	36.45±0.005	74.6±0.0043
600	46.32±0.0056	43.41±0.009	76.5±0.0092
800	49.03±0.005	49.65±0.0039	81.1±0.056
1000	51.002±0.008	54.62±0.0052	86.4±0.015

The values are expressed as Mean±SEM, n=3

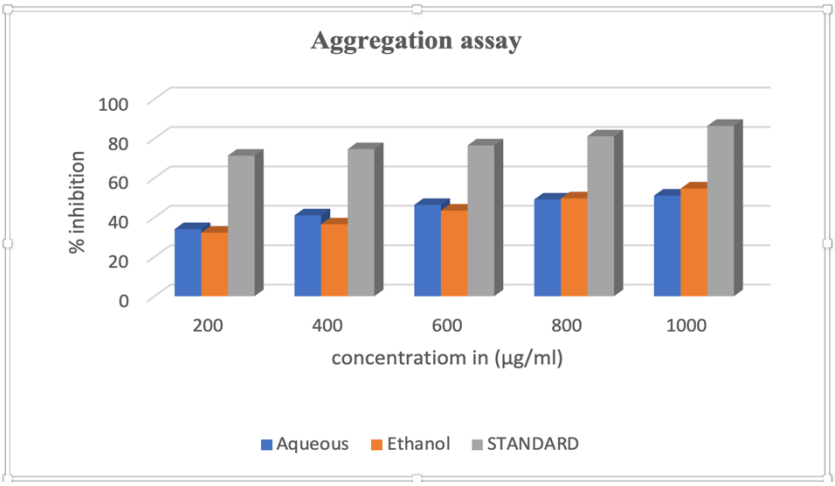

Figure 5: Aggregation assay on the root extract of *Homonoia retusa*.

RESULTS AND DISCUSSION

Preliminary phytochemical investigations of extracts were assessed to reveal the presence of different secondary metabolites. Petroleum ether and chloroform extract revealed the presence of steroids, whereas ethanol and aqueous extracts indicated the presence of flavonoids, saponins, phenols, and steroids.

Total Phenolic Content

The total phenolic content for aqueous, ethanol, chloroform, and petroleum ether extracts of *Homonoia retusa* was estimated by Folin Ciocalteu's method using gallic acid as standard. The reagent is formed from a mixture of phosphotungstic acid and phosphomolybdic acid, which, after oxidation of the phenols, is reduced to a mix of blue oxides of tungsten and molybdenum. The blue colouration produced has maximum absorption in the region of 750 nm and is proportional to the total quantity of phenolic compounds initially present. The gallic acid solution of concentration (10-100 ppm) conformed to Beer's Law at 750 nm with a regression coefficient (R2) =0.9905. The plot has a slope (m) = 0.011 and intercept =0.1017. The equation of the standard curve is y = 0.011x + 0.1017. (Table 1)

Total Flavonoid Content

The total flavonoid content for aqueous, ethanol, chloroform and petroleum ether extracts of *Homonoia retusa* were measured with the aluminium chloride colourimetric assay using quercetin as standard. Aluminium chloride forms acid-stable complexes with the C-4 keto groups and the C-3 or C-5 hydroxide group of flavones and flavonols. In addition, it also forms liable complexes with ortho dihydroxide groups in A/B rings of flavonoids. The quercetin concentration solution (100-1000 ppm) conformed to Beer's Law at 510 nm with a regression coefficient (R^2)= 0.9973. The plot has a slope (m) = 0.0105 and intercept = 0.0891. The equation of the standard curve is y = 0.0105x + 0.0891. (Table 1)

Antioxidant Activity

The antioxidant activity of all extracts is measured by three methods viz. DPPH scavenging assay, Ferric reducing antioxidant power assay and H_2O_2 reducing assay were compared with the standard ascorbic acid. The percentage inhibition of extracts of *Homonoia retusa* is given in Table 2.

DPPH reducing assay

Figure 1 shows the scavenging effects of samples on DPPH radicals in the following order: Water Extract > Ethanolic Extract> Chloroform extract> Pet ether extract. The IC_{50} values of scavenging DPPH radicals for the water and ethanolic extract were 80.07 and 98.552 µg/ml, respectively (Table 2). The presence of phenolic compounds could be the reason for the antioxidant property of *Homonoia retusa.*

Ferric-reducing power assay (FRAP)

Figure 2 shows the dose-response curves for the reducing powers of all extracts (10-100 µg/ml) from *Homonoia retusa*. It was found that the reducing power increased with the concentration of each sample. The ranking order for reducing power was Water Extract > Ethanolic Extract> Chloroform extract> Pet ether extract. Significantly higher reducing power (63.03±0.01at 100 µg/ml) was evident in the water extract. (Table 3)

H_2O_2 reducing assay

The scavenging effect of different extracts of *Homonoia retusa* on hydrogen peroxide was concentration-dependent (10-100 µg/mL), as shown in Figure 3. The water extract displayed strong H_2O_2 scavenging activity (IC_{50} 50.054 µg/mL). Whereas ethanolic extract exhibited IC_{50} 103.31µg/mL (Table 4). The significant difference in percentage inhibition of H_2O_2 of all

extracts was compromised in Figure 3. Among various extracts of the *Homonoia retusa*, the aqueous extract exhibited better H_2O_2 scavenging activity. The naturally occurring H_2O_2 in the air, water, human body, plants, microorganisms and food is at low concentration levels. It is quickly decomposed into oxygen (O_2) and water (H_2O) and may create hydroxyl radicals (OH) that can initiate lipid peroxidation and cause DNA damage. The water extract of *Homonoia retusa* capably scavenged hydrogen peroxide, which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralising it into H_2O.

***In Vitro* Anti-urolithiatic Activity**

Nucleation Assay Method

The effects of inhibition of nucleation activities for different extracts are presented in Figure 4. The results in Table 5 indicate a significant difference between two factors, the type of extract and concentration of the extract, in terms of the percentage of inhibition activity of the samples. The highest inhibition of nucleation activity was obtained from the aqueous root extract from *Homonoia retusa*).

Aggregation Assay

In the aggregation assay, the ethanol root extract showed significant inhibition (54.62%) compared to Cystone (86.4%) at 1000 µg/ml concentration. The microscopic assay indicated that ethanol extracts of the root of *Homonoia retusa* are capable of dissolving the calcium oxalate crystals. (Table 6, Figure 5)

CONCLUSION

The results of this study explored the antioxidant capabilities of *Homonoia retusa* extracts and the *in-vitro* inhibition of calcium oxalate stones. The phenolic and flavonoid contents of the root section were highly linked with antioxidant and anti-urolithiatic effects. The antioxidant activity of all extracts was measured using three methods, viz. DPPH scavenging assay, Ferric reducing antioxidant power assay and H_2O_2 reducing assay were compared with the standard ascorbic acid. It was observed that the water extracts of *Homonoia retusa* have higher antioxidant activity than ethanol, chloroform and pet ether extracts. The anti-urolithiatic activity of root extracts was carried out by nucleation and aggregation assay. The study shows a significant increase in the percentage of inhibitory activity in a concentration-dependent manner. Thus, it is concluded that our study provides the data that helps to isolate, identify, and characterise the various medicinal potentials, such as the anti-urolithiatic and antioxidant potential of *Homonoia retusa* root. The traditional usage of *Homonoia retusa* roots in the breakdown of stones may have biological justification. However, this study found that the *Homonoia retusa* roots possess antioxidant and anti-urolithiatic effects.

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