

Phytochemical Screening and *in vitro* Anti-urolithiatic Activity of Fruit-seed Extracts of *Melia azedarach*

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ABSTRACT

Melia azedarach L. has been utilized in traditional systems of medicine for the treatment of various diseases including urolithiasis. The study aimed to perform phytochemical studies and anti-urolithiatic potential of fruit-seed extracts of *Melia azedarach*. Sequential extraction was performed using chloroform, methanol and water. The extracts of the plant were then subjected to quantitative tests for phytochemical analysis. An anti-urolithiatic activity was investigated via *in vitro* nucleation and aggregation assay using spectrophotometer. The preliminary phytochemical analysis showed the presence of proteins ($31.97 \pm 0.56\%$), lipids ($2.95 \pm 0.03\%$) and carbohydrates ($64.90 \pm 0.56\%$). The chloroform extract contained the maximum quantity of polyphenols (77.65 ± 0.53 mg/g) and flavonoids (74.71 ± 0.67 mg/g). At a concentration of 5 mg/ml, the chloroform extract exhibited the maximum inhibitory activity in both aggregation and nucleation assay ($55.85 \pm 1.43\%$; $56.42 \pm 4.49\%$) respectively. All extracts showed substantial anti-urolithiatic activity by inhibiting the crystallization of calcium oxalate. Due to presence of primary and secondary metabolites, the plant could serve as a source of useful drugs.

Keywords: *Melia azedarach*, phytochemical analysis, anti-urolithiatic, aggregation assay, nucleation assay.

1. INTRODUCTION

Urolithiasis or renal stone disease remains a serious issue in the adult population, with serious medical consequences throughout life (1). The prevalence of urolithiasis is quite high around the globe and more than 80% of urinary calculi are calcium oxalate stones alone or calcium oxalate mixed with calcium phosphate (2, 3). Despite modest progress in the pathophysiology and treatment of urolithiasis, there is still no effective drug being utilized in clinical therapy (4). Extracorporeal shock-wave lithotripsy (ESWL) and endoscopic stone removal are well-recognized procedures but it is expensive and recurrence is quite common with these procedures (5).

However, an effective drug for the treatment of this disorder or its recurrence would be of great importance. Medicinal plants have played a substantial role in various ancient traditional systems of medicine. Several pharmacological investigations on the medicinal plants used in traditional anti-urolithiatic therapy have revealed their therapeutic potential in the *in vitro* models. Various medicinal plants exhibiting anti-urolithiatic activity have been well documented in previous studies (6-9).

Melia azedarach, a deciduous tree is derived from Greek words; *Melia* means “flowering ash or manna ash” and *azedarach* means “poisonous tree” (10). It is commonly used in Chinese, Iranian and Indian traditional medicines (11). All parts of the plant i.e. root, bark, leaves, seeds, flowers, and fruits have shown pesticidal as well as pharmacological activities (10). It is well-known for its antioxidant, cardioprotective, anti-inflammatory, anti-

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microbial, anti-ulcer and anti-cancer properties which are proven by clinical and experimental studies (12-15). The literature review of *Melia azedarach* revealed that this plant contains many phytochemicals that possess various pharmacological activities (16). Various studies have been focused on leaves, bark, flower, and seeds but fruit-seed is not so much focused. However, more exploratory research is required to evaluate safety, efficacy and therapeutic potential of this plant. Therefore, the aim of this study is to assess phytochemical studies and anti-urolithiatic activity of fruit-seed extracts of *Melia azedarach*.

2. MATERIALS AND METHODS

2.1. Chemical and reagents

Chloroform, methanol, acetone, potassium acetate, aluminium nitrate crystals, Bovine Serum albumin (BSA), copper sulphate, potassium tartrate, sodium bicarbonate (Merck, Germany), Cystone (Himalaya, India), sodium oxalate, calcium chloride (Duksan Reagents, Korea), Tris-HCl (Sigma, Aldrich, Germany), hydrochloric acid, sulphuric acid (BDH, England).

2.2. Plant material and extraction

In December 2019, the fruit-seeds were collected from Akbari Mandi, Lahore, Pakistan. This plant was then authenticated by taxonomist Prof. Dr. Zaheer-ur-Din Khan, Department of Botany, Government College University Lahore (GCU), Pakistan. A voucher specimen (GC Herb. Bot. # 3645) was deposited in the herbarium of GCU. The fresh fruit-seeds were separated, cleaned and air-dried under shade for 2 weeks at room temperature. The dried fruit-seeds were compressed into a fine powder and stored in an air-tight bag. It was then subjected to proximate analysis.

The powdered material (50 g) was extracted sequentially using solvents of different polarities i.e. chloroform, methanol and water. All the extracts were dried with the help of a rotary evaporator, keeping the temperature less than the boiling point of the respective solvent. The extracts were collected in tarred, cleaned and labeled storage vials, and allowed to dry in the oven at 40

°C until the solvent disappeared.

2.3. Phytochemical analysis

2.3.1. Estimation of Primary Metabolites

The different extracts of *Melia azedarach* fruit seed were evaluated for the quantification of primary metabolites such as carbohydrates, proteins, and total lipids.

2.3.1.1. Estimation of total lipids

Continuous hot extraction of the plant was performed to determine the lipid content (17). Petroleum ether was used as a solvent. Before extraction, the thimble was macerated in an organic solvent for about 12 hours and then extraction was performed in Soxhlet apparatus at 40°C - 60°C for 24 hours. The extract was then filtered and dried in the rotary evaporator at 50°C up to 10 ml. It was then stored in an oven so that extract was dried. After drying, the weight of the extract was determined to calculate the total lipid contents.

2.3.1.2. Estimation of total proteins

Total protein contents of *Melia azedarach* fruit seed were evaluated according to the protocol prescribed by Lowry et al. (1951) (18). About 1 g of fruit-seed powder was weighed accurately. The sample was then mixed with 10 ml of distilled water along with 2-3 drops of Triton-X and macerated for about thirty minutes. It was then centrifuged at 2700 rpm for at least 10 minutes. A 100 µl of the supernatant was collected in a falcon tube and made-up volume up-to 1 ml with distilled water. About 3 ml of reagent C that was prepared by mixing 50 ml of reagent A (composed of mixing 2% sodium carbonate in 0.1N sodium hydroxide) and 1ml of reagent B (composed of mixing 0.5% copper sulphate in 1% potassium sodium tartrate)) was added in the falcon tubes. After adding reagent C, 200 µl of Folin-Ciocalteu reagent was added in the respective falcon tubes and incubated for 30 minutes at room temperature. To plot the standard curve, Bovine Serum Albumin (BSA) of different concentrations was used as a standard. Blank was prepared using the same method that except standard and sample. The absorbance

of plant and standard was measured at 600 nm. Total protein was performed in triplicated and evaluated from standard curve by using linear regression equation.

2.3.1.3. Estimation of total carbohydrates

Total carbohydrates were then evaluated by the following formula as mentioned in Al-Hooti (2008) (19).

Total carbohydrates (%) = 100 - (total protein + total ash + total fat + moisture content)

2.3.2. Estimation of Secondary Metabolites

2.3.2.1. Estimation of polyphenols

Total polyphenol contents of fruit seed of *Melia azedarach* were evaluated according to protocol prescribed by Singleton and Slinkard (1997) (20). A methanolic solution of Gallic acid (mg/ml) of different concentrations was used as a standard to plot standard calibration curve. Accordingly, 200 µl of standard/test solution was taken into falcon tubes. A 200 µl of Folin-Ciocalteu reagent was added in the respective falcon tubes and mixed the solution homogeneously. After five minutes, 1ml of 7.5% of sodium carbonate was added into the falcon tubes and the final volume was made up to 3 ml with methanol. Blank was treated like test solution/standard with the exception of standard or test solution which is replaced by methanol. The reaction mixture was incubated at room temperature for about 2 hours and absorbance was then measured at 760 nm. Standard curve of gallic acid was plotted and polyphenol content was determined from the calibration curve of the standard. The phenolic contents were expressed as mg/g of gallic acid equivalent by the linear regression equation.

2.3.2.2. Estimation of total flavonoids

Pavun *et al* (2018) method was used for the determination of the total flavonoids with little modification (21). A methanolic solution of quercetin (mg/ml) of different concentration was used as a standard to plot the standard calibration curve. A reaction mixture contains 200 µl of standard/test solution, 100 µl 10% aluminium nitrite and 100 µl of 1M potassium acetate and final volume was made up to 5 ml with distilled water. Falcon tubes were then

incubated for 30 minutes at room temperature and absorbance was measured at 415 nm. The standard curve of quercetin was plotted and flavonoid content was determined from the calibration curve of standard.

2.3.2.3. Estimation of polysaccharides

To determine the total polysaccharide content, the protocol recommended by Hussain *et al.*, was used with little modification (22). A 200 mg of each extract was mixed with 7ml of hot ethanol (80%) in falcon tubes to eliminate any soluble sugars. The reaction mixture was vortexed for 5 minutes and then centrifuged at 2700 rpm for 10 minutes. The supernatant layer was discarded. The residue was washed with anthrone reagent (0.2% in concentrated sulphuric acid) until the color disappeared and placed into the water bath until the residue was dried. After that, 5 ml of HCl (25%) and 5 ml of distilled water were added into respective falcon tubes. The falcon tubes were then incubated at 0°C for 25 minutes and centrifuged at 2700 rpm for 10 minutes. This process was repeated three times and the supernatant was collected in a 100ml volumetric flask final volume was made with distilled water.

Then, 100 µl of supernatant was transferred into the test tubes and the final volume was made up to 1 ml with water and 4 ml of anthrone reagent was then added. The reaction mixture was then heated in a boiling water bath for eight minutes and allowed it to cool. The absorbance of the reaction mixture was measured at 630 nm. The glucose was used as standard and total polysaccharides were evaluated by multiplying the glucose content by 0.9.

2.3.2.4. Estimation of glycosaponins

Total glycosaponins were estimated according to the protocol as prescribed by Hussain *et al.* 2008 (22). Accordingly, 1 g of different extracts was refluxed in 50 ml methanol for 30 minutes. The process was repeated until all the glycosaponins were extracted. The extracts were then concentrated up to 10 ml through a rotary evaporator. After that, the concentrated extract was added into the beaker containing 50ml of acetone. The precipitates were obtained and dried in an oven at 60°C.

The weight of precipitates was measured to calculate the total glycosaponins by using the following formula;

$$\text{Total glycosaponins} = \frac{\text{Weight of precipitates} \times 100}{\text{Weight of sample}}$$

2.4. Anti-urolithiatic activity

2.4.1. Aggregation assay

The anti-urolithiatic activity was determined using aggregation assay (23). Calcium oxalate was prepared by mixing equimolar calcium chloride solution (50 mM) and sodium oxalate solution (50 mM) and allowed it to stand overnight for the formation of calcium oxalate crystals. The crystals were then washed with ethanol twice to remove any impurities. It was then filtered and placed in the oven at 60 °C for 1 hour. Calcium oxalate solution was prepared in Tris-HCl buffer at a concentration of 8 mg/ml at pH 6.5. The reaction mixture contains 1 ml of extracts (5 mg/ml) and 2 ml of calcium oxalate solution. The test was performed at 37 °C. The absorbance was measured at 620 nm for 30, 60, 180 and 360 minutes. Cystone was used as a standard. Control is treated like the test solution except standard/extract solution which was replaced by the solvent used. The turbidity was calculated by using the following formula;

$$\text{Turbidity (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

2.5. Nucleation assay

Nucleation assay was assessed according to the published protocol with little modification (23). The

solution of calcium chloride (4 mM) and sodium oxalate (7.5 mM) was prepared in tris HCL buffer (50 mM Tris-HCl and 150 mM NaCl) at pH 6.5. A volume of 1 ml extract (5 mg/ml) was mixed with 1ml of calcium chloride solution and incubated at 37 °C for 5 minutes. A volume of 1 ml sodium oxalate solution was then added to induce the reaction of crystallization. The test solution was maintained at 37°C. The absorbance was measured at 620 nm for 30, 60, 180 and 360 minutes. Cystone was used as a standard. A control is treated like test solutions with the exception of standard/extract solution which is replaced by solvent used. The turbidity was calculated by using the following formula;

$$\text{Turbidity (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

2.6. Statistical analysis

All the tests were performed in triplicate and the means were calculated. All the values were expressed as means \pm standard deviation (SD). A t-test was utilized to test for comparing two groups. A p-value <0.05 was considered to indicate statistical significance.

3. RESULTS AND DISCUSSION

3.1. Phytochemical screening

The standard curves of Bovine Serum Albumin (BSA), quercetin, gallic acid and glucose were plotted for the estimation of total proteins, flavonoids, polyphenols and polysaccharides (Figure I-IV).

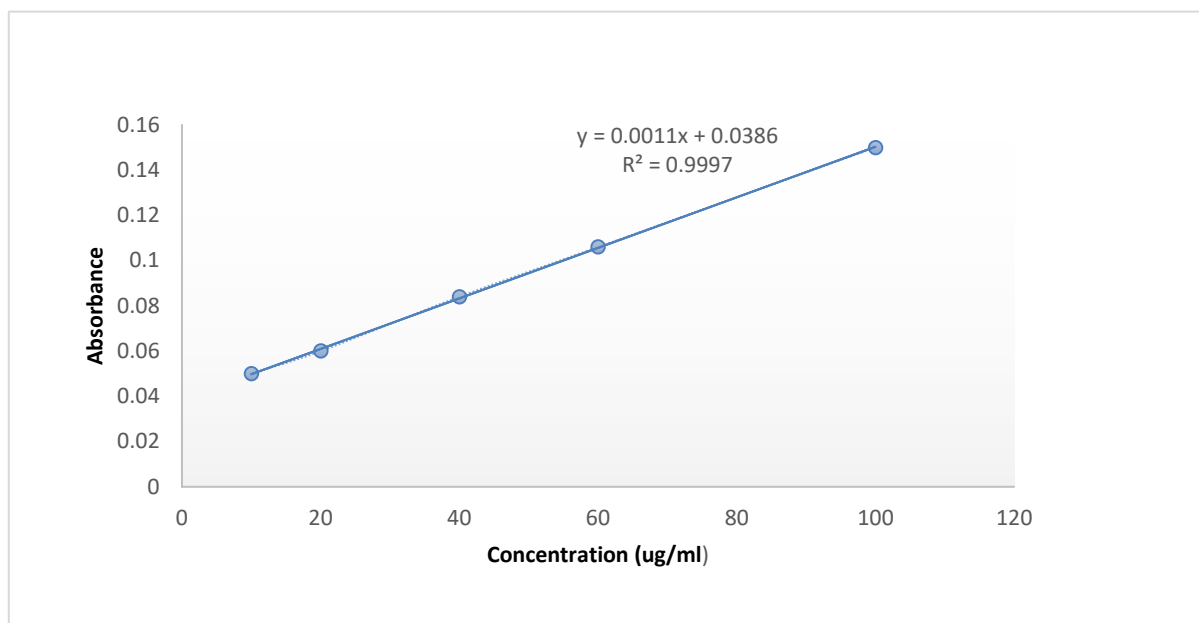


Figure I: Standard curve of Bovine Serum Albumin (BSA) for the estimation of proteins.

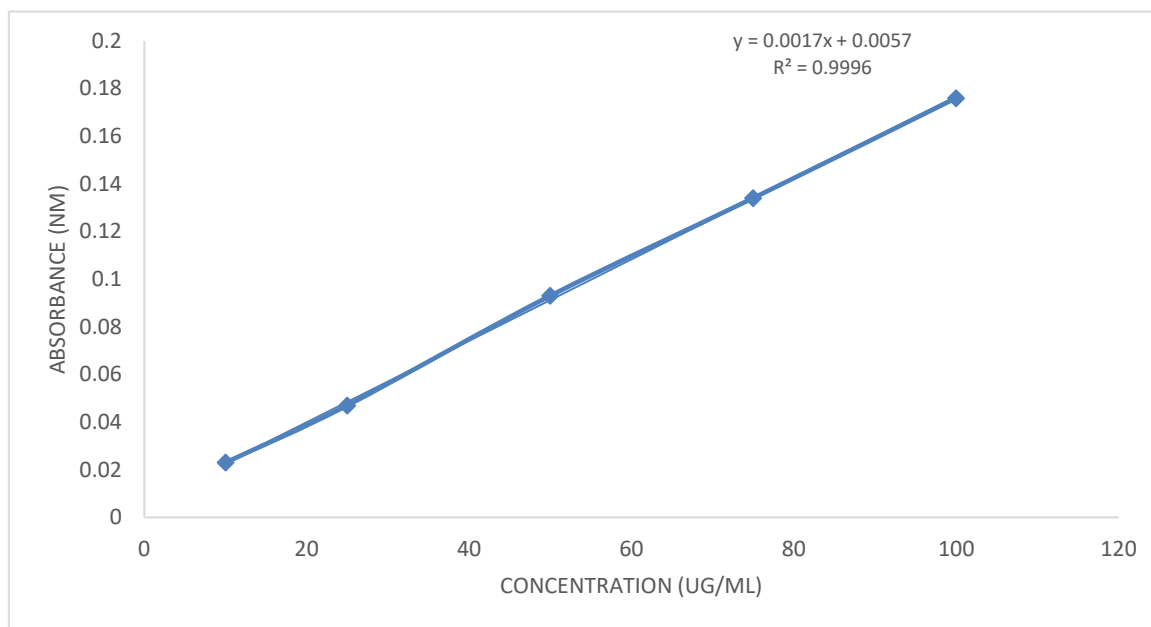


Figure II: Standard curve of Quercetin for the estimation of flavonoids.

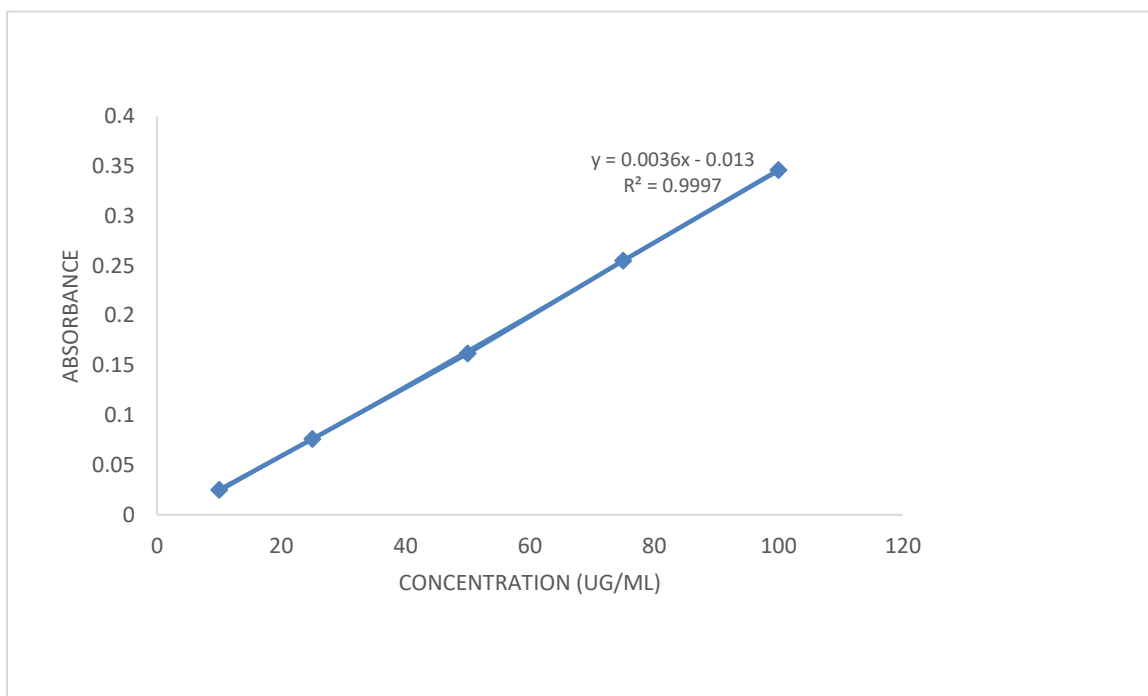


Figure III: Standard curve of Gallic acid for the estimation of polyphenols.

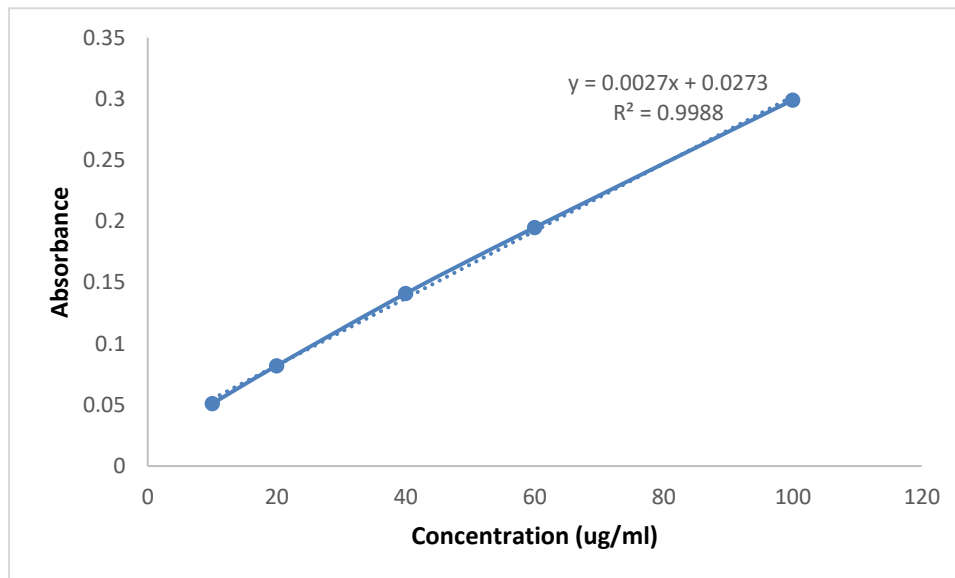


Figure IV: Standard curve of glucose for the estimation of polysaccharides.

Melia azedarach reported the presence of total proteins (31.97 ± 0.56%), total lipids (2.95 ± 0.03%) and total

carbohydrates (64.90 ± 0.56%) as shown in Table I. The findings of secondary metabolites (total polyphenols, total

flavonoids, total polysaccharides and total glycosaponins) were summarized in the Table II. The chloroform extract showed the maximum quantity of polyphenols (77.65 ± 0.53 mg/g) and flavonoids (74.71 ± 0.67 mg/g). However, a large amount of glycosaponins (42.71 ± 0.53 mg/g) and polysaccharides (125.27 ± 0.41 mg/g) were observed in aqueous extract. Phytochemical screening is essential for the identification of lead components that possess pharmacological activities (24). These include primary (proteins, carbohydrates, and lipids) and secondary metabolites (Polyphenols, tannins, flavonoids, alkaloids and saponins) (25). Our present study reported the

presence of phytochemical constituents in *Melia azedarach*. Another study reported the presence of proteins, carbohydrates, lipids, glycosides, flavonoids, and polyphenolic compounds in plant (26). The maximum quantity of polyphenols and flavonoids was present in chloroform extract. However, Ahmed and his colleagues documented that the alcoholic extract had large quantity of polyphenolic compounds, followed by aqueous and petroleum ether extracts (27). A variety of phytochemical constituents was found in plants that exhibited pharmacological and therapeutic activities.

Table I. Total primary metabolites in fruit-seed powdered material of *Melia azedarach*.

Primary Metabolites	Percentage content \pm SD
Total protein	31.97 ± 0.56
Total lipids	2.95 ± 0.03
Total carbohydrates	64.90 ± 0.56

Table II. Total content (mg/g) of secondary metabolites of fruit-seed extracts of *Melia azedarach*.

Extracts	Total Polyphenols (mg/g)	Total Flavonoids (mg/g)	Total Glycosaponins (mg/g)	Total Polysaccharides (mg/g)
Chloroform	77.65 ± 0.53	74.71 ± 0.67	3.86 ± 0.04	6.38 ± 0.42
Methanol	43.02 ± 0.72	23.06 ± 0.69	11.21 ± 0.03	86.47 ± 0.49
Aqueous	23.32 ± 0.53	10.31 ± 0.33	42.71 ± 0.53	125.27 ± 0.41

3.2. Anti-urolithiatic activity

The effect of inhibition of aggregation and nucleation activities for various plant extracts were illustrated in Figure V and VI. All the extracts showed increasing trends with an increase in incubation time. In aggregation assay, the chloroform extract ($55.85 \pm 1.43\%$) possessed more potency in the dissolution of calcium oxalate crystals,

followed by methanolic extract ($48.58 \pm 3.48\%$) and aqueous ($45.74 \pm 5.37\%$) after the incubation of 360 minutes (Figure V). Similarly, in nucleation assay, chloroform extract exhibited highest inhibitory activity ($56.42 \pm 2.39\%$), followed by methanolic ($54.10 \pm 4.49\%$) and aqueous ($39.70 \pm 0.76\%$) extract (Figure VI).

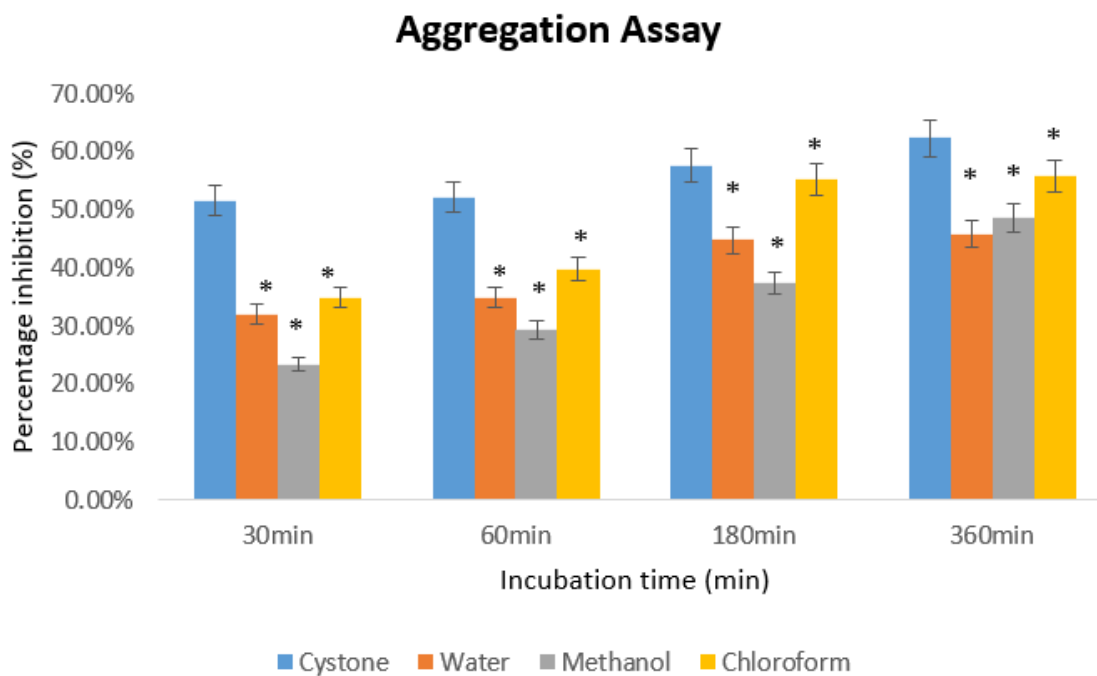


Figure V: Aggregation assay of different extracts of fruit-seed of *Melia azedarach* and positive control (cystone). Bars with asterisk (*) are significantly different from standard (Cystone), $p < 0.05$.

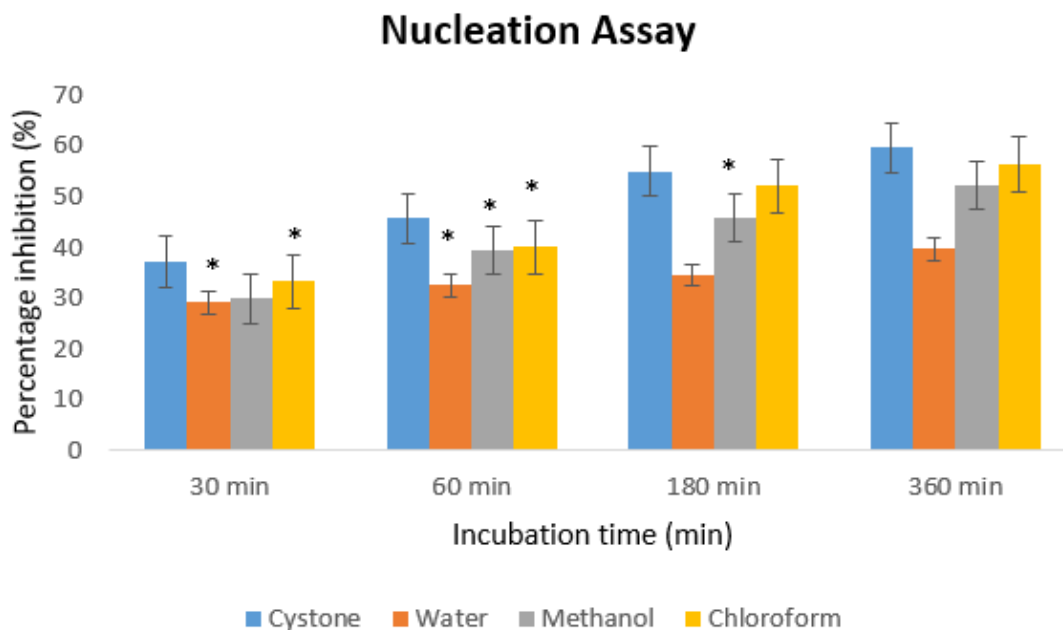


Figure VI: Nucleation assay of different extracts of fruit-seed of *Melia azedarach* and positive control (cystone). Bars with asterisk (*) are significantly different from standard (Cystone), $p < 0.05$.

In aggregation assay, the difference in activity between methanolic extracts and control was statistically significant ($p < 0.05$). However, in nucleation assay, the activity between extracts and control was not statistically significant after the incubation of 360 minutes ($p > 0.05$). The order of inhibitory activity of various plant extract were cystone > chloroform > methanol > aqueous.

Due to presence of various phytochemicals, *Melia azedarach* showed the anti-urolithiatic activity. However, a study reported that alcoholic and aqueous extracts of plant leaves exhibited highest inhibitory activity against ethylene-glycol-induced calcium oxalate urolithiasis in male albino Wistar rats (1). It increased the urine volume and urinary magnesium levels and minimized the level of urinary phosphate, oxalate and calcium. Similarly, another study reported the anti-urolithiatic activity in alcoholic extracts of plant leaves (3). The results may differ due to geographical origin of the plant. However, the mechanism of action is still not clear. It could be due to the presence of antioxidant, antilithiatic, diuretic, and anti-inflammatory constituents in plant. There is no data available on fruit-seed extract of *Melia azedarach*.

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4. CONCLUSION

M. azedarach was found to be rich in primary as well as secondary metabolites which represents that there will be a high content of potential bioactive compounds to treat number of diseases. The chloroform extract showed the high concentration of polyphenols and flavonoids. The outcomes of the present study reveals that *M. azedarach* has a potential to prevent the aggregation and nucleation of calcium oxalate crystals. However, further investigation is needed to assess the mechanism of action in animal models of lithiasis. The positive outcomes may be due to the presence of phytochemicals, thus, further characterization, isolation of bioactive components from plant extracts are needed. Extensive research is required to characterize and isolate bioactive constituents and evaluate other mechanisms of action.

Conflicts of interests

None.

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فحص كيميائي نباتي ونشاط مضاد لمجرى البول في المختبر لمستخلصات بذور الفاكهة من ميليا أزداراش

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ملخص

تم استخدام ميليا أزداراش في أنظمة الطب التقليدية لعلاج العديد من الأمراض بما في ذلك تحص بولي. هدفت الدراسة إلى إجراء دراسات كيميائية نباتية وإمكانات مقاومة المسالك البولية لمستخلصات بذور الفاكهة من ميليا أزداراش. تم إجراء الاستخراج المتسلسل باستخدام الكلوروفورم والميثانول والماء. ثم خضعت المستخلصات النباتية لاختبارات كمية لتحليل الكيمياء النباتية. تم التحقيق في نشاط مضاد لمجرى البول عن طريق التنوي في المختبر ومقايسة التجميع باستخدام مقياس الطيف الضوئي. أظهر التحليل الكيميائي النباتي الأولي وجود بروتينات (31.97 ± 0.56%)، دهون (2.95 ± 0.03%) وكربوهيدرات (64.90 ± 0.56%). يحتوي مستخلص الكلوروفورم على أقصى كمية من البوليفينول (77.65 ± 0.53 جم / جم) والفلافونويد (74.71 ± 0.67 جم / جم). بتركيز 5 ملغ / مل، أظهر مستخلص الكلوروفورم أقصى نشاط مثبط في كل من التجميع ومقايسة النواة (55.85 ± 1.43% ؛ 56.42 ± 4.49%) على التوالي. أظهرت جميع المستخلصات نشاطا كبيرا في مقاومة المسالك البولية عن طريق تثبيط تبلور أكسالات الكالسيوم. بسبب وجود المستقلبات الأولية والثانوية، يمكن أن يكون النبات بمثابة مصدر للأدوية المفيدة.

الكلمات الدالة: ميليا أزداراش، التحليل الكيميائي النباتي، مضاد للجراثيم، فحص التجميع، فحص النواة.

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