EVALUATION OF INVITRO AND INSILICO ANTILITHIATIC AND ANTIOXIDANT POTENTIAL WITH THE ACETONIC EXTRACT OF PUNICA GRANATUM WHOLE FRUIT

Abstract

Development of stones anywhere in the urinary sections, are referred to as urolithiasis. The Punicaceae plant family comprises Punica granatum. Pomegranate fruit is utilised in the ailment of diarrhoea, dysentery, and intestinal parasites and has anti-inflammatory and antibacterial qualities. The objectives of our study is to evaluate the antilithiatic activity by *in vitro* and *in silico* molecular analysis of an whole fruit acetonic extract of *Punica granatum* Cystone used as a standard drug in the present study. Plant screening resulted the presence of tannins, terpenoids, alkaloids, glycosides, carbohydrates, saponins and steroids. The acetonic extract of *Punica granatum* was proved to possess prominent antioxidant activity by reducing power assay. By reducing the concentration of reactive oxygen species i.e. measured by the transformation of Ferric ion to Frrousion, increased absorption of reaction mixture indicates increased reducing power. The Nucleation and aggregation processes of CaC2O crystallisation were both hindered by the administration of PGAE and Cystone coupled with calcium chloride dihydrate; the decrease in solution turbidity in both phenomena indicated percentage inhibition. *In vitro* anti-lithiatic activity by Homogenous and precipitation method, PGAE and Cystone dissolved calcium oxalate precipitate. The compounds present in *Punica granatum* whole fruit, acetonic extract they are docked with protein 5G3N. Ellagic acid, Alpha tocopherol, Brevifolin, Kaempferol, Maslinic acid shown good docking score Overall results explain us that PGAE has proven *in-vitro* anti-lithiatic activity and antioxidant activity.

Keywords: *Punica granatum*, Homogenous and precipitation method, docking.

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I. INTRODUCTION

The term "urolithiasis," which is drawn out from a Greek term "ouron" (for urine) plus "lithos" (for stone), describes the buildup of tough, solid, & non-metallic particles within in the urinary system. Aphorisms of Hippocrates describe the clinical development and expression of this chronic and widespread ailment (1). Defined also as an kidney calculus, a renal stone is a hard crystalline that develops in the kidney via minerals within urine. The preponderance of stone formation is made up of calcium salt crystals (2).

Five years after the first episode with symptomatic renal lithiasis, recurrence chances have been found to be approximately to 50% (3). The Nucleation & aggregation assay and the homogenous precipitation method are *in vitro* models for anti-lithiatic action. Cystone is the standard medication employed in the trial. Allopathic drugs could eliminate healthy microorganisms and have unfavourable side effects that make other therapies less successful (4). Traditional medical systems are still used extensively on many fronts. For a broad range of ailments in human were treated with herbal materials as a source that has received more attention as a result of factors including population growth, insufficient drug supply, the restrictive cost of therapies, adverse reactions of many pharmaceutical chemicals, and resistance emergence to presently utilised medicine for infectious ailments.

Punicaceae belong to plant family which comprises the pomegranate, scientific name is *Punica granatum* L. High therapeutic and nutritional qualities make it a significant fruit. The pomegranate plant's various elements have medicinal benefits, including the fruit's anti-inflammatory and antibacterial properties, the seed oil's inhibitory effect on skin and breast cancers, and it shows an presence of phytoestrogen compounds. The fruit is also abundant in phenolic content, which have potent antioxidant properties. It is employed against dysentery, intestinal parasites, diarrhoea, heart and throat tonic. It is used to treat haemorrhoids and halt nose and gum bleeding (5).

A crucial technique in molecular biology and computer-aided drug design is *in silico* analysis of molecular docking. Predicting the dominant linkage mode(s) of a receptor with a protein with a recognized three-dimensional configuration is objective ligand-protein docking.

The goal of study to perform *in-vitro* analysis of anti-lithiatic activity and antioxidant activity by *Punica granatum* whole fruit extract.

II. MATERIALS AND METHODS

The process of establishing a technique entails a number of processes carried out in a methodical manner in order to accomplish the desired outcomes in accordance with the established standards and rules.

1. **A Phytochemical screening of plant:** The herb serves as a laboratory of biosynthetic producing a variety of chemicals, including glycosides, alkaloids, volatile oils, tannins, and others that have physiological & medicinal effects in addition to chemical molecules like carbohydrates, protein, and lipids.

2. **Evaluation of antioxidant assay by *in vitro* method:** *Punica granatum* whole fruit acetonic extract was used to test the antioxidant activity *in vitro* using a reducing power assay methodology.

- **Reducing power assay:** Enhanced absorbance is a sign that antioxidant activity has elevated. The methodology is based on the idea that reaction combinations absorb additional light. Potassium ferricyanide ($Fe^{3+)}$ combines with substances that have the ability to reduce it to make potassium ferrocyanide (Fe^{ii+}), that eventually interacts with fecl3 to create ferric complex, which contains absorption peak at 700 nanometre. Phosphate buffer pH 6.6, Potassium dihydrogen phosphate (0.2 M) solution, Sodium hydroxide solution (0.2M) solution, Potassium ferric cyanide (1% w/v) solution and Ferric chloride solution (0.1% w/v) are the reagents required for the assay.

  - Add potassium ferricyanide 2.5 mL (1 percent by weight) and the phosphate buffer of 205 mL pH 6.6 to 1 mL of the standard and test substances, and then incubate at 500 C for about 30 minutes.
  - For stated supernatant liquid of 2.5 mL, distilled water 205 mL, and 0.5 mL of a 0.1 percent w/v $FeCl_3$ solution were added.
  - Employing an Ultra violet - visible analyser, the absorbance of complex (ferric ferrous) was determined utilising a phosphate buffer at pH 6.6 as a reference control at 700 nanometre and the increase in absorbance was calculated (6).

The present increase in reducing power is calculated using the following equation,

Where 'Abs_{test}' is absorbance of test solution: 'Abs_{blank}' is absorbance of blank.

3. **Evaluation of anti lithiatic activity by in vitro method:** *Punica granatum* whole fruit's acetonic extract was used to test the anti lithiatic activity *in vitro* using the Nucleation and aggregation assay and homogenous precipitation technique.

- ***Nucleation and aggregation assay:*** A freshly made combination of 200 mM NaCl and 10 mM sodium acetate trihydrate, together with 10 mM $CaCl_2$ dihydrate and 1.0 mM sodium oxalate, was brought to pH 5.7. A flowing water bath was used to conduct each experiment at 37 °C. Sodium oxalate of 25 mL solution was allowed

$$\text{Percentage increase in reducing power (\%)} = Abs_{test} - Abs_{blank} \,/\, Abs_{blank} * 100$$

into a beaker and maintained at 37 C by placing on a hot plate magnetic stirrer (Model 2MLH,REMI) upon stirring continuously at about 800 rpm, to perform crystallisation studies.

Before adding 25 mL of calcium chloride solution, an additional 1 mL of distilled water, the standard (Cystone), and the extract is added. After the addition of a calcium-containing solution, the (OD) optical density is examined at 620 nanometre using a spectrophotometer, first every 15 s over 5 minutes followed by every 1 minute over 10 minutes. The studies were all carried out in triplicate. To evaluate the intensity of constituted crystals in finished solutions, they were observed under a light microscope (Olympus, the USA).

With the use of following formula, the percentage of inhibition by the influence of Cystone or PGAE was compared to control. The formula used to determine the percentage inhibition was

$$\boxed{1 - \frac{T_{si}}{T_{sc}} * 100}$$

Where T_{sc}, the turbidity slope of control; and T_{si}, the turbidity slope in the presence of the inhibitor (7)

- **The Homogenous method of precipitation**

Step 1: The generation of experimental calcium oxalate kidney stones using homogeneous precipitation: Equimolar solutions of sodium oxalate (AR) in 10 mL of 2 N H_2SO_4 and $CaCl_2$ dihydrate in distilled water are combined and permitted to respond in a beaker with enough distilled water. Calcium oxalate was the precipitate that was obtained. Ammonia solution cleans precipitate of sulphuric acid residue. It was rinsed in distilled water and dried for four hours at 60 degrees Celsius.

Step-2: Semi-permeable membrane preparation employing farm eggs: A semi-permeable layer over an egg sits between its calcified outside shell and it's inside yolk and albumin. By soaking the eggs using 2M Hydrochloric acid for quite a night that resulted in full decalcification, the shell being chemically removed from the eggs. Additionally, the egg was gently poked with a pointed tip, thoroughly washed using distilled water, then had the contents strained out apart out from a decalcified egg. After properly washing the layer of egg membrane with water, it was immersed in ammonia solvent and kept moist for a time before being completely rinsed. Preserved in a refrigerator with a pH of 7.3–7.4.

Step-3: Titrimetry determination of calcium oxalate: 1 mg of calcium salt of oxalic acid and 10 mg of an extract, chemical, or standard were precisely weighed, suturing them together in a membrane obtained from eggs. A titration flask holding 0.1M TRIS buffer (100 mL) was used to suspend this. One group served as the negative control (contained calcium oxalate of 1 mg). All conical flasks in an incubator were warmed for 2 hours, or for roughly 7-8 hours, to 37 C. Each group's semi-permeable membrane's contents were taken out and placed in a test tube. The endpoint is reached by adding 2 millilitres of 1N sulphuric acid and titrating with 0.9494 N $KMnO_4$.

1 mL of 0.9494N $KMnO_4$ = 0.1898 mg of 4 Calcium

To determine how much calcium oxalate really dissolved the test chemical, the quantity of undissolved calcium oxalate was deducted from the total quantity utilised in the experiment at the beginning (s) (8).

4. **Insilico analysis: Molecular docking studies:** A specific type of bioinformatic simulation called molecular docking includes the combination of more than one molecule

to produce a stable compound. It makes predictions about the three-dimensional configuration of every complex based on the binding characteristics of ligand as well as target. Utilizing the score feature of the mCULE programme, distinct potential compound structures obtained using molecular docking are graded and categorised. Biochemical docking needs a dataset to search for targets with the correct PDB format and a mechanism to design compounds in the mCULE. In a ligand-receptor complex, intermolecular reactions are crucial and challenging modelling exercises. Typically, the ligand molecules are permitted to vary while the receptor is kept stiff or somewhat rigid (9). Through the discovery studio visualizer, the resulting docking visuals are seen. The glide score technique was used to choose the best docked structures. The binding is more favourable, greater negative is the score.

III. RESULTS

Using appropriate *in-vitro* models and *in silico* studies, an extract of *Punica granatum* whole fruit by acetone was investigated for its antioxidant and anti-lithiatic activity. All the data achieved in the study were presented below.

1. **Phytochemical screening of plant:** The acetonic extract contained tannins, terpenoids, alkaloids, glycosides, carbohydrates, saponins, and steroids, according to phytochemical analysis.

2. **Evaluation of antioxidant assay by *in vitro* method**

   - **Reducing power assay*:*** This assay was used to measure the antioxidant activity by *Punica granatum* of acetone extract.

Table 1: Antioxidant activity of *Punica granatum* acetone extract whole fruit by using reducing power assay

S. No	Compounds	Concentration (µg / mL)	% Inhibition (Mean±SEM)	IC_{50} value (µg / mL)
1	PGAE	10 20 30 40 50	18.33±0.98 23.33±0.72 36.66±0.54 58.6±0.72 68.3±0.72	34
2	Ascorbic acid	10 20 30 40 50	21±0.47 34.3±0.98 56.6±0.98 69±0.47 73.3±0.98	29

With increasing doses, PGAE has demonstrated a greater percentage of free radical inhibition, and IC50 value was discovered to be 40 µg/mL. The extract's potential was still on track with that of reference ascorbic acid, and IC50 value was determined to be 29 g/mL.

3. Evaluation of anti lithiatic activity by in vitro method

- **Nucleation and aggregation assay:**

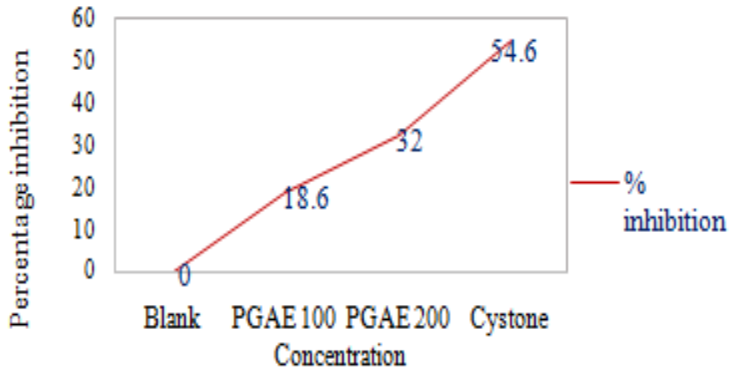


Figure 1: The effect of PGAE and Cystone in *in-vitro* nucleation and aggregation assay method.

The anti-lithiatic activity of the acetone extract of *Punica granatum* is carried out by employing *in-vitro* nucleation and aggregation assay. The blank group showed high turbidity so the percent inhibition was found to be 0 %. PGAE has shown an increase in percent inhibition, a decrease in turbidity with an increase in dose. PGAE (100) shown 18.6 % and PGAE (200) shown 32 % of percent inhibition**.** The potential of the extract was comparable with standard Cystone and percent inhibition value was found to be 54.6.

- ***The Homogenous method of precipitation***

Step 1: The generation of experimental calcium oxalate kidney stones using homogeneous precipitation

A - Preparation of equimolar solutions of sodium oxalate & $cacl_2$,

B - Mixer of Equimolar solutions in distilled water

C – Calcium oxalate ppt filtered,

Step 2: Semi-permeable membrane preparation employing farm eggs

D –Eggs covered with HCl overnight

E – Squeezing of the contents from an egg

F – Egg membrane in ammonia solution

Step 3: Titrimetry determination of calcium oxalate

G – CaC_2O_4 + 10 mg control/PGAE/Cystone sutured in membrane and immersed in a conical flask with Tris buffer and

H - Estimation of percent dissolution of calcium oxalate by titrimetric.

Figure 2: *In-vitro* anti-lithiatic activity by the Homogenous method of precipitation

Table 2: The outcome of PGAE on percent dissolution CaC_2O_4 by the homogenous method of precipitation.

Groups	$KMnO_4$ (mL)	Dissolved Calcium (mg)	Undissolved calcium (mg)	Percent dissolution calcium oxalate (%)
Blank	-	-	1	0
PGAE	2.5	0.45	0.55	45
Cystone	3.2	0.57	0.42	57.6

The homogeneous precipitation technique was used to test *Punica granatum*'s acetonic extract's *in-vitro* anti lithiatic activity. The solubility of calcium oxalate in the blank group was determined to be zero percent. Calcium oxalate dissolution has increased to 45%, according to PGAE. The PGAE potential was comparable with regular Cystone, and 57.6 percent of calcium oxalate was observed to dissolve.

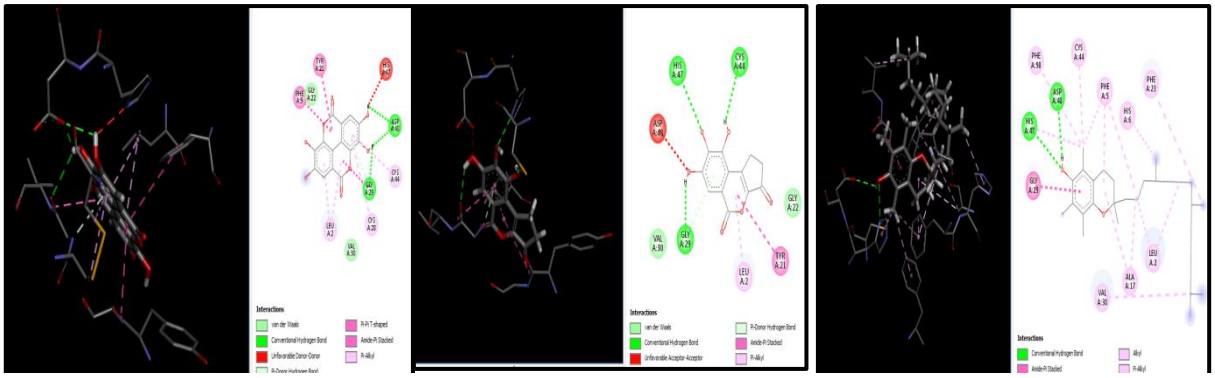
4. **Insilico analysis: Molecular docking studies:** Initially the protein is downloaded from PDB and prepared by removing extra chains. Attributes of spheres are prepared and noted. Molecules identified from GC-MS were selected. Later molecules drawn in mCULE and ligprep is created. Protein is uploaded with sphere attributes and the structures were docked against 5G3N protein. Docking indicated that some of our compounds have good binding ability with phospholipase A2 inhibitor protein (PDB ID:

5G3N). Following are the ligand interactions of compounds present in *Punica granatum* whole fruit with 5G3N protein.

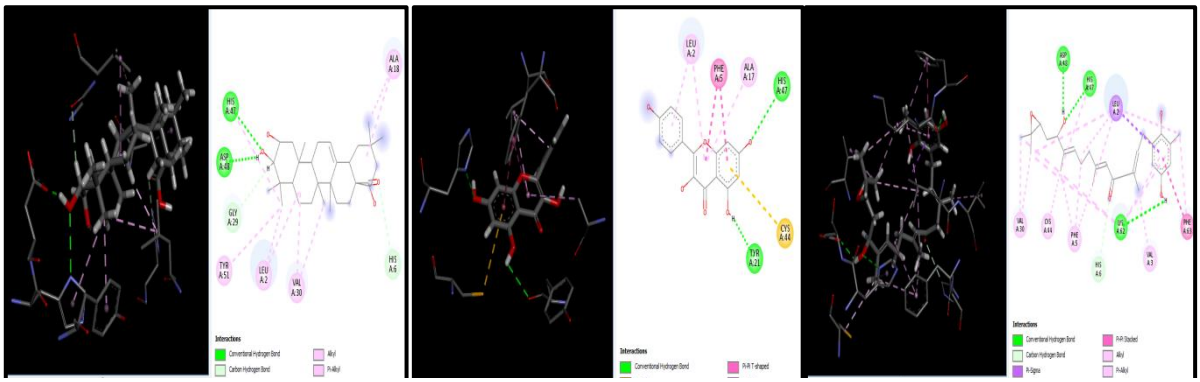
Table 3: Mcule docking scores

Compounds	Score
Ellagic acid	-8.8
Gallic acid, Punicalin	-5.5
Oleic acid	-5.6
Linoleic acid, Vanilic acid	-5.9
Cafffeic acid	-6.2
Ferulic acid	-6.4
Alpha tocopherol	-7.4
Beta carotene	-5.5
Kaempferol	-7.1
Brevifolin	-8.1
Maslinic acid	-7.2
Cystone A	-7.8

The greater negative the score the more favourable the binding.



a) Ellagic acid score -8.8 **b) Brevifolin -8.1** **c) Alpha tocopherol -7.4**



d) Maslinic acid -7.2 **e) Linoleic acid -5.9** **f) Cystone A-7.8**

Figure 3: *Insilico* analysis by molecular docking studies

IV. DISCUSSION

As urine is indeed a metastable flow containing a number of coexisting components that might precipitate to produce urinary calculi, variables related to urine composition are crucial in crystal growth (10). According to reports, Cystone is effective in treating urolithiasis since it balances crystalloid and colloid levels as well as operates by dissolving crystals (11). The present work objective is of phytochemical analysis with anti-lithiatic activity of *Punica granatum* whole fruit, acetone extract with anti-lithiatic and antioxidant activity. Initial phytochemical analysis of PGAE revealed the presence of terpenoids, alkaloids, glycosides, steroids, flavonoids, carbohydrates, and saponins.

By lowering the quantity of superoxide anion, which is assessed by the conversion of iron (III) to iron (II) the acetonic extract of *Punica granatum* has shown to exhibit significant antioxidant properties. Enhanced absorption of the reaction mixture denotes increased reducing power was shown to exhibit significant antioxidant properties. Enhanced absorption of the reaction mixture denotes increased reducing power.

Upon the presence of $CaCl_2$ dihydrate, the turbidity gradually increased up to 5 min (nucleation phenomena) and then declined linearly for up to fifteen minutes (aggregation) in an in vitro calcium oxalate coalescence investigation. The nucleation and aggregation approaches of CaC_2O_4 crystallisation are suppressed by the application of PGAE, Cystone, plus calcium chloride dihydrate. The inhibition of *in-vitro* crystallization of CaC_2O_4 suggests that PGAE has influence on the formation of crystals from sodium oxalate and calcium chloride and/or their aggregation.

The involvement of PGAE and Cystone in disintegrating the previously developed stones in the renal system is ascertained with an *in vitro* anti-lithiatic efficacy by homogenous precipitation method. Calcium oxalate and calcium phosphate are dissolution procedures. Comparing Cystone and PGAE to a blank, the percentage solubility of CaC_2O_4 is higher.

The compounds present in *Punica granatum* whole fruit, by acetone extract they are docked with protein 5G3N. In order to alleviate or counteract the generated mucosal inflammatory response that result in edema development due to the presence of stones, steroids have been utilized (12). Rich terpenoid content, which impeded the emergence of calcium oxalate deposits by any of the methods, enhanced bioavailability of nitric oxide, which inhibits the calcium influx by cyclic guanosine mono phosphate pathways, and its nephron-protective effect may have all contributed to the potential urolithiasis preventive effect (13).

Herbal flavonoids can indeed in vitro and in vivo successfully prevent the development of CaOx stones (14). Ellagic acid, Alpha tocopherol, Brevifolin, Kaempferol, and Maslinic acid had good docking scores. The reliability of our docking investigation is been supported in the current study by the superposition of Ellagic acid, Maslinic acid, and an brevifolin structure from both crystal structure and docking, including the comparable interactions discovered in the cytosolic PLA2 binding site (15).

Overall findings show that PGAE has demonstrated in-vitro antioxidant activity and anti-lithiatic action. The usage of molecular docking studies to substances found in PGAE

with protein 5G3N is regarded as being quite beneficial and shown to have anti-lithiatic activity by phospholipase A2 inhibitory action.

V. CONCLUSION

The anti-lithiatic and antioxidant potential of PGAE was assessed in the current study. The initial phytochemical screening of PGAE showed the appearance of Carbohydrates, Alkaloids, Terpenoids, Glycosides, Saponins, Steroids, tannins and Flavonoids. *Punica granatum*'s acetonic whole fruit extract neutralised free radicals and screened for *in vitro* antilithiatic activity by nucleation and aggregation assay showed a decrease in nucleation and aggregation of crystals, PGAE shown an increase in percent inhibition, decrease in turbidity with an increase in dose. In the homogenous precipitation method PGAE has shown enhanced percent dissolution calcium oxalate lower growth of crystal. The compounds' ability to inhibit PLA2 in the cytosol is validated by molecular docking experiments since they occupied the binding site and displayed interactions. The experimental PLA2 inhibitory action was represented in the docking score order for all compounds.

In the future, to understand an active component responsible for anti-lithiatic action and to investigate the mechanism, *in-vivo* activity and the isolation of active ingredients are required.

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