

PRELIMINARY PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT AND ANTI GOUT POTENTIAL OF AQUEOUS SEED EXTRACT OF *PUNICA GRANATUM* - AN IN VITRO STUDY

Running title: Preliminary phytochemical analysis, antioxidant and anti gout potential of aqueous extract of *punica granatum*, Innovative technology, Novel method

ABSTRACT

Background: *Punica granatum* belongs to the family puniceae. Pomegranate has a reservoir of secondary metabolites which possess free radical scavenging activity. Xanthine oxidase (XO) plays an important role in the regulation of production of uric acid. Many research has been done in the extract to analyse the pharmacological characteristics and its beneficial uses. Pomegranate is widely consumed and used as preventive and therapeutic agents.

AIM: The aim of this study is to analyse phytochemical constituents, antioxidant and anti gout activity of aqueous seed extract of *Punica granatum*.

METHODS: Aqueous seed extract of *Punica granatum* was prepared and analysed for its phytochemical, antioxidant and anti gout potential by using statistical methods.

RESULTS: Phytochemicals such as flavonoids, amino acids, alkaloids, terpenoids, steroids were present in aqueous extract of *Punica granatum*. IC₅₀ of antioxidant activity of aqueous seed extract of *Punica granatum* was found to be 280µg/ml. The extract also exhibited anti gout potential with an IC₅₀ = 310 µg/ml. P value is < 0.05 so, it is significant

CONCLUSION: aqueous seed extract of *punica granatum* can be used to treat gout and to combat various other disorders and also have antioxidant and phytochemical activity. It will be an alternative for synthetic drugs. Herbal extracts are more preferred for its accessibility, cost effectiveness and less side effects. The combined effects of antioxidant and xanthine oxidase inhibitors will be helpful in hyperuricemia treatment

KEYWORDS: *Punica granatum*, phytochemical, antioxidant, anti gout, xanthine oxidase, Innovative technology, Novel method,

INTRODUCTION

Punica granatum commonly known as pomegranate. *Punica granatum* is a shrub. It has multiple seeds. It grows up to 1.8 - 4.6 m tall. It is rich in vitamin A,C and E. Pomegranate juice contains a significant amount of anthocyanins and anthoxanthins that support good heart health. Pomegranate has many pharmacological characteristics (1). Synthetic drugs are effective but sometimes cause side effects and are also expensive. Thus herbs can be used as an alternative for the synthetic drug as it is effective and safe (2).

Phytochemicals are natural bioactive compounds. The phytochemicals from natural products contain various chemical compounds such as polyphenols, flavonoids, steroids, saponins etc . It reduces the risk of cardiovascular diseases and cancer (3). Pomegranate peel contains bioactive compounds such as phenolic acid, tannins, flavonoids etc (4). Ligands, triterpenoids, phytosterols, fatty acids and also numerous phytochemicals are identified in different parts of the plants. Aromatic phytochemicals that help to convert the androgen to estrogen in therapeutic targets for treating the hormone sensitive types of breast cancer are also reported. It also helps in colon cancer treatment (5).

Pomegranate has high antioxidants content, which helps in the delayed progress of atherosclerosis, cancer and ageing. The large amount of phenolics present in the peel extract may be a reason for its strong antioxidant ability. The antioxidant activity was evaluated by four different methods like ABTS, DPPH and FRAP. The methanolic extract from the pomegranate exhibits 93.7% inhibition using the thiobarbituric acid methanol, hydroxyl, radical scavenging activity and LDL oxidation (6).

Gout is a chronic metabolic disease. It is caused due to increased deposition of monosodium urate crystals in joints. It is caused due to increased intake of purine rich food. Xanthine oxidase converts hypoxanthine to uric acid. Uric acid crystals accumulate in joints,

causing gouty arthritis (7),(8),(9) The most important treatment of hyperuricemia is the development of an xanthine oxidase inhibitor (10),(11),(12). Research in medicinal plants is conducted to isolate the active ingredients for the treatment of hyperuricemia. Our team has extensive knowledge and research experience that has translate into high quality publications (13),(14),(15),(16),(17),(18),(19),(20),(21),(22),(23),(24),(25),(26),(27),(28)The aim of this study is to analyse the phytochemical constituents, evaluate the antioxidant and anti gout activity in aqueous seed extract of *Punica granatum*.

MATERIALS AND METHODS

Preparation of aqueous seed extract of *Punica granatum*:

Punica granatum was purchased from a farm in Chennai. Peeled ,fruit was crushed and filtered to get the seed extract. The extract was prepared by a hot percolation method.Antioxidant and Anti gout potential of the extract was evaluated.

Phytochemical screening test

1. Test for phlobatannin

1ml of the extract was treated with 1ml of 1% HCl and boiled for 10 mins. The formation of red color precipitate indicates the presence of phlobatannin.

2. Test for carbohydrates

Three to five drops of Molisch reagent was added with 1 mL of the extract and then 1 mL of concentrated sulphuric acid was added carefully through the side of the test tube. The mixture was then allowed to stand for two minutes and diluted with 5 mL of distilled water. The development of a red or dull violet ring at the junction of the liquids showed the presence of carbohydrates.

3. Test for flavonoids

Few drops of 1% liquid ammonia were taken in a test tube and along with it 1ml of the extract was added resulting in the formation of yellow color thereby indicating the presence of flavonoids.

4. Test for alkaloids

2ml of sample was mixed with 2ml of HCl. Then 6 drops of HCN was added and further 2 drops of picric acid was added that resulted in a creamish pale yellow ppt indicating the presence of alkaloids.

5. Test for Terpenoids

2 ml of sample along with 2ml of chloroform and 3ml of con. H₂SO₄ was added. Red color ppt obtained indicates the presence of terpenoids.

6. Test for Proteins

One milliliter of ninhydrin was dissolved in 1 mL of acetone and then a small amount of extract was added with ninhydrin. The formation of purple colour revealed the presence of protein.

7. Detection of saponins

Foam test: A fraction of the extract was vigorously shaken with water and observed for persistent foam.

8. Test for steroids

One ml of chloroform was mixed with 1 mL of extract and then ten drops of acetic anhydride and five drops of concentrated sulphuric acid were added and mixed. The formation of dark red colour or dark pink colour indicates the presence of steroids.

Assessment of antioxidant activity

DPPH free radical scavenging activity

Scavenging of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radicals was assessed by the method of Hatano et al, (1989). DPPH solution (1.0 ml) was added to 1.0 ml of extract at different concentrations (0.1 to 0.5mg/ml). The mixture was kept at room temperature for 50 minutes and the activity was measured at 517 nm. Ascorbic acid at the same concentrations was used as standard. The capability to scavenge the DPPH radical was calculated and expressed in percentage (%) using following formula:

$$\text{DPPH radical scavenging (\%)} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

Control OD

***In vitro* xanthine oxidase inhibitory activity of aqueous seed extract of Punica granatum**

In vitro Xanthine oxidase inhibitory of the extract was assessed as per the method of (Nguyen et al, 2004; Umamaheswari et al., 2007) (20). Briefly, the assay mixture consisted of 1 ml of the fraction (0.1 to 0.5g/ml), 2.9 ml of phosphate buffer (pH 7.5) and 0.1 ml of xanthine oxidase enzyme solution (0.1 units/ml in phosphate buffer, pH 7.5), which was prepared immediately before use. After preincubation at 25°C for 15 min, the reaction was initiated by the addition of 2 ml of the substrate solution (150 M xanthine in the same buffer). The assay mixture was incubated at 25°C for 30 min. The reaction was then stopped by the

addition of 1 ml of 1N hydrochloric acid and the absorbance was measured at 290 nm using a UV spectrophotometer. Allopurinol (0.1 to 0.5mg/ml), a known inhibitor of XO, was used as the positive control. One unit of XO is defined as the amount of enzyme required to produce 1 mmol of uric acid/min at 25°C. XO activity was expressed as the percentage inhibition of XO in the above assay system

calculated as percentage of inhibition as follows.

Inhibitory activity (%) = $(1 - A_s/A_c) \times 100$ Where,

A_s – absorbance in presence of test substance, A_c – absorbance of control

Statistical analysis

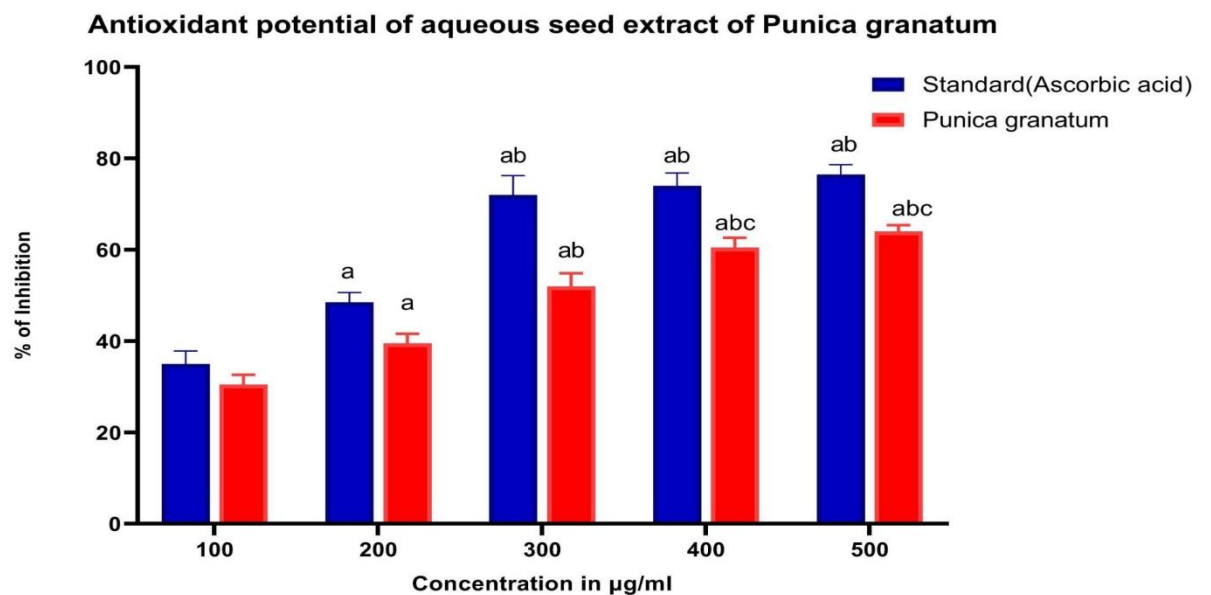
The data were subjected to statistical analysis using two-way analysis of variance (ANOVA) and Tukey's multiple range test to assess the significance of individual variations between the groups. In Tukey's test, significance was considered at the level of $p < 0.05$.

RESULTS AND DISCUSSION:

Table 1: Phytochemical screening of aqueous seed extract of *Punica granatum*

Phytochemicals	Presence / Absence
Amino acid	+++
Flavonoids	++
Alkaloids	++
Terpenoids	++
Saponins	+
Steroids	+
Carbohydrate	+
Protein	+

Antioxidant potential of aqueous seed extract of *Punica granatum*

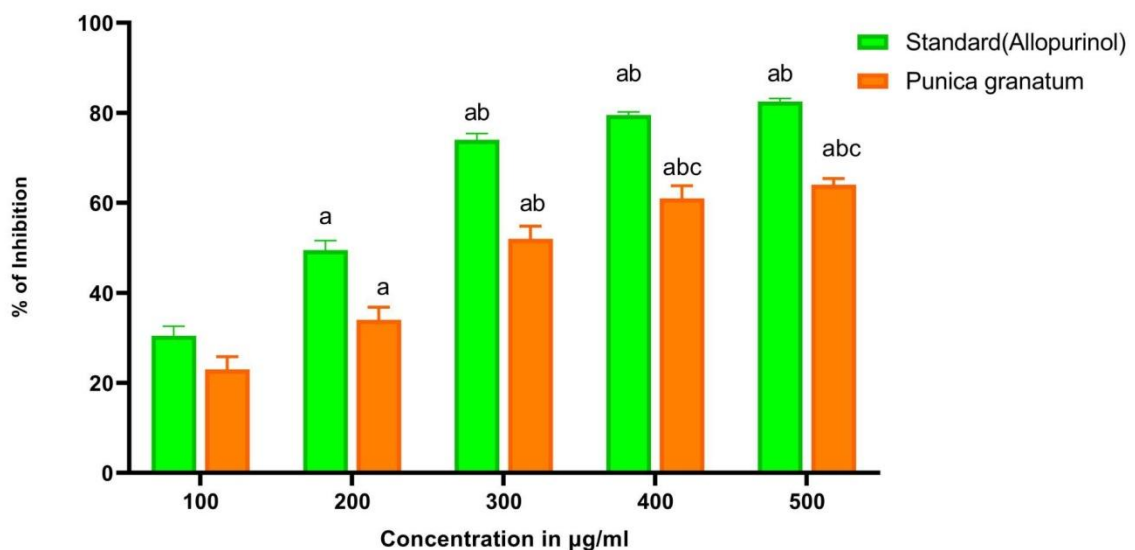


Graph 1: represents antioxidant activity of aqueous seed extract of *punica granatum*. X axis represents the concentration in µg/ml and Y axis represents the inhibitory potential of the extract. Blue indicates standard (vitamin c) and red indicates aqueous seed extract of *punica*

granatum Each bar represents Mean \pm SEM of 3 independent observations . Significance at $p < 0.05$.

Anti gout activity of aqueous seed extract of *punica granatum*

Xanthine oxidase inhibitory potential of aqueous seed extract of *Punica granatum*



Graph 2: Anti gout activity of aqueous seed extract of *Punica granatum*- Xanthine oxidase inhibitory potential X axis represents the concentration in $\mu\text{g/ml}$ and Y axis represents the inhibitory potential of the extract. Green indicate standard (allopurinol) and orange indicate aqueous seed extract of *punica granatum*. Each line represents Mean \pm SEM of 3 independent observations . Significance at $p < 0.05$.

From the results, it was evident that the plant extract was rich in phytochemicals such as steroids, amino acids, flavonoids, alkaloids and terpenoids.(Table 1). Phytochemical constituents are responsible for the medicinal property of the plant extract. The aromatic

phytoconstituents help in scavenging the free radicals, as it can donate or accept electrons. (29)

Antioxidant potential of aqueous seed extract of *Punica granatum* was determined by DPPH Free radical scavenging assay. IC₅₀ of antioxidant activity of aqueous seed extract of *Punica granatum* was found to be 280µg/ml (Graph 1) and increases in a dose dependent manner as compared to the standard Vitamin C. Antioxidant potential of the extract may be due to the presence of phytochemicals such as flavonoids and alkaloids. Free radical scavenging activity of the extract significantly increased in a dose dependent manner

The extract also exhibited antigout potential (IC₅₀ = 310 microg/ml)(Graph 2) which increased in a dose dependent manner as compared to the control(allopurinol). Though the antigout potential of the herbal extract is significantly less than the standard allopurinol, it possesses other advantages like reduced side effects, cost effective and easy availability. (30). Allopurinol is a synthetic drug used in the treatment of gout. Long term use of allopurinol has side effects like nephrolithiasis, nausea and therefore, medicinal plants may be considered as an alternative for the treatment of gout.(31)

In a previous study antioxidant rich fractions were extracted from pomegranate (*Punica granatum*) peels and seeds using ethyl acetate, methanol, and water. The extracts were screened for their potential as antioxidants using various in vitro models, such as beta-carotene-linoleate and 1,1-diphenyl-2-picryl hydrazyl (DPPH) systems(32). Future studies may be needed to explore the potential health benefits in the prevention of generation of free radicals and the extract's scavenging ability (33).

Free radical scavenging ability of the plant is attributed to the aromatic phytochemicals which can quench the unpaired electrons produced during various metabolic reactions..(30). In previous studies, pomegranate seed oil has powerful anticancer agent particularly potential in breast and prostate cancer . Physiological parameters of *punica granatum* 19.23% of alcohol soluble extractives, 28.16% of water soluble extractives, 0.21% Of chloroform soluble extract, 0.83% of pet-ether double extract, 0.90% of water soluble ash, 2.82% of water soluble ash (16).

The present research was carried out to prove the wound healing ability of the pomegranate peel extract on dead tissue, incisional and excisional wounds, which can be related to its antioxidant potential (17). Gout is a metabolic disorder which has shown its increase in the past 50 years. The increased prevalence can be due to the high living and increased alcoholism (34). Since, the clinical complications associated with gout are highly

unmanageable (35) and the available synthetic drug does not act as a complete cure, alternative measures to explore natural remedies are taking an upscale.

CONCLUSION

The aqueous seed extract of *Punica granata* was able to scavenge free radicals and inhibit the xanthine oxidase enzyme in a concentration dependent manner. Treating the gout with medicinal plants is gaining new interest. Further *in vivo* studies and human cell line models may be required to validate and develop the extract into an alternative drug for the treatment of gout.

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