

THROMBOLYTIC ACTIVITY OF *PUNICA GRANATUM* FRUIT AND PEEL EXTRACTSAMPATH R¹, RENUKA SARAVANAN¹, BRINDHA PEMIAH², SIVAKUMAR RAMALINGAM^{1*}¹Department of Chemistry and Biosciences, SASTRA University, Srinivasa Ramanujan Centre, Kumbakonam, Thanjavur, Tamil Nadu, India.²Department of Chemistry and Biosciences, CARISM, SASTRA University, Thanjavur, Tamil Nadu, India. Email: rsiva@src.sastra.edu

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ABSTRACT

Objective: The present study was undertaken to investigate the thrombolytic activity of *Punica granatum* fruit and peel extract.**Methods:** Phytoconstituents and free radical scavenging activity of *P. granatum* extract were performed. Ethanolic and aqueous extract of fruit and peel of the plant was used for *in vitro* clot lysis activity using streptokinase (SK) as standard. In a series of pre-weighed eppendorf tubes, equal volume of venous blood was collected and serum was removed and the tubes were weighed. The tubes containing clot were incubated with fruit, peel extract (100 µg/ml), and SK individually at 37°C for 60 minutes. After incubation, fluid obtained was removed, and tubes were again weighed to observe the difference in weight after clot disruption. The percent of clot lysis was determined.**Results:** Free radical scavenging activity of the extracts was increased in a dose-dependent manner. Of the extracts, ethanolic extract showed maximum activity as compared with aqueous extract, whereas the ascorbic acid exerted maximum activity as compared with the extracts. Maximum thrombolytic activity (59%) was observed with ethanolic extract of peel of the fruit.**Conclusions:** The present study concluded that the aqueous and ethanolic extracts of *P. granatum* fruit and peel possess thrombolytic activity.**Keywords:** *Punica granatum*, Antithrombolytic activity, Antibacterial, Antioxidant, Phytochemicals.

INTRODUCTION

A blood clot (thrombus) developed in the circulatory system due to the failure of hemostasis causes vascular blockage and while recovering leads to serious consequences in atherothrombotic diseases such as myocardial or cerebral infarction, at times leading to death [1]. Thrombolytic agents that include tissue plasminogen activator, urokinase (UK), and streptokinase (SK) are used all over the world for the treatment of these diseases. In India, though SK and UK are widely used due to lower cost [2], as compared to other thrombolytic drugs, their use is associated with hyper risk of hemorrhage [3] severe anaphylactic reaction and lacks specificity. Moreover, as a result of immunogenicity multiple treatments with SK in a given patient are restricted [4]. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs.

Herbal products are often perceived as safe because they are "natural" [5]. In India, in recent years, there is increased research on traditional ayurvedic herbal medicines on the basis of their known effectiveness in the treatment of ailments for which they have been traditionally applied.

Although only recently has pomegranate (*Punica granatum*) been acclaimed for its health benefits, this fruit has long been cultivated and consumed, as fresh fruit or in the form of beverage, especially in the Mediterranean region. Pomegranate fruit, juice and peel possess a marked antioxidant capacity [6] with a high content in polyphenols, in particular, ellagitannins, condensed tannins, and anthocyanins [7]. Some of these antioxidant molecules has been shown to be bioavailable and safe [8]. Pomegranate juice has been proposed as chemopreventive, chemotherapeutic, anti-atherosclerotic, and anti-inflammatory [9], and accordingly its consumption has grown tremendously [8]. However, it would be important to confirm and characterize pomegranate juice *in vivo* effects on biological systems. The aim of the present work is to investigate the antithrombolytic activity of *P. granatum* fruit and peel extract.

METHODS

P. granatum fruit and peel were collected from Kumbakonam, identified and authenticated by the Department of Botany, Government Arts College (Autonomous), Kumbakonam, Tamil Nadu, India.

Preparation of extracts

Fruit extract

The fresh fruits were washed thoroughly to remove adhered materials. 10 g of mesocarp was ground with 200 ml of distilled water and ethanol separately and allowed to stand for 2 hrs. It was filtered through Whatmann No.1 filter paper. The filtrate was then centrifuged at 3000 rpm for 10 minutes. Then the supernatant was saved and evaporated to dryness in a water bath with reduced temperature.

Peel extract

The *P. granatum* peel was manually removed; shade dried for 3 days and finely powdered using a blender. 10 g of peel powder was mixed with 200 ml of distilled water and ethanol separately. This was allowed to stand in a dark place for 24 hrs at room temperature with occasional stirring. Then, the solution was filtered through Whatmann No.1 filter paper followed by centrifugation at 3000 rpm for 10 minutes. Then the supernatant was saved and evaporated to dryness in a water bath with reduced temperature.

Preliminary phytochemical screening

Preliminary phytochemical screening was performed in the extract using the standard methods [10,11].

In vitro antioxidant activity

Antioxidant activity of the extract was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging method [12] and reducing power assay [13]. Tests were carried out in triplicate for separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, IC₅₀ was graphically estimated using a non-linear regression algorithm.

In vitro thrombolytic activity

Blood samples were collected from healthy volunteers after obtaining their consent for the study. Commercially available lyophilized streptokinase (Biocon Bangalore) of 15,00,000 I.U., 5 ml of sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µl (30,000 I.U) was prepared for *in vitro* thrombolysis. Experiments for clot lysis were carried as reported earlier [27]. Venous blood drawn from healthy volunteers was transferred in different pre-weighed sterile eppendorf tube (500 µl/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completed removed (aspirated out without disturbing the clot formed). Each tube having clot was again weighed to determine the clot weight (clot weight=weight of clot containing tube-weight of tube alone). Each eppendorf tube containing clot was properly labeled, and 100 µl of plant extract was added to the tubes. All the tubes were then incubated at 37°C for 60 minutes and observed for clot lysis. After incubation, fluid obtained was removed, and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as a percentage of clot lysis. Streptokinase and water were used as a positive and negative (non-thrombolytic) control, respectively. The experiment was repeated several times with the blood samples of different volunteers.

$$\% \text{ clot lysis} = (\text{Weight of the lysis clot} / \text{Weight of clot before lysis}) \times 100$$

Table 1: Preliminary phytochemical screening of *Punica granatum* fruit and peel extract

Phytochemicals	Fruit		Peel	
	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract
Alkaloids	-	-	-	-
Flavanoids	+	+	+	+
Amino acids	-	-	-	-
Proteins	-	-	-	-
Tannins	-	+	-	+
Carbohydrates	+	+	+	+
Terpenoids	-	+	-	-
Glycosides	-	+	+	-
Vitamins	-	+	+	-
Saponins	+	+	+	-
Steroids	-	+	-	+

Table 2: In vitro thrombolytic activity of *Punica granatum* of fruit and peel (aqueous, ethanol) extract

Serial number	Extract	Percentage of clot lysis
1	Water (negative control)	10
2	Streptokinase (positive control)	72
3	Fruit extract (aqueous)	33
4	Fruit extract (ethanol)	38
5	Peel extract (aqueous)	34
6	Peel extract (ethanol)	60

Table 3: DPPH radical scavenging activity of *Punica granatum* fruit and peel extract

Concentration (µg/ml)	Ascorbic acid	Fruit		Peel	
		Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract
50	7.55	6.25	7.25	5.44	4.21
100	10.22	8.27	9.21	7.41	6.43
150	15.24	14.67	15.43	10.11	9.43
200	18.23	16.43	18.32	13.40	12.23
250	28.21	19.57	22.70	16.11	15.12

DPPH: 1,1-diphenyl-2-picryl-hydrazyl

RESULTS

Preliminary phytochemical investigation of the aqueous and ethanolic extract of *P. granatum* fruit extract revealed the presence of various bioactive components. Aqueous extract showed the presence of flavanoids, carbohydrates, and saponins, whereas ethanolic extract showed the presence of alkaloids, flavanoids, tannins, carbohydrates, triterpenoids, steroids, glycosides Vitamin C, and saponins. Similarly, aqueous and ethanolic extract of *P. granatum* peel extract showed the presence of flavanoids, glycosides, and Vitamin C. Both the extracts of fruit and peel did not show the presence of amino acids and proteins (Table 1).

Thrombolytic activity

Table 2 represents clot lysis by the aqueous and ethanolic extract of fruit and peel. Maximum thrombolytic activity (60%) was observed in ethanolic extract of peel. Streptokinase and water and positive and negative control showed 72% and 10%, respectively.

Antioxidant

Table 3 represents DPPH free radical scavenging activity of *P. granatum* fruit extracts peel extract. The free radical scavenging activity of the extracts is expressed as percent inhibition. Ethanolic extract of fruit at 250 µl/ml showed maximum free radical scavenging activity as compared to peel extract, however, lesser than the standard ascorbic acid. A dose-dependent increase in free radical scavenging activity of fruit and peel extract was observed.

Determination of Na and K in *P. granatum* fruit and peel extract

The minerals content of pomegranate peel and fruit (aqueous, ethanol) extracts were shown in Table 4. The Na concentration was found to higher (13.9 ppm) as K (19.9 ppm) in ethanolic extract of the peel.

Reducing power

Table 5 exhibits the reducing power assay of *P. granatum* fruit and peel extract. Reducing power assay is a tool to determine the antioxidant capacity of extracts or any other sample. The reducing power of both fruit and peel was observed. The ethanolic extract of *P. granatum* fruit and peel showed the highest reducing ability than water extract.

The transformation of Fe³⁺ into Fe²⁺ in the presence of various extract was measured to determine the reducing ability. The reducing ability of a compared generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom [24]. The antioxidant

Table 4: Level of Na and K in *Punica granatum* fruit and peel (aqueous, ethanol) extract

Serial number	Content	Sodium (ppm)	Potassium (ppm)
1	Fruit extract (aqueous)	6.5	4.8
2	Fruit extract (ethanol)	4.1	12.8
3	Peel extract (aqueous)	8.7	11.9
4	Peel extract (ethanol)	13.9	19.9

Table 5: Reducing power assay of *Punica granatum* of fruit and peel (aqueous, ethanol) extract

Concentration ($\mu\text{l/ml}$)	Percentage of inhibition				
	Standard ascorbic acid	Fruit extract (aqueous)	Fruit extract (ethanol)	Peel extract (aqueous)	Peel extract (ethanol)
100	29.88	12.5	25	34.37	18.75
200	42.33	31.25	31.25	22.23	32.25
300	49.57	46.87	46.87	31.62	55.25

principles present in the fractions of *P. granatum* caused the reduction of Fe^{3+} /ferricyanide complex to the ferrous form, and thus proved the reducing ability.

DISCUSSION

The preliminary phytochemical screening tests may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development. Further, these tests facilitate their qualitative separation of pharmacologically active chemical compounds [18].

Secondary metabolites in various plants and microorganisms are generally evolved in self-defense to avert the toxicity due to adverse environmental/physiological conditions. Subsequently, these intermediary metabolites also provided a natural platform for the development of numerous drugs leading to the treatment of various diseases. Methanol extracts of leaf and flower of *P. granatum* were reported to have glycosides, triterpenoids and steroids, saponins, alkaloids, flavanoids, tannins, carbohydrate, and Vitamin C. The fruit extract showed the abundant occurrence of flavanoids [19]. The presence of phytoconstituents in our fruit extracts well agrees with the previous reports.

These phytoconstituents might be responsible for different biological activities and medicinal importance of this peel. A variety of herbal extracts contains different phytochemicals with biological activity that can be of valuable therapeutic index [20].

Flavanoids and tannins are phenolic compounds, and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers. Since these compounds were found to be present in the extracts, it might be responsible for the potent antioxidant capacity of pomegranate. The secondary metabolites and other chemical constituents of medicinal plants account for their medicinal value. For example, saponins have hypotensive and cardio depressant properties [21]. Glycosides are naturally cardioactive drugs used in the treatment of congestive heart failure and cardiac arrhythmia.

In our study of thrombolysis, we have tried pomegranate which have been using for treating *P. granatum* was reported to have antithrombotic activity, blood purifying property [24].

The prevention of lifestyle-related atherothrombotic diseases such as myocardial infarction and stroke is an important and urgent social task in many developed countries. Epidemiological studies have provided irrefutable evidence for the causative role of inappropriate diet in the development and clinical outcome of thrombotic diseases [25]. The development of natural products from various plant and animal sources which have anti-platelet, anti-coagulant, and thrombolytic activity is being studied intensively [26,28].

Reported that recently consumption of phytochemical-rich plant foods, including whole grains, vegetables, and fruits, was reported to reduce the risk for acute coronary disease incidence, which was closely associated with thrombosis [27]. Likewise, *P. granatum* has a high clot lysis (59%) effect. From the results, we concluded that *P. granatum* has antithrombolytic activity.

DPPH radical scavenging activity of an aqueous and ethanolic extract of *P. granatum* fruit extract was found to increase with increasing concentration of the extract. This assay was based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants, which denotes the plant has the antioxidant potential [25].

Sodium is present in most food, and its deficiency is rare. Distorted enzymatic activity and poor electrolyte balance of the body fluid are related to inadequate Na and K as they are the most required elements of living cells [15]. Sodium is an extracellular cation involved in the regulation of plasma volume, acid-base balance nerve, and muscle contraction. High dietary sodium has been associated with essential hypertension [23].

Reducing power is one of the mechanisms for the possible antioxidant activity and may serve as a significant indicator of potential antioxidants. Ethanolic extract of *P. granatum* fruit extract showed good concentration dependent manner with a perfect reducing power, thus indicating good electron donors and ability to terminate radical scavenging reaction [28]. The ethanolic extract of fruits of the *P. granatum* has a significant reducing power, which indicates the antioxidant potential of the plant.

CONCLUSION

In our present study, crude extracts of *P. granatum* fruit and peel were screened for phytochemicals; it revealed the presence of flavanoids, tannins, and carbohydrates. Both the fruit and peel extracts showed antioxidant activity (*in vitro*) which could be due to the presence of phytoconstituents in the extracts. The thrombolytic activity of peel extracts was more than the fruit extracts. However, the fruit and peel could be used as a better antioxidant and thrombolytic agents.

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