



PHYTOCHEMICAL INVESTIGATION AND ANTIOXIDANT ACTIVITIES OF METHANOL PEEL EXTRACT OF *PUNICA GRANATUM* L.

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ABSTRACT

Peels of *Punica granatum* L., a well-known medicinal plant, has extensively been used in ayurvedic medicines to treat various diseases. The *Punica granatum* is usually found growing on river banks or near dry river beds in West Bengal and South and Central India. Phenols and flavonoids content were analyzed using Folin-Ciocalteu reagent and aluminium chloride colorimetric methods. Methanol extract of peels of *Punica granatum* was evaluated for the antioxidant activity using DPPH[•] radical, superoxide radical (O_2^-), Fe^{2+} chelation assay, Fe^{3+} reducing power and phosphomolybdenum reduction assay. The IC_{50} of methanol extract of peels of *Punica granatum* for DPPH[•] radical, superoxide radical (O_2^-) and Fe^{2+} chelation was 181.09, 94.66 and 51.69 μ g/mL concentration. The RC_{50} of methanol extract of peels of *Punica granatum* for Fe^{3+} reduction and phosphomolybdenum reduction was 15.00 and 16.92 μ g/mL concentration respectively. The IC_{50} of methanol extract of peels of *Punica granatum* for antidiabetic activity carried out by α -amylase enzyme inhibition assay was found to be 37.18 μ g/mL concentration. From the present investigation it is concluded that methanol extract of peel of *Punica granatum* L. was found to be good source of natural antioxidants and can be used effectively to inhibit radicals.

KEY WORDS

Punica granatum, DPPH[•], superoxide (O_2^-), Fe^{2+} chelation, Fe^{3+} reducing power, α -amylase, Phenols, flavonoids.

INTRODUCTION

Fruits and vegetables containing antioxidant molecules such as tannins, phenols, flavonoids and ascorbic acid in dietary system prevent several diseases. [1,2]. Consuming higher fruits and vegetables daily provides healthier benefits due to presence of phytoconstituents. Phenolic compounds from medicinal plants exhibit several biological properties such as antioxidant, antimicrobial, anti-thrombotic, cardio-protective effects. [2-4]. Naturally available antioxidant molecules in food products provide increasing consumer acceptability and improve product stability. [3-6]. Waste products from agricultural commodities (processing steps) such as fruit peels and vegetable peels offer practicable and economic source of rich

antioxidant molecules thereby replacing the synthetic molecules [7-11]. This made the interest to carry out antioxidant evaluation from several food products. Therefore, the crude extracts and purified compounds from peels could be used in preservation of foods and in pharmaceutical industries.

A pomegranate tree has also been considered as "The Tree of Life" and is native from Iran to the Himalayas in northern India and has been cultivated since ancient times throughout the Mediterranean region of Asia, Africa and Europe [12]. Fruits are nearly round 2 to 5 inches wide and crowned at the base by a prominent calyx. They have tough, leathery skin, generally yellow with a touch of pink or deep red. They have great nutritional values and numerous health benefits. The dried pericarp is decocted with other herbs and used in

the treatment of colic dysentery, leucorrhoea [13]. In recent times, Chinese researchers have claimed that the pomegranate peel does hold immense potential as it contains double the antioxidants than the fruit pulp contains. Compared to the pulp, the inedible pomegranate peel belongs to the Punicaceae family contains thrice the total amount of polyphenols [14] including condensed tannins [15] catechins, gallo catechins and prodelphinidins [16]. The peels are effective against heart disease and are a rich source of vitamin C. Clinical research shows that pomegranates,

when part of a healthy diet, might help prevent heart disease, heart attacks and strokes.

Fruits rind and the bark of the pomegranate tree are used as an excellent remedy against diarrhoea, dysentery and intestinal parasites. Pomegranate juice is used as eye drops to slow down cataract development. Also, the pomegranate peel extracts possess anti-mutant properties due to higher polyphenolic content such as tannins, ellagic and gallic acids. Anthocyanidins and flavan-3-ols are responsible for bright red colour of pomegranate peel, also flavones and flavonols are major flavonoids of pomegranate peel [17,18]

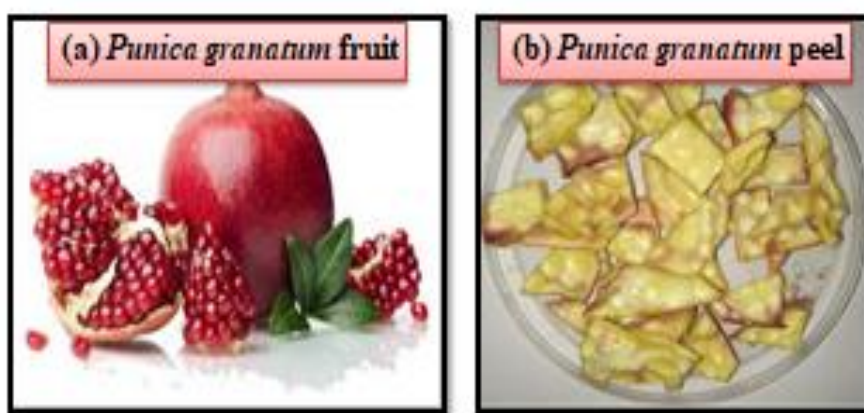


Fig.1: Habitat of *Punica granatum*

MATERIALS AND METHODS

Collection and preparation of plant extract

The *Punica granatum* L. were collected from the market at Chennai. The peels were removed, washed and shade dried for 10 days. The dried peels were cut into small pieces and soaked in methanol for 72 hours. The supernatant was filtered, condensed at room temperature and brown gummy mass was obtained.

Determination of total phenols

The total phenolic content of methanol extract of peels of *Punica granatum* was determined by Folin-Ciocalteu reagent method [19]. One hundred μ L of methanol extract of *Punica granatum* (1mg/mL) was mixed with 0.5 mL of methanol, 1 mL of Folin-Ciocalteu reagent (1:10 diluted with water) and 1 mL of 20% Na_2CO_3 (w/v). The reaction mixture was incubated at 37°C for 30 minutes. The absorbance of the sample was measured at 765 nm. The result was expressed as milligram of gallic acid equivalent.

Determination of total flavonoids

The total flavonoid content of methanol extract of peels of *Punica granatum* was determined using aluminium

chloride colorimetric method with slight modifications [20]. Five hundred μ L of methanol extract of *Punica granatum* (1mg/mL) was mixed with 0.5 mL of methanol, 0.5 mL of 5% Sodium nitrite (w/v), 0.5 mL 10% aluminium chloride (w/v) was added and incubated for 5 min at 37°C. Sodium hydroxide solution 1mL (1M, w/v) was added and the absorbance was measured at 510 nm using spectrophotometer. The results were expressed as mg of quercetin equivalent.

DPPH[•] radical scavenging assay

The antioxidant activity of methanol extract of the peels of *Punica granatum* was measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical [21] with slight modifications. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of methanol extract of various concentrations (50 - 300 μ g/mL). Ascorbic acid was used as reference standard. Mixture of 1 mL methanol and 1 mL DPPH solution was used as control. The decrease in absorbance was measured at 517 nm after 30 min in dark using UV-Vis spectrophotometer.

The percentage of DPPH[•] radical inhibition was calculated as

$$\% \text{ of DPPH}^{\bullet} \text{ radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Superoxide anion radical (O₂⁻) scavenging assay

The assay of superoxide anion radical scavenging activity was analyzed by riboflavin-light-NBT system [22]. Briefly, 1 mL of methanol extract of the peels of *Punica granatum* was taken at different concentrations (20 - 120 µg/mL) and mixed with 0.5 mL of phosphate buffer (50 mM, pH 7.6), 0.3 mL riboflavin (50 mM), 0.25

mL PMS (20 mM), and 0.1 mL NBT (0.5 mM). Reaction was started by illuminating the reaction mixture using a fluorescent lamp. After 20 minutes of incubation, the absorbance was measured at 560 nm. Ascorbic acid was used as standard. The scavenging ability of the peel extract was determined by the following equation.

$$\% \text{ of O}_2^{\bullet-} \text{ radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Fe²⁺ chelation assay

The basic principle of this test is based on the capacity to decolorize the iron-ferrozine complex. Ferrozine is a substance which can quickly act on iron to form a coloured complex. This test was done by adding 1 mL of methanol extract of the peels of *Punica granatum* in varying concentrations (10 - 60 µg/mL) and 0.05 mL of

FeCl₂ (2 mM, w/v) was added. After 30 seconds, 0.1 mL ferrozine (5 mM, w/v) was added in it. Ferrozine can act on divalent iron to form stable magenta complex-soluble in water. After 10 minutes at room temperature, the absorbance of the complex was measured at 562 nm spectrophotometrically [23, 24].

$$\% \text{ of Fe}^{2+} \text{ chelation} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Fe³⁺ Reducing power assay

The reducing power assay of methanol extract of the peels of *P. granatum* was determined according to the method of [25]. One mL each of various concentrations of methanol extract (10 - 60 µg/mL) was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% (w/v) solution of potassium ferricyanide. The mixture was incubated at 50°C for 20 min during which period

ferricyanide was reduced to ferrocyanide. Then 1 mL of 1% trichloroacetic acid (w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min if necessary. The upper layer of the solution was mixed with distilled water and 0.5 mL of 0.1% FeCl₃ (w/v) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates the increase in reduction of ferricyanide.

$$\% \text{ of Fe}^{3+} \text{ reduction} = \frac{\text{Sample} - \text{Control}}{\text{Sample}} \times 100$$

Phosphomolybdenum reduction assay

The reduction activity of methanol extract of peels of *Punica granatum* was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto et al [26]. 1mL of methanol extract (10 - 60 µg/mL) was combined with 1 mL of reagent

solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in test tubes. The tubes were capped and incubated in a water bath at 95°C for 90 min. After the samples were cooled to room temperature and the absorbance of the mixture was measured at 695 nm against a blank.

$$\% \text{ of Phosphomolybdenum reduction} = \frac{\text{Sample} - \text{Control}}{\text{Sample}} \times 100$$

Antidiabetic activity by alpha amylase inhibition method

The methanol extract of peels of *Punica granatum* (10-60 µg/mL) were added to 500 µL of 0.02 M phosphate buffer (pH 6.9) which containing α-amylase (1 mg/mL) solution in test tubes. The contents were incubated at 25°C for 10 min. Then, 500 µL of 1% starch solution (w/v) and 0.02 M sodium phosphate buffer (pH 6.9) were

added to the test tubes containing the reaction mixture. The reaction mixtures were then incubated at 25°C for 10 min and the reaction was stopped by adding 1.0 mL of 3,5- dinitro salicylic acid reagent (w/v). The test tubes were then cooled to room temperature and the reaction mixture was diluted by adding 10 mL of distilled water. The absorbance was measured at 540 nm [27, 28].

$$\% \text{ of } \alpha\text{-amylase enzyme inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

RESULTS AND DISCUSSION

Phytochemical estimations

The total phenolic content (estimated by Folin-Ciocalteu method) in methanol extract of peels of *Punica granatum* was 50.55 mg/g and the total flavonoid content (estimated by AlCl₃ method) was 68.91 mg/g (Table 1). Free radicals are produced during normal cellular function in the body, these molecules are missing an electron, giving them an electric charge. To neutralize this charge, free radicals try to withdraw an electron from or donate an electron to a neighbouring molecule [29]. The newly created free

radicals, in turn, looks out for another molecule and withdraws or donates an electron, setting off a chain reaction that can damage hundreds of molecules. Antioxidants such as phenolic compounds and flavonoids halt this chain reaction. These antioxidants are themselves free radical, donating electrons to stabilize and neutralize the dangerous free radicals. The phenolic compounds and flavonoids present in the methanol extract of peels of *Punica granatum* work against the molecules that form free radicals, destroying them before they can begin the domino effect that leads to oxidative damage [30].

Table 1: Quantitative phytochemical estimation of phenols and flavonoids of methanol extract of peels of *Punica granatum*.

Phytochemical parameters	<i>Punica granatum</i> extract (µg/mg)
Phenol content	50.55±0.28
Flavonoid content	68.91±0.14

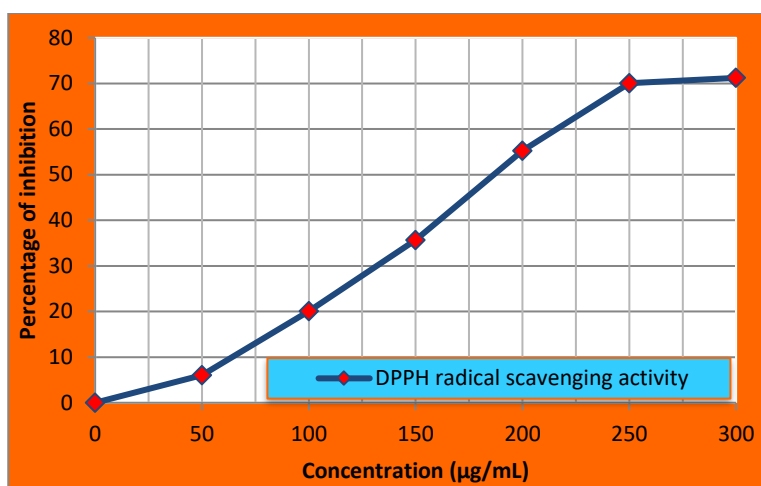
DPPH' radical scavenging assay

DPPH assay is based on the measurement of the scavenging ability of antioxidants present in the methanol extract of peels of *Punica granatum* towards the DPPH free radical. The method is based on the reduction of purple colored methanol solution of DPPH radical in the presence of methanol extract of, peels of *Punica granatum* is having hydrogen donating antioxidants, by the formation of yellow colored non-radical form of DPPH. Lower absorbance indicates

higher DPPH free radical scavenging activity [31]. The methanol extract of peels of *Punica granatum* was able to reduce purple colored 1,1-diphenyl-2-picryl hydrazyl (DPPH) to yellow colored 1,1-diphenyl-2-picryl hydrazine. The maximum DPPH radical scavenging activity was 71.21±0.18 (Graph 1) at 300µg/mL concentration (Table 2) and was compared with standard (Ascorbic acid, IC₅₀ value as 13.95 µg/mL concentration).

Table 2: DPPH' radical scavenging activity of methanol extract of peels of *Punica granatum*

S.No	Concentration (µg/mL)	% of inhibition DPPH' radical
1	50	6.06±0.42
2	100	20.07±0.20
3	150	35.69±0.39
4	200	55.22±0.16
5	250	70.02±0.35
6	300	71.21±0.18



Graph 1: DPPH[•] radical scavenging activity of methanol extract of peels of *Punica granatum*

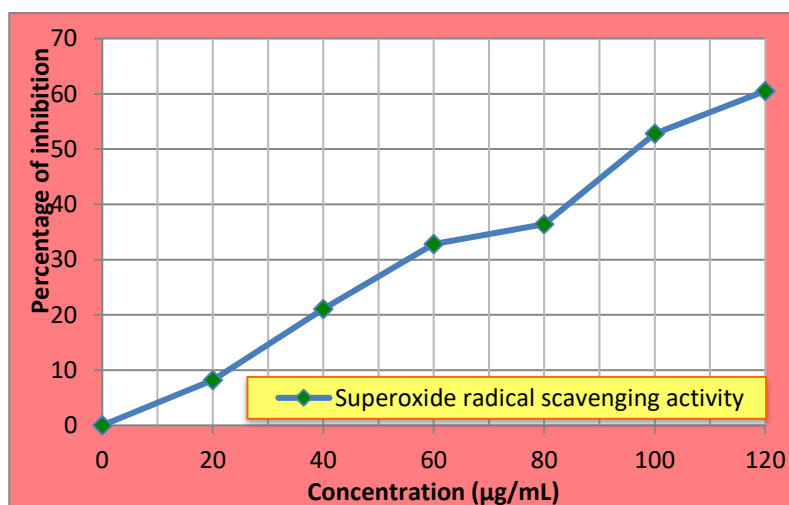
Superoxide anion radical (O₂^{•-}) scavenging assay

Superoxide radical is considered a major biological source of reactive oxygen species [32]. Although superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress [33]. The results of our study revealed

that the methanol extract of peels of *Punica granatum* have effective capacity of scavenging for superoxide radical thus suggesting its antioxidant potential. The percentage of inhibition is 60.51±0.23 (Graph 2) at 120 µg/mL concentration of the extract (Table 3) and was compared with standard (Ascorbic acid, IC₅₀ value as 9.15µg/mL concentration).

Table 3: Superoxide anion radical (O₂^{•-}) scavenging activity of methanol extract of peels of *Punica granatum*

S.No	Concentration (µg/mL)	% of inhibition Superoxide radical (O ₂ ^{•-})
1	20	8.21±0.37
2	40	21.03±0.21
3	60	32.82±0.29
4	80	36.41±0.44
5	100	52.82±0.19
6	120	60.51±0.23



Graph 2: Superoxide anion radical (O₂^{•-}) scavenging activity of methanol extract of peels of *Punica granatum*

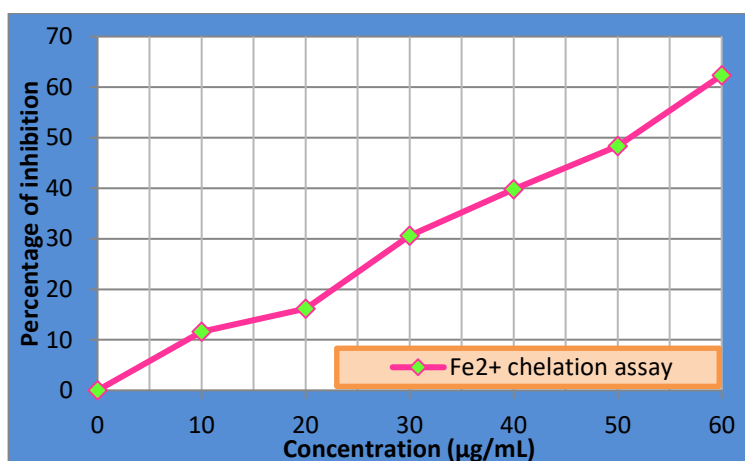
Fe²⁺ chelation assay

Ferrozine can quantitatively form complexes with Fe²⁺ ion. However, in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity in the presence of coexisting chelator. The transition metal ion, Fe²⁺ possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive

radicals [34]. The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions. The active methanol extract of peels of *Punica granatum* interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The maximum Fe²⁺ chelating activity was 62.36±0.36 (Graph 3) at 60µg/mL concentration (Table 4) and was compared with standard (Ascorbic acid, IC₅₀ value as 10.76 µg/mL concentration).

Table 4: Fe²⁺ chelating activity of methanol extract of peels of *Punica granatum*

S.No	Concentration (µg/mL)	% of inhibition Fe ²⁺ chelation
1	10	11.59±0.31
2	20	16.19±0.15
3	30	30.63±0.14
4	40	39.82±0.38
5	50	48.36±0.48
6	60	62.36±0.36



Graph 3: Fe²⁺ chelating activity of methanol extract of peels of *Punica granatum*

Fe³⁺ reducing power assay

The reducing properties are generally associated with the presence of reductones, such as flavonoids and phenolic compounds, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react directly with peroxides and also with certain precursors of peroxides, thus preventing peroxide formation [35]. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of methanol extract of peels of *Punica granatum*. Presence of reducers causes the conversion of the Fe³⁺/ferricyanide

complex to ferrous form. Fe³⁺ reduction is often used as an indicator of electron- donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties. By measuring the formation of Pearl's Prussian blue at 700 nm, it is possible to determine the concentration of ferrous ions. Increase in absorbance of the reaction mixture indicated increase in reducing power of the extract and maximum activity was found to be 88.23±0.30 (Graph 4) at 60 µg/mL concentration. The RC₅₀ of methanol extract of peels of *Punica granatum* was found to be 15.00 µg/mL concentration

(Table 5) and was compared with the standard (25.71 μ g/mL concentration) Ascorbic acid.

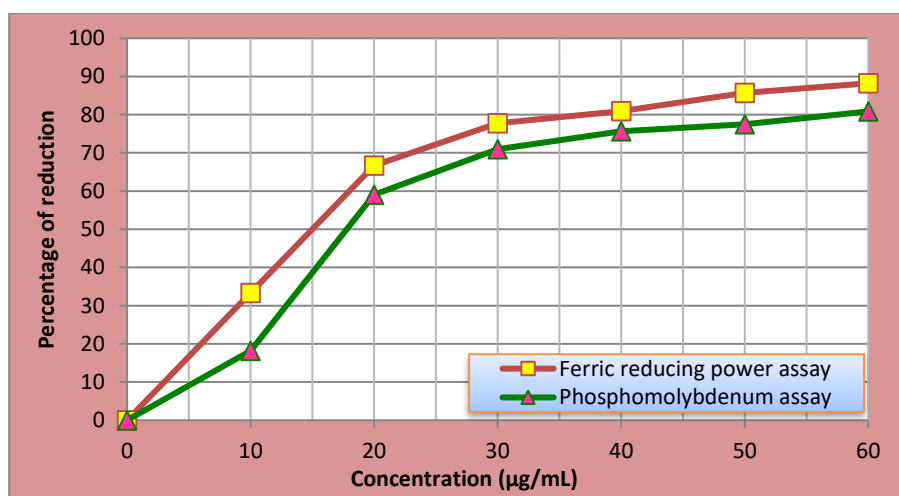
Phosphomolybdenum assay

Phosphomolybdenum assay revealed the reduction of Mo (VI) to Mo (V) by the methanol extract of peels of *Punica granatum* and formation of a Mo (V) complex at acidic pH. Increase in absorbance of the reaction mixture indicates increase in reducing power [36]. The significant increase of methanol extract was found to be 80.85 \pm 0.18 (Graph 4) at 60 μ g/mL concentration. The result obtained was confirmed by the high potency of

the methanol extract of peels of *Punica granatum* towards the reduction of transition metal ions. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants. The experiment demonstrated higher antioxidant activity the RC₅₀ of 16.92 μ g/mL concentration for methanol extract of peels of *Punica granatum* (Table 5) and was compared with standard Ascorbic acid (RC₅₀ value as 14.28 μ g/mL concentration).

Table 5: Fe³⁺ reduction and Phosphomolybdenum reduction of methanol extract of peels of *Punica granatum*

S.No	Concentration (μ g/mL)	% of reduction	
		Fe ³⁺ reduction	Phosphomolybdenum reduction
1	10	33.33 \pm 0.32	18.18 \pm 0.20
2	20	66.66 \pm 0.41	59.09 \pm 0.31
3	30	77.77 \pm 0.16	70.96 \pm 0.35
4	40	80.95 \pm 0.27	75.67 \pm 0.19
5	50	85.71 \pm 0.46	77.5 \pm 0.29
6	60	88.23 \pm 0.30	80.85 \pm 0.18



Graph 4: Fe³⁺ reduction and Phosphomolybdenum reduction of methanol extract of peels of *Punica granatum*

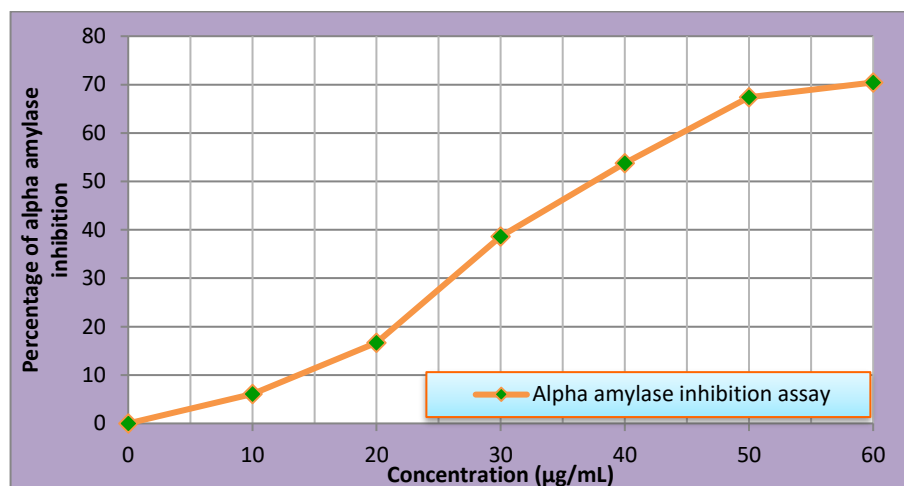
Antidiabetic activity by alpha amylase inhibition method

Diabetes mellitus is a chronic metabolic disorder identified by hyperglycemia due to insulin insufficiency and/or insulin resistance contributing to excess blood glucose. [37]. Blood glucose level management is an essential approach in the control of diabetes complications. Inhibitors of carbohydrates hydrolysing enzymes (alpha amylase and alpha glucosidase) have been helpful as oral hypoglycemic medicines for the control of hyperglycemia exclusively in patients with type-2 diabetes mellitus [38-41]. Inhibition of the

enzymes holds of carbohydrate digestion and extends the total carbohydrate digestion time, leading to a decrease in the rate of glucose absorption and therefore reducing the postprandial plasma glucose rise [42, 43]. From the present study, it is evident that the methanol extract of peels of *Punica granatum* displayed a good inhibitory activity on alpha amylase. The percentage of inhibition is 70.45 \pm 0.32 (Graph 5) at 60 μ g/mL concentration of the extract (Table 6) and was compared with standard (Acarbose, IC₅₀ value as 20.16 μ g/mL concentration).

Table 6: Alpha amylase inhibition activity of methanol extract of peels of *Punica granatum*

S. No	Concentration ($\mu\text{g/mL}$)	% of alpha amylase inhibition
1	10	6.06 \pm 0.28
2	20	16.66 \pm 0.16
3	30	38.63 \pm 0.42
4	40	53.79 \pm 0.27
5	50	67.42 \pm 0.16
6	60	70.45 \pm 0.32


Graph 5: Alpha amylase inhibition activity of methanol extract of peels of *Punica granatum*

The various antioxidant assays conducted are useful indices of evaluating the effective antioxidant properties of the methanol extract of peels of *Punica granatum*. Reactive oxygen species [ROS], sometimes called as active oxygen species, are various forms of activated oxygen, which include free radicals such as superoxide ions ($\text{O}_2^{\cdot-}$) and hydroxyl radicals (OH^{\cdot}) as well as non-free radical species such as hydrogen peroxide (H_2O_2). Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against these free radicals and protect us from various diseases. They exert their action by interfering with the oxidative processes by scavenging free radicals, chelating free catalytic metals and by acting as electron donors [44]. The therapeutic effects of several medicinal plants are usually attributed to their antioxidant phytochemicals. It has been suggested that there is an inverse relationship between dietary intake of antioxidant rich foods and incidence of human diseases. Antioxidants derived from plants are more effective than the synthetic ones because they are non-toxic and have more than one mode of action [45]. These lead to the screening of plants for their antioxidant properties and other medicinal properties.

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