



## ANTIOXIDANT AND TYROSINASE INHIBITORY ACTIVITY OF AQUEOUS EXTRACT AND OIL OF SEEDS OF *PUNICA GRANATUM* L. (PUNICACEAE)

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### ABSTRACT

More than 60 botanicals are marketed in cosmeceutical formulations. The most important botanicals pertaining to dermatologic uses as cosmeceuticals includes green and black tea, soya, pomegranate *etc.*, All are documented to treat dermatologic conditions. The aim of the present study is investigate the role of seeds of *Punica granatum* L. as skin cosmeceuticals in cosmetic preparation. The effect of seed extract and oil of *Punica granatum* L was done by antioxidant activity against free radicals causing aging and wrinkle and depigmentation activity by inhibition of mushroom tyrosinase enzyme. The invitro mushroom tyrosinase inhibitory activity shows significant results when compared to standard drug Kojic acid ( $p < 0.001$ ). So it may be included as an ingredient in formulation of cosmeceuticals for skin depigmentation problems.

**KEY WORDS:** *Punica granatum* L. Antioxidant, Depigmentation, Kojic acid, Mushroom tyrosinase.

### INTRODUCTION

More than 60 botanicals are marketed in cosmeceutical formulations. The most important botanicals pertaining to dermatologic uses as cosmeceuticals, include teas, soya, pomegranate, dates, grape seed *etc.* All are documented to treat dermatologic conditions. Only green and black tea, pomegranate and dates have been taken into clinical trials for the treatment of parameters of extrinsic aging<sup>1</sup>. Also several studies have shown that the pomogranate seed contain phenolic compounds and can be a good source of natural antioxidants<sup>2</sup>, so the fruit of pomegranate was selected for the present study. Pomegranate is the fruit of *Punica granatum* L. (Fam: Punicaceae) which is widely grown in mediterranean countries and has been introduced to most parts of the tropics and subtropics. The seeds are the edible part of the fruit which are normally consumed when fresh<sup>3</sup>. Many human diseases are caused by oxidative stress that results from imbalance between the formulation and neutralization of pro-oxidants. Oxidative stress initiated by free radicals such as superoxide anions, hydrogen peroxide, nitric oxide and peroxynitrite which plays a vital role in damaging various cellular macromolecules<sup>4</sup>. Melanin is the major pigment for color of human skin. It is secreted by melanocyte cells in basal layer of the epidermis. Melanin may be over produced with chronic sun exposure, melasma, or other hyper pigmentation diseases. Therefore, a number of depigmenting agents have been developed for cases of undesirable skin discoloration. Tyrosinase, a copper-containing monooxygenase, is a key enzyme that catalyzes melanin synthesis in melanocytes. It catalyzes two major reactions, including hydroxylation of tyrosine oxidation of the O-diphenyl product, L-Dopa. Dopa oxidation produces a highly reactive intermediate that is further oxidized to

form melanin by free radical-coupling pathway<sup>5</sup>. In this study, an effort was undertaken to examine the free radical scavenging and tyrosinase inhibition activities of aqueous extract and oil of seeds of *Punica granatum*L.

### MATERIALS AND METHODS

#### Chemicals

Folin-ciocalteu reagent, Gallic acid, Ascorbic acid were purchased from Sigma chemical Co., Pvt. Ltd. Kojic acid, Mushroom tyrosinase, griss reagent, Sodium nitroprusside, L-Dopa were purchased from Fluka Enterprises, Chennai and various chemicals and solvents like Sodium thiosulphate, Potassium iodide, Iodine, Bromide, Chloroform, Glacial acetic acid, Petroleum ether (60-80<sup>0</sup> C) and Ethanol were purchased from Sigma-Al-drich Co., Ltd. Mumbai, India.

#### Collection of specimen

The plant specimens for the purpose of the study were collected from the surrounding of SRM University campus. The specimen was identified and authenticated by Prof. Dr. P. Jayaraman, Director, Plant Anatomy Research Center (PARC), Thambaram, Chennai. Care was under taken to select healthy plant and normal organs.

### PREPARATION OF TEST DRUG

#### a) Preparation of aqueous extract from seed

The seeds were dried under shade with occasional shifting and then powdered with a mechanical grinder and stored in an air tight container. The dried powdered seeds were defatted with petroleum ether (60-80<sup>0</sup>C) in a soxhlet apparatus. The defatted powder material thus obtained was further extracted with water in a large beaker by cold maceration process. The solvent was removed by

distillation under the resulting semisolid mass was dried in a dessicator for further use.

#### b) Extraction of oil from seed

The seeds were recovered from healthy looking fruits. Unwanted material like pulps and other parts were removed. Seeds were weighed, dried under shade with occasional shifting and then powdered with a mechanical grinder. The powdered seed material was spreaded in trays and kept under shade at room temperature to remove extra moisture in the seeds. The powdered seed material was packed in a thimble of a soxhlet assembly. Then it was extracted with n-Hexane as a solvent for 6 hours. Then the solvent was removed and oil was separated by vaccum distillation. Thus the oil obtained was kept in an air container in a refrigerator for further use.

#### Quantitative analysis of seed oil

The separated oil was quantitatively analysed by standard methods<sup>6</sup>. The parameters evaluated are Acid value, Saponification value, Iodine value and Peroxide value for the oil.

#### Determination of total phenolic content (TPC)

The total phenolic content in the extracts was determined using Folin-Ciocalteu's phenol reagent. Briefly, 1ml of extract was mixed with 5ml of Folin-Ciocalteu's phenol reagent. After mixing for 3min, 5ml of (75g/L) sodium carbonate was added. The mixtures were agitated and allowed to stand for a further 30 min in the dark. The absorbance of seed extract, standard gallic acid and a prepared blank were measured at 765nm using a spectrophotometer. The concentration of total phenolic compounds in seed extract and standard was expressed in microgram. All determination was performed in triplicate<sup>7</sup>.

#### Determination of antioxidant activity<sup>8-10</sup>

The total free radical-scavenging capacity of seeds of *Punica granatum* aqueous extract and oil was determined by using the DPPH<sup>+</sup>, H<sub>2</sub>O<sub>2</sub>, SO and NO methods.

#### DPPH free radical scavenging activity

A stock solution of DPPH (33mg/l) was prepared in methanol and 5ml of this stock solution was added to 1ml of the test solution at different concentration (100, 200, 300, 400 and 500µg/ml). After 30 min, absorbance was measured at 517nm and compared with the standard i.e. Ascorbic acid. Scavenging activity was expressed as percentage inhibition. Percent inhibition was calculated using the following formula;

Radical Scavenging (%) =  $(A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$

Where A is the absorbance. The percentage of radical scavenging activity was plotted against corresponding concentration of extract to obtained IC<sub>50</sub> value.

#### Hydrogen peroxide radical scavenging (H<sub>2</sub>O<sub>2</sub>) assay

A solution of hydrogen peroxide (40mM) was prepared in Phosphate buffer (50mM, pH 7.4). Different concentrations of seed extract and oil were added to the hydrogen peroxide (40mM). Absorbance of hydrogen peroxide at 230nm was determined after 10min against a blank solution containing phosphate buffer without hydrogen peroxide. Percentage scavenging of hydrogen peroxide of the seed extract, seed oil and standard compound was calculated.

#### Superoxide anion radical scavenging (SO) assay

The reaction mixture consisting of 0.5ml of nitro blue tetrazolium (NBT) solution 0.3mM in Tris-HCL buffer, pH 8.0), 0.5ml NADH solution (0.936mM) and 1ml of sample solution of extract was mixed. The reaction was started by adding 0.5ml of phenazine methosulfate (PMS) solution 0.12mM in Tris-HCL buffer, pH 8.0) to the mixture. The reaction mixture was incubated at 25°C for 5min and the absorbance was measured at 560nm against blank sample and compared with the standard. Decreased absorbance of the reaction mixture incubated superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated.

#### Nitric oxide scavenging activity (NO)

Nitric oxide scavenging activity was measured by the spectrophotometry method. Sodium nitropruside (10mM) in phosphate buffered saline pH 7.4 was mixed with different concentrations of the extract prepared in ethanol and incubated at 25°C for 30 min. After incubation, 0.5ml of Griess reagent (1% sulphonilamide 2% phosphoric acid and 1% naphthyl ethylene diamine di-hydrochloriide) was added and the absorbance was measured at 546nm using UV-visible spectrophotometer and the percentage activity was measured with reference to the standard using the following formula;  $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$ . All tests and analysis were carried out in triplicate and averaged.

#### Determination of tyrosinase inhibition activity<sup>11-13</sup>

Mushroom tyrosinase was used for the bioassay because it is readily available. Since the mode of inhibition depends on the structure of both the substrate and inhibitor, L-DOPA was used as the substrate in this experiment, unless otherwise specified. Therefore, inhibitors discussed in this paper are inhibitors of diphenolase activity of mushroom tyrosinase, and their effect on the enzyme was determined by spectrophotometry, based on dopachrome formation at 475nm. All the samples were first dissolved in dimethyl sulfoxide (DMSO) and used for the experiment at 30 times dilution. L-DOPA solution (0.87ml, 4.5mM) was mixed with 0.9ml of 0.1M phosphate buffer (pH6.8) and incubated at 30°C for 5min. Then 0.9ml of various concentrations of sample solutions followed by 0.03ml of the aqueous solution of mushroom tyrosinase (4000 units) was added to the mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475nm (30°C), corresponding to the formation of dopachrome, for 25min at 1min intervals. Controls, without inhibitor but containing 3.3%DMSO, were routinely determined. The percent inhibition of the enzyme by the active compounds was calculated as follows; inhibition (%) =  $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$ . The inhibitory effect (%) of the compounds was expressed as the inhibitor concentration causing 50% loss of enzyme activity (ID<sub>50</sub>). All the studies were carried out at least in triplicate.

## RESULTS & DISCUSSION

The oil was quantitatively analysed by various methods with the help of standard procedures. The results were tabulated in table no: 1.

**TABLE 1:** Quantitative analysis of seed oil

S.No	Parameter	Observed value
1	Specific gravity	0.956
2	Iodine value	102.32
3	Sap. value	186.5
4	Acid value	2.41
5	Peroxide value	4.2

**Determination of total phenolic content**

From the results, the total phenolic content was found that, the 0.5 mg/ml of aqueous extract of *Punica granatum* seed contain 103.50µg of phenolic compound which is 20.7% equivalent to gallic acid.

**Antioxidant activity**

The free radical scavenging activity was evaluated by using various Invitro assays. DPPH radical was used as a substrate to evaluate the free radical scavenging activity of aqueous extract and oil of seeds of *Punica granatum*. The scavenging effects of aq. extract and oil of seeds of *Punica granatum* on the DPPH radical was 54.29% for aqueous extract and 65.44% for oil at the concentration of 1000 µg/ml, compared to the scavenging effects of ascorbic acid.

Hydroxyl radicals are the major active oxygen species that cause lipid oxidation and enormous biological damage. The percentage of H<sub>2</sub>O<sub>2</sub> scavenging activity of aqueous extract and oil of seeds of *Punica granatum* were found to be 62.67% and 69.46 at the concentration of 1000 µg/ml compared to the scavenging effects of ascorbic acid.

The superoxide anion radical scavenging activity of aqueous extracts and oil of seeds of *Punica granatum* was assayed using the PNS – NADH system. The percentage of superoxide generation by *Punica granatum* seed extracts and seed oil at 1000 µg/ml concentration were found to be 58.69% and 68.0% inhibition of the superoxide radical.

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and retgulation of cell mediated toxicity. The percentage inhibition of nitric oxide generation by aqueous seed extracts and seed oil of *Punica granatum* at 1000 µg/ml concentration were found to be 60.92 and 66.55. From the results it can be known that seed oil possessing much free radical scavenging activity when compared to seed extract (Table 2).

**TABLE 2:** comparison of IC<sub>50</sub> value of antioxidants activity of seed extract and oil of P. granatum. L

S.No	Antioxidant method	Seed extract (µg/ml)	Seed oil (µg/ml)	Standard (µg/ml)
1.	DPPH	703.9	277.5	46.23
2.	Hydrogen peroxide	389.6	262.2	28.52
3.	Superoxide anion	331.14	206.5	14.19
4.	Nitric oxide	388.3	251.1	58.66

**TYROSINASE INHIBITION ACTIVITY**

Tyrosinase plays an important role in the formation of melanins because it facilitates melanization by catalyzing reaction from tyrosinase to dopa and from dopa to dopa quinine. Tyrosinase inhibitors have become increasingly important as cosmetic and medicinal product, primarily to control melanin pigmentation. Melanin synthesis inhibitors are topically used for treating localized hyperpigmentation in humans such as lentigo, nevus, ephelis, postinflammatory state and melanoma of pregnancy. Mushroom tyrosinase was used for the bioassay because it is readily available. Since the mode of inhibition depends on the structure of both the substrate and inhibitor, L-DOPA was used as the substrate in this

experiment, unless otherwise specified. Therefore, inhibitors discussed in this method are inhibitors of diphenolase activity of mushroom tyrosinase, and their effect on the enzyme was determined by spectrophotometry, based on dopachrome formation at 475nm.

In the present study both seed extract and seed oil exhibited almost equal percentage of inhibition for mushroom tyrosinase at 250µg/ml i.e. 62.52% & 75.52 % respectively. The IC<sub>50</sub> values were found to be 85.54µg/ml & 39.73µg/ml for seed extract and seed oil respectively. It clearly indicates that both the extracts may be included for formulating a herbal cosmeceuticals for various skin pigmentation problem (Table 3&4).

**TABLE 3:** Mushroom Tyrosinase Inhibitory activity of seed extract and oil of P. granatum.L

S.no	Conc. (µg/ml)	Seed Extract		Seed Oil	
		% Inhibition	IC <sub>50</sub> (µg/ml)	% Inhibition	IC <sub>50</sub> (µg/ml)
1	250	62.52	85.54	75.52	39.73
2	125	53.05		63.67	
3	62.50	46.52		52.83	
4	31.25	38.52		47.25	
5	15.62	33.20		39.40	

**TABLE 4.** Mushroom Tyrosinase Inhibitory activity of Standard Kojic acid

S.no	Conc. ( $\mu\text{g/ml}$ )	Kojic Acid	
		% Inhibition	IC <sub>50</sub> ( $\mu\text{g/ml}$ )
1	25	91.4	
2	12.5	79.2	
3	6.25	58.5	4.02
4	3.12	41.2	
5	1.56	29.5	

### CONCLUSION

The *in-vitro* antioxidant and mushroom tyrosinase inhibitory activity shows significant results when compared to standard drugs respectively. The promising results encourages us to use the extract and oil of seeds of *P.granatum* L. may be included as an ingredient in formulation of cosmeceuticals for skin depigmentation problems.

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