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In Vitro Antioxidant and Anti-inflammatory Activity of Isolated Compound from Methanolic flowers Extract of *Punica granatum* Linn.

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Abstract: The aim of present study was to investigate *in vitro* antioxidant and anti-inflammatory potential of methanolic extract of flowers of *Punica granatum* Linn. (Family: Punicaceae). Antioxidant activity was studied using 1, 1-Diphenyl-2-Picrylhydrazyl radical scavenging activity (DPPH), superoxide radical scavenging activity (SRSA), total reducing power (TRP) assay, nitric oxide radical scavenging activity (NOSA), anti-lipid peroxidation (ALPO) activity, ABTS radical scavenging Activity and hydrogen peroxide scavenging activity (HPSA). Ascorbic acid was used as standard antioxidant. *In•vitro* anti-inflammatory activity was evaluated using membrane stabilization assay and at different concentrations. Diclofenac was used as a standard drug for the study of anti- inflammatory activity. Linear regression analysis was used to calculate IC_{50} value. Results showed that, the extract exhibited significant total antioxidant, DPPH, SRSA, TRP, NOSA, ABTS and HPSA with IC_{50} value. Extract also showed *in•vitro* anti-inflammatory activity by inhibiting the Red Blood Cells membrane stabilization with the IC_{50} values. This study revealed that the methanolic flower extracts of *Punica granatum* Linn. Has demonstrated significant antioxidant and anti inflammatory activity.

Keywords: *Punica granatum* Linn, Punicaflavone, Antioxidant and Anti inflammatory activity.

INTRODUCTION

Antioxidant compounds in food play an important role as a health protecting factor. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables.

The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species can initiate degenerative diseases. Antioxidant compounds like phenolic acids, polyphenols and flavonoids are commonly found in plants have been reported to have multiple biological effects, including antioxidant activity [1]. Currently, the possible toxicity of synthetic antioxidants has been criticized. Thus interest in natural antioxidant, especially of plant origin has greatly increased in recent years [2]. Excess free radicals can result from tissue damage and hypoxia, over exposure to environmental factors (eg: smoking, ultraviolet radiation, and pollutants) and due to lack of antioxidants or destruction of free radical scavengers. When the production of damaging free radicals exceed the capacity of the body's antioxidant defenses to detoxify them, a condition known as oxidative stress occurs [3]. As free radicals play an important role in the diseases scenario of an individual, a thorough understanding of the various physiologically significant free radicals is of paramount importance [4]. Consequently, antioxidants that can neutralize direct ROS attacks and terminate free radical mediated oxidative reaction would have beneficial effect in protecting the human body from various diseases [5, 6]. The researchers have focused on natural antioxidants and numerous crude extracts and pure natural compounds have been reported to possess antioxidant properties [7].

Inflammation is the reaction of living tissues to injury, infection or irritation. Lysosomal enzymes released during inflammation produce a variety of disorders which leads to the tissue injury by damaging the macromolecules and lipid peroxidation of membranes which are assumed to be responsible for certain pathological conditions as heart attacks, septic shocks and rheumatoid arthritis etc. The extra cellular activity of these enzymes is said to be related to acute or chronic inflammation. Stabilization of lysosomal

membrane is important in limiting the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release or by stabilizing the lysosomal membrane [8]. HRBC or erythrocyte membrane is analogous to the lysosomal membrane [9] and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of human red blood cell membrane (HRBC) by hypo tonicity induced membrane lysis can be taken as an in vitro measure of anti inflammatory activity of the drugs or plant extracts.

In many inflammatory disorders there is excessive activation of phagocytes, production of o2, oh radicals as well as non-free radical species (H_2O_2) [10] which can harm surroundings tissue either by powerful direct oxidizing action or indirectly with hydrogen peroxide (H_2O_2) and OH radicals formed from O_2 which initiates lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors [11]. The reactive oxygen species are also known to activate matrix metalloproteinase (e.g. collagenase) causing increased destruction of tissues e.g. collagenase damage seen in various arthritic reactions [12].

Punica granatum L. (Punicaceae), known as pomegranate, is a deciduous small tree, up to 8 m in height with attractive reddish scarlet edible fruits. The species originated in Iran, Afghanistan and Baluchistan, found wild in the warm valleys of the Himalayas and is cultivated throughout India [13]. The dried flowers, known as Gulnar, are efficacious to treat haematuria, haemoptysis, diarrhoea, dysentery, nasal hemorrhage [14] and in Unani literature as a remedy for diabetes [15-16]. Flower juice is recommended as a gargle for sore throat, in leucorrhoea, hemorrhages and ulcers of the uterus and rectum. The root bark and stem bark of the plant are astringent and used as anthelmintic especially against tapeworms. Fruit rind is valued as an astringent in diarrhea and dysentery. The powdered flower buds are useful in bronchitis. The seeds are reputed as stomachic and the pulp as cardiac and stomachic. The green leaf paste is applied to relieve conjunctivitis [17]. In addition *Punica granatum* L. is considered as "a pharmacy unto itself" in ayurvedic medicine and is used as an antiparasitic agent, a blood tonic, and to ulcers [18].

The aim of this study was to investigate the antioxidant and anti inflammatory properties of isolated compound (Punicaflavone) from the methanol extract of flowers of *Punica granatum* L. through different *in vitro* assays.

MATERIALS AND METHODS

Collection and authentication of plant material

The flowers of *Punica granatum* L. were collected from in and around the Mannargudi, Thiruvarur DT, Tamilnadu, India. They were identified and authenticated by Dr. S. John Britto, Department of Botany, Rabiant Herbarium and center for Modular Systematics, St. Joseph's College, Trichurappalli, and Tamilnadu, India.

Preparation of plant material

Collected plant material were thoroughly washed with distilled water and then dried under shade at room temperature for few days. The dried plant samples were ground well into a fine powder using blender. The powdered samples were then stored in airtight containers for further use at room temperature.

Preparation of extract

The residue was exhaustively extracted in a Soxhlet apparatus for at least 12 h with alcohol (methanol) and extract was used for experiment. The solvent from extract was removed under reduced pressure and controlled temperature (40-50 ºC). The yield of the extract was 12.28% w/w. The extract was kept in tightly closed container in refrigerator for further analysis.

Isolation, puriification and characterization of compound

The isolation, purification and characterization of bioactive compounds were carried out using repeated silica gel column chromatography and thin layer chromatography (TLC) [19]. The purified bioactive compound was characterized by subjected to UV, IR, NMR and MS spectroscopy studies.

In vitro studies

The isolated compound (Punicaflavone) from methanolic extract of *Punica granatum* L. was tested for its free radical scavenging and anti inflammatory property using different invitro models. All the experiments were performed thrice and the results averaged. The results were expressed as mean ± standard deviation. Linear regression analysis was used to calculate IC_{50} value.

Antioxidant activity

The antioxidant activities of the isolated compound (10-30 µg/ml) was investigated by using seven different *in vitro* assays Viz., Total Antioxidant activity [20], DPPH radical scavenging activity (DPPH) [21], Superoxide radical scavenging activity (SRSA) [22], total reducing power (TRP) assay [23], nitric oxide scavenging activity (NOSA) [24], ABTS radical scavenging Activity [25], and hydrogen peroxide scavenging activity (HPSA) [26]. Ascorbic acid was used as standard antioxidant. Results were expressed as IC_{50} value, which represents the sample concentration require to show 50% antioxidant activity.

Anti-inflammatory activity

The HRBC membrane stabilization has been used as a method to study the anti inflammatory activity [27].

RESULTS

Antioxidant activity

Antioxidant activity of Punicaflavone was performed by various *in vitro* models like TAC, DPPH, and Superoxide radical ion, reducing power assay, Nitric acid scavenging and ABTS assay at three different concentrations. Similarly standard ascorbic acid was also involved for the evaluation of antioxidants potential. The results of antioxidant properties by various models and IC $_{50}$ values were depicted in table-1 and figure 1-7.

Table-1: In-vitro- Antioxidant Activity of Punica flavone

S.No	Particulars	Concentration (µg /ml)	Punicaflavone	IC50 Value (µg)	Ascorbic acid	IC50 Value (µg)
1.	Total Antioxidant activity (%)	10	20.73 ± 0.86	200	23.04±0.08	170
		20	24.66 ± 0.47		29.69±0.07	
		30	29.45 ± 0.03		37.64±0.78	
2.	DPPH radical scavenging activity (%)	10	29.48 ± 0.46	260	30.69±0.07	210
		20	41.55 ± 0.12		49.44±0.48	
		30	65.72 ± 0.73		61.88±0.49	
3.	Superoxide radical anion scavenging activity (%)	10	26.47 ± 0.16	250	22.43±0.08	220
		20	39.49 ± 0.03		47.44±0.91	
		30	53.43 ± 0.09		61.88±0.49	
4.	Reducing power assay (%)	10	29.40 ± 0.27	270	28.49±0.48	180
		20	47.98 ± 0.45		49.44±0.48	
		30	71.19 ± 0.15		70.18±0.13	
5.	Nitric oxide radical Scavenging activity (%)	10	53.45 ± 0.72	150	61.73±0.77	210
		20	69.84 ± 0.09		73.04±0.45	
		30	75.04 ± 0.44		78.39±0.18	
6.	ABTS radical scavenging Activity(%)	10	27.43 ± 0.07	200	24.66±0.45	240
		20	59.49 ± 0.03		65.71±0.78	
		30	78.81 ± 0.05		80.65±0.20	
7.	Hydrogen peroxide scavenging activity (%)	10	33.81±1.60	170	34.26±0.94	190
		20	38.73±0.79		46.55±2.09	
		30	47.08±1.06		56.72±0.75	

All values are expressed as mean ± SEM for three determinations

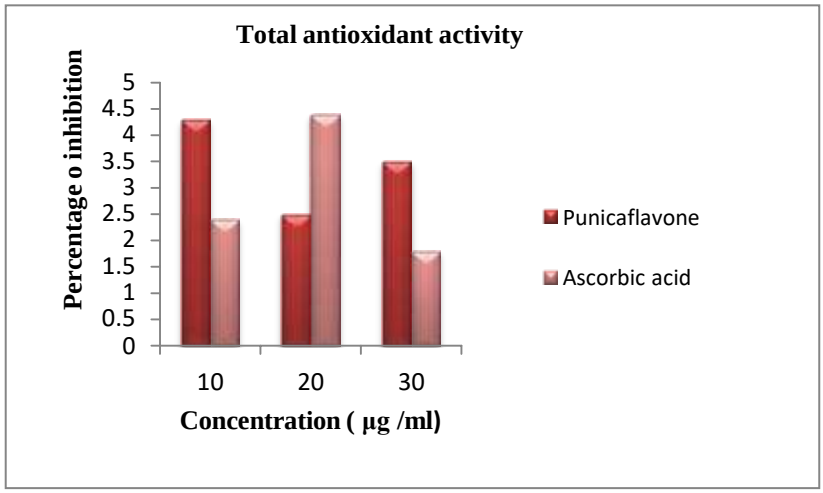

Fig-1: Total antioxidant activity

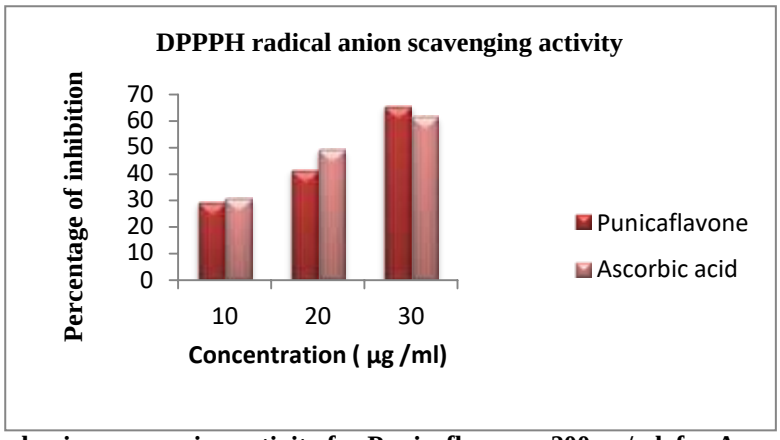

Fig-2: DPPPH radical anion scavenging activity for Punicaflavone =200 µg/ml, for Ascorbic acid =170 µg/ml

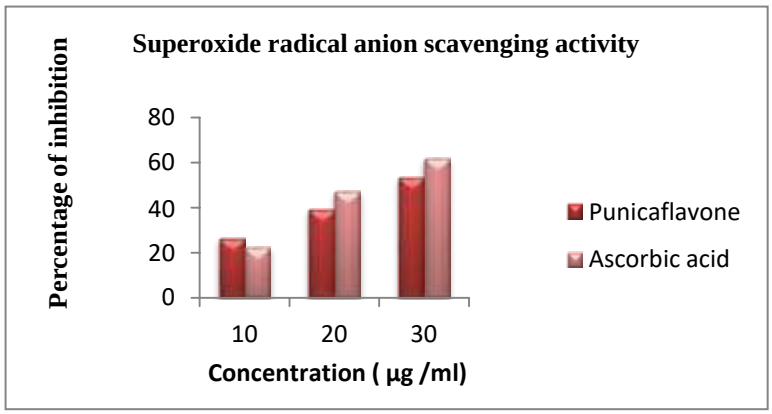

Fig-3: Superoxide radical anion scavenging activity for punicaflavone =260 µg/ml, for ascorbic acid =210 µg/ml

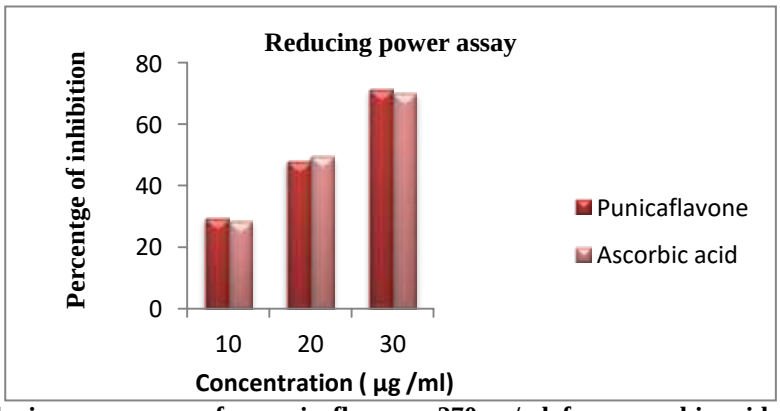

Fig-4: Reducing power assay for punicaflavone =270 µg/ml, for s ascorbic acid =180 µg/ml

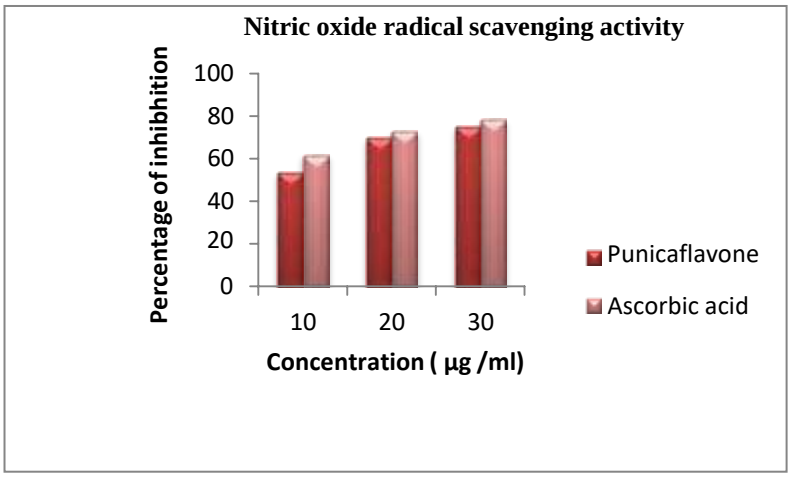

Fig-5: Nitric oxide radical scavenging activity for punicaflavone =150 µg/ml, for ascorbic acid =210 µg/ml

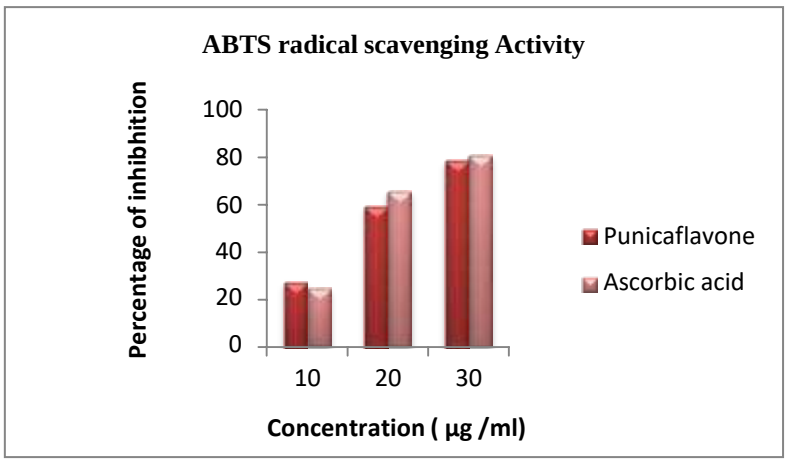

Fig-6: ABTS radical scavenging Activity for punicaflavone =200 µg/ml, for ascorbic acid =240 µg/ml

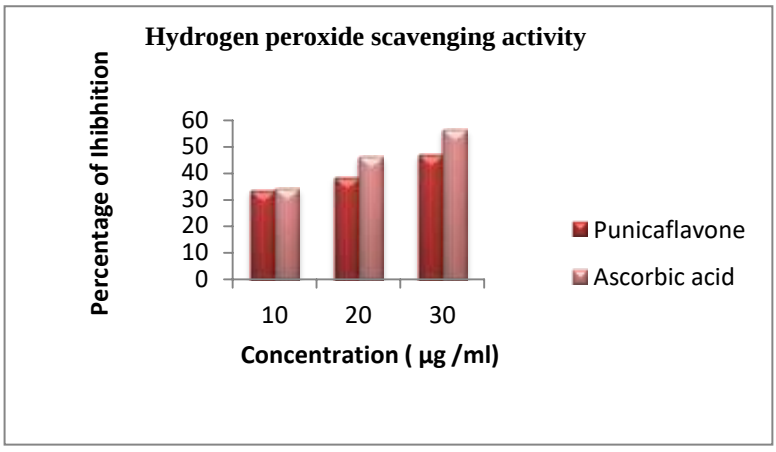

Fig-6: Hydrogen peroxide scavenging activity for punicaflavone =170 µg/ml, for s ascorbic acid =190 µg/ml

Anti inflammatory activity

In vitro anti-inflammatory activity of plant drug was evaluated by HRBC membrane stabilization method. Percentage of protection by the plant drug was compared with standard Diclofenac. The results were recorded in table-2 and figure-2.The *in vitro* anti inflammatory activity of the Punicaflavone was concentration dependent, with the increasing concentration, the activity is also increased. The concentration of 30µg/ml showed 75.48 % protection of HRBC in hypotonic solution. All the results were compared with standard Diclofenac which showed 82.47 % protection.

Table-2: In-Vitro- Anti-Inflammatory Activity of Punicaflavone

S.No	Concentration (µg/ml)	Punicaflavone		Standard (Diclofenac Sodium)	
		Optical density at 560 mm	% Protection	Optical density at 560 nm	% Protection
1	10	0.39 ±0.06	61.70	0.25 ± 0.03	75.65
2	20	0.34 ± 0.04	66.27	0.22 ± 0.07	78.82
3	30	0.25 ± 0.01	75.48	0.18 ± 0.05	82.47

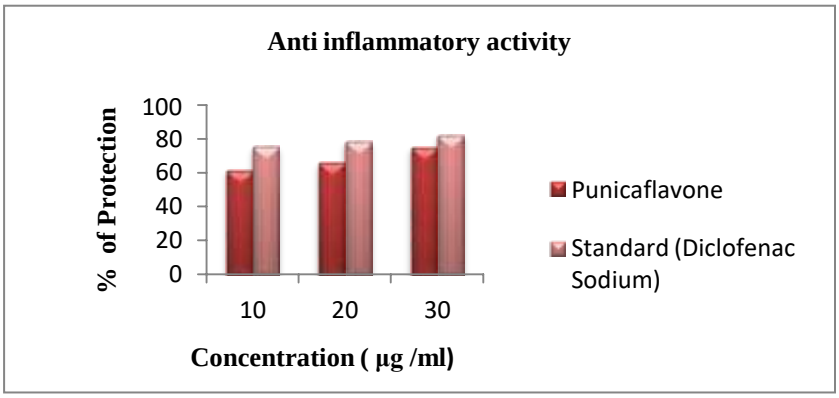

Fig-8: *In-Vitro* Anti inflammatory activity of Punicaflavone

DISCUSSION

Antioxidant activity

The antioxidant properties of the Punicaflavone were significantly corroborated by the phytochemical constituents of the flowers of Punica grantum L.

Total antioxidant activity

The total antioxidant activity of the methanol plant extract was found to be concentration dependent that is with the increasing concentration, the activity is also increased. Highest antioxidant activity of Punicaflavone and standard were found to be 29.45 and 37.64% respectively at 30µg/ml. IC_{50} value for Punicaflavone and standard drug were 800 and 650µg respectively. These findings showed that Punicaflavone exhibited good antioxidant activity which was similar to that of standard. The phospho molybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid [28].

DPPH radical scavenging activity

The DPPH radical is a stable free radical, which has been widely used as a sensitive and rapid tool to estimate the free radical scavenging activity of both hydrophilic and lipophilic antioxidants [29]. Antioxidants neutralize the free radicals on interaction with DPPH by transferring electrons or hydrogen atoms to DPPH. This method determines the antiradical power of an antioxidant by measuring the decrease in the absorbance of DPPH, resulting in a colour change from purple to yellow, through the donation of hydrogen to form a stable DPPH molecule [30]. DPPH shows strong absorbance in the radical form at a wavelength of 517 nm, which disappears after the acceptance of an electron or a hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule . In the present study, the percentage of scavenging effect on the DPPH radical was concomitantly increased with the increase in the concentration of Punicaflavone from 10to 30µg/ml. The percentage of inhibition is existing from 29.48 at 10µg/ml to 65.72 at 30µg/ml.

Superoxide radical ion scavenging activity

Superoxide radicals, which are highly toxic ROS, are generated in the body by various biological and metabolic reactions. Although the relatively weak superoxide oxidants have only limited chemical reactivity, they are potential precursors of highly reactive species including hydrogen peroxide, hydroxyl radicals and singlet oxygen, causing lipid peroxidation [31]. Superoxide radical scavenging capacity is the first line of defense against oxidative stress in humans. The superoxide anion is an oxygen-centered radical with selective reactivity. It has been reported that the antioxidant properties of some plant products are effective mainly *via* scavenging of superoxide anion radicals [32].

In this assay, superoxide anions derived from dissolved oxygen by the PMS/NADH system reduce NBT. Superoxide radical reduces the yellow dye (NBT^{2+}) to produce blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants inhibit the formation of blue NBT. The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion radical in the reaction mixture. Table 11 indicated the concentration-dependent inhibition of superoxide radical generation by all concentrations of Punicaflavone and the standard ascorbic acid. At the concentration of 30µg/ml, the superoxide radical inhibitory effects of Punicaflavone and ascorbic acid were 53.43% and 61.88%,

respectively. Study suggested that the flavonoids may be involved in the dismutation of superoxide anion radical [33].

Reducing power assay

Several studies have shown that the electron donation capacity reflects the reducing power of biologically active compounds in relation to their antioxidant activity [34]. The reduction of Fe^{3+} is often used as an indicator of electron donating ability, which is an important mechanism of phenolic antioxidant action. In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of Fe^{3+} to Fe^{2+} by the donation of an electron. The amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue ferric ferrocyanide ($Fe_4[Fe(CN)_6]_3$) at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. It was found in this assay that the reducing power of Punicaflavone and ascorbic acid increased with the increase in their concentrations. At the concentration of 30µg/ml, the reducing power efficacies of Punicaflavone and ascorbic acid were 71.19 and 70.18, respectively. Similarly, various plant extracts containing different phytochemicals have shown antioxidant activity through their reductive capacity in a Fe^{3+}–Fe^{2+} system [35]. The results obtained in this study indicate that the marked reducing power of Punicaflavone may be attributable to its antioxidant activity.

Nitric Oxide radical scavenging activity

As a cell signaling molecule, nitric oxide has been associated with a variety of physiological processes in the human body. It transmits signals from vascular endothelial cells to vascular smooth muscle cells, resulting in vasodilatation [36]. It plays an important role in vital physiological functions in the respiratory, immune, neuromuscular and other systems. A number of related reports suggest that nitric oxide may modulate iron-catalyzed oxidation reactions such as the superoxide-anion driven Fenton's reaction, resulting in the production of strong oxidants such as the hydroxyl radical and organometallic complexes [37]. The mechanisms by which nitric oxide may inhibit lipid peroxidation have not been studied in depth; however, one possible mechanism relates to its ability to terminate the propagation of lipid peroxidation reactions. The results were statistically significant ($p < 0.05$) and concentration dependent. The nitric oxide radical scavenging activities of various plant-based products and extracts have been reported previously [38].

Free radical scavenging activity (ABTS•+)

The decolorization of the ABTS+ was measuring through the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm [39]. ABTS•+ was generated by incubating ABTS+ chromophore through the reaction [40]. The presence of specific chemical compounds in the Punicaflavone may inhibit the potassium per sulfate activity and hence reduced the production of ABTS+. This study reports that the isolated Punicaflavone has highest antioxidant activity (78.81) and that of its standard ascorbic acid was 80.65 (Table 1).

Hydrogen peroxide scavenging activity

Hydrogen peroxide is weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with Fe 2+ and possibly Cu 2+ ions to form hydroxyl radicals and these may be the origin of many of its toxic effects [41]. From the results it appeared H2O2 scavenging activity of the methanol extract is comparatively very high to that of the standard ascorbic acid (Table 1 and Fig-7).

ANTI INFLAMMATORY ACTIVITY

Inflammation is a normal protective response to tissue injury and it involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair [42]. It is a complex process, which is frequently associated with pain and involves occurrences such as: the increase in vascular permeability, increase of protein denaturation and membrane alterations [43].

The HRBC membrane stabilization has been used as a method to study the *invitro* anti inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane [44] and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation produce a various disorders. The extra cellular activity of these enzymes are said to be related to acute or chronic inflammation. The non steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane.

Anti-inflammatory refers to the property of a substance that reduces inflammation or swelling. The inflammatory response is coordinated by a large range of mediators that form complex regulatory networks. They activate specialized sensors, which then elicit the production of specific sets of mediators. The mediators, in turn, alter the functional states of tissues and organs (which are the effectors of inflammation) in a way that allows them to adapt to the conditions indicated by the particular inducer of inflammation [45]. Membrane damage refers to the damage of cell membranes which disturb the state of cell electrolytes, which when constantly increased, induces apoptosis. RBC is

essentially a bag of hemoglobin (Hb). The RBC is unique among eukaryotic cells in that it is a-nuclear, has no cytoplasmic structures and organelles. Structural properties are linked to the membrane. RBCs take up oxygen in the lungs and release it into tissues while squeezing through the body's capillaries. Anesthetics tranquilizers and nonsteroidal anti-inflammatory drugs stabilize erythrocytes against hypotonic hemolysis at low concentration. When RBC is subjected to hypotonic stress the release of Hb from RBC is prevented by anti inflammatory agents because of membrane stabilization. So, the stabilization of HRBC membrane by drugs against hypotonicity-induced hemolysis serves as a useful in vitro method for assessing the anti-inflammatory activity of plant extracts [46].

CONCLUSION

In conclusion, the results of the present investigation infer that the isolated compound (Punicaflavone) possess potent antioxidant and anti-inflammatory property. The present study was attempted for the first time to investigate the antioxidant and anti inflammatory activity of Punicaflavone from *Punica granatum* L. search for newer, safer and more potent antioxidant agent and we herein delineate the results of our study. This analysis revealed that the flowers of *Punica granatum* L. contained higher value of different secondary metabolites, which are used in different disease.

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