IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 13, Issue 2 Ver. I (Mar. – Apr. 2018), PP 58-67
www.iosrjournals.org

Evaluation of Phytochemical Constituents and *In vitro* Anti-inflammatory Activity of Kashmiri Pomegranate (*Punica granatum* Linn.) Flower Extract

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Abstract: *Punica granatum Linn. is popularly known as pomegranate (Anar). It is a member of Punicaceae family, have been used in folk medicine for centuries in the Middle East, India, and China, and it has been used to treat ailments ranging from inflammation to the pain of a simple sore throat. Different part of Pomegranate like bark, leaves, immature fruits, and fruit rind have some medicinal significance. The pericarps of Punica granutam L. contains seven highly active inhibitors of carbonic anhydrase (CA) i.e., punicalin, punicalagin, granatin B, gallagyldilactone, punicalagin,pedunculagin and tellimagrandin.The four weakly active inhibitors, gallic acid, granatin A, corilagin and ellagic acid, are known to exhibitant microbial, antifungal, antimutagenic activity. Other traditional uses have included treatments for snakebite, diabetes, burns and leprosy. The fresh fruit itself has been used to lower fever. The anti-inflammatory activity of methanolic and aqueous extracts of Punica granatumL. was determined using Inhibition of albumin denaturation, Membrane stabilization, Antiproteinase and Antilipoxygenase methods. Aspirin, Diclofenac sodium and Indomethacin were used as standards. The results showed that Indomethacin has the highest inhibition value.Preliminary Phytochemical Screening of flowers of Punica granatumL. showed that methanolic and aqueous extracts are rich source of phytochemicals as compared to ethyl acetate extract. Methanolic and aqueous extracts showed positive results for various phytoconstituents, both methanolic and aqueous extracts showed the presence of maximum constituent's viz., sterols and triterpenoids, glycosides, saponins, alkaloids, flavonoids, tannins, proteins, amino acids and carbohydrates. Methanolic extract of Punica granatumL. flowers showed negative result for glycosidesand proteins. Ethyl acetate showed positive result for glycosides.*

Keywords;*Punica granatum, antimicrobial, anti-inflammatory, anticancer, Aspirin, Diclofenac sodium, Indomethacin.*

Date of Submission: 26-02-2018 Date of acceptance: 17-03-2018

I. Introduction

Inflammation is the body's reaction to invasion by an infectious agent, antigen challenge or even just physical, chemical or traumatic damage. It is a stereotype response that is identical irrespective of the stimuli **(pathogenic organism, foreign body, ischemia, physical trauma, ionizing radiation, electrical energy, or extreme temperature).**

The effect of inflammation is divided into two fold i.e.

- To destroy or remove the causative agent.
- To repair the damage tissue.

An inflammatory response can also be divided into two major categories acute and chronic based on timing and pathological features. An acute inflammatory response starts within minutes and lasts for days or a few weeks, while a chronic inflammation could last for months or even years and it may arise from acute inflammation or it starts to be chronic from the very beginning. Sepsis, severe trauma and major surgery all have major acute inflammatory components. It is characterized by vasodilatation, the exudation of protein-rich fluid (plasma) and a migration of cells (primarily neutrophils) into the site of injury. Vasodilatation is a classic feature of acute inflammation. The purpose of the vasodilatory response is to facilitate the local delivery of soluble mediators and inflammatory cells. An acute inflammatory response may be triggered by activation of tissue resident phagocytes through LPS, a cell wall component of gram-negative bacteria **(Koj, 1985).** This provokes the secretion of quite a number of proteins and other agents, among them the proinflammatory cytokines IL-1, IL-6 and TNF-α **(Matsushima and Oppenheim, 1989).** Subsequently, IL-land TNF-α leads to an activation of fibroblasts and endothelial cells and a secondary release of cytokines by these cells. The combined action of these proinflammatory agents mediates the above mentioned local changes, thus initiating focal leukocyte recruitment **(Moser *et al*., 1992; Moser *et al*., 1989; Muir, 1992).**

*Punica granatum*L.is the male abortive flower of wild rumman plant which belongs to the family Punicaceae. Though the entire plant has medicinal value however, its flowers and rind are more commonly used as therapeutic agents for diverse pathological conditions. Since the Persian variety is supposed to be superior so it is more famous. The flowers of this species are large in size and red, pink, white, and black in colour. The flowers are astringent and hence used medicinally.

Several studies focusedon prevention and treatment of cancer, cardiovasculardisease, diabetes, dental conditions, erectiledysfunction and skin allergy investigations were carriedout to determine antioxidant, anticarcinogenic andanti-inflammatory properties of pomegranate constituents**(Ahmed *et al.*, 2013). Singh *et al.* (2002)** evaluated theconstituents of *Punica granatum* L.which includedgallocatechins, delphinidin, cyanidin, gallic acid andsitosterol, which had therapeutic properties. Numerousphytochemical constituents have been reported to be present in different parts of the pomegranate plantmaking it pharmacologically precious **(Prakash andPrakash, 2011).Barzegarl *et al.* (2007)** studied the peelextract of *Punica granatum* L.and reported substantial amountsof polyphenols such as ellagic tannins, ellagic acid andgallic acid. Preliminary phytochemical screening of theaqueous extract of *Punica granatum* L.peels gave positive testsfor tannins, flavonoids, and alkaloids and showed thatthe aqueous extract of *P. granatum* L.peels may containsome biologically active principles that may be the basisfor its traditional use **(Qnais *et al.*, 2007).Prakash and Prakash (2011)** showed significantvariations in organic acids, phenolic compounds, sugars, water-soluble vitamins and mineral composition ofpomegranates in their study. Keeping the above facts inview, this study investigated phytochemical constituentsof methanolic, ethyl acetate and aqueous extracts of *Punicagranatum*L flowers.

II. Materials and Methods

Collection and Identification of Plant Material

The flowers of *Punica granatum*L.were collected from Chandilora, Tangmarg,Kashmir in the month of May and were identified and authenticated by Mr. Akhtar, Curator, at theCentre for Biodiversity and Taxonomy, Department of Botany, University of Kashmir.The Voucher specimen has been retained in the herbarium of The Centre for Biodiversityand Taxonomy, Department of Botany, University of Kashmir for future reference underherbarium number: **2428 (KASH).**The flower material was cleaned, reduced to smallfragments, air dried under shade at room temperature and coarsely powdered in agrinding mill. The powdered material was stored or taken up for extraction process.

Preparation of Plant Extracts

The powdered plant material was successively extracted in 250ml of methanol and distilled water by using Soxhlet extractor. The plant material was suspended in the main chamber of Soxhlet extractor which was then placed onto a flask containing the extraction solvent. The Soxhlet was then equipped with a condenser. The flask was heated; the solvent evaporated and moved up into the condenser where it was converted into a liquid that trickled into the extraction chamber containing the sample. The chamber containing the solid material was slowly filled with warm solvent. When the Soxhlet chamber was almost full, the chamber was automatically emptied, with the solvent running back down to the distillation flask. This cycle was repeated many times, over hours or a few days, until the colour of the solvent in the siphon of the Soxhlet faded away. At the end of the hot extraction process each extract was filtered. The filtrate was concentrated and the solvent was recovered using rotary evaporator. The extracts were then kept in desiccators to remove remaining moisture, if present, and finally stored in air tight containers at 4^{o}C for further use.

Preliminary Phytochemical Analysis of the Extracts

The *Punica granatum* L.flower extracts were tested for various chemical constituents with the help of different chemical tests. The fallowing extracts were subjected to preliminary phytochemical screening for the detection of various plant constituents:

- Methanolic extract
- Ethyl acetate extract
- Aqueous extract

The extracts subjected to preliminary phytochemical investigation for the detection of following **(Anonymous, 1998; Anonymous, 1985).** Following standard procedures were used.

Test for Steroids and Triterpenoids:

Liebermann Burchard test - Crude extract was mixed with few drops of acetic anhydride, boiled and cooled concentrated sulphuric acid was then added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. Green coloration of the upper layer and the formation of deep red color in the lower layer would indicate a positive test for steroids and triterpenoids respectively.

Test for Glycosides:
Killiani Test – Test solution was treated with few drops of glacial acetic acid and Ferric chloride solution and mixed. Concentrated sulphuric acid was added, and observed for the formation of two layers. Lower reddish brown layer and upper acetic acid layer which turns bluish green would indicate a positive test for glycosides.
Bromine water test - Test solution was dissolved in bromine water and observed for the formation of yellow precipitate to show a positive result for the presence of glycosides.

Test for Saponins:
Foam Test – Test solution was mixed with water and shaken and observed for the formation of froth, which is stable for 15 minutes for a positive result.
Test for Alkaloids:
Hager's Test– Test solution was treated with few drops of Hager's reagent (saturated picric acid solution).Formation of yellow precipitate would show a positive result for the presence of alkaloids.
Test for Flavonoids:
Ferric chloride test – Test solution when treated with few drops of Ferric chloride solution would result in the formation of blackish red color indicating the presence of flavonoids.
Alkaline reagent Test – Test solution when treated with sodium hydroxide solution, shows increase in the intensity of yellow color which would become colorless on addition of few drops of dilute Hydrochloric acid, indicates the presence of flavonoids.
Lead acetate solution Test – Test solution when treated with few drops of lead acetate (10%) solution would result in the formation of yellow precipitate.
Test for Tannins:
Gelatin Test – Test solution when treated with gelatin solution would give white precipitate indicating the presence of tannins.
Test for Proteins:
Biuret Test – Test solution was treated with 10% sodium hydroxide solution and two drops of 0.1% copper sulphate solution and observed for the formation of violet/pink color.
Test for Free Amino Acids:
Ninhydrin Test – Test solution when boiled with 0.2% solution of Ninhydrin, would result in the formation of purple color suggesting the presence of free amino acids.

Test for Carbohydrate:
Benedict's test – Test solution was mixed with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and boiled in water bath, observed for the formation of reddish brown precipitate to show a positive result for the presence of carbohydrate.

III. Evaluation of *in vitro* anti-inflammatory assay

1. Inhibition of albumin denaturation
The anti-inflammatory activity of methanolic and aqueous extract of flowers of *Punica granatum*L.was studied by using inhibition of albumin denaturation technique which was studied according to **(Mizushima *et al* .,1968 and Sakat *et al.*,2010)** followed with minor modifications. The reaction mixture was consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37ºC for 20 min and then heated to 51ºC for 20 min, after cooling the samples the turbidity was measured at 660nm. The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows: Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control
2. Membrane stabilization
Preparation of Red Blood Cell (RBC_S) suspension (Sakat *et al*., 2010; Sadique *et al*., 1989)
The Blood was collected from healthy human volunteer who has not taken any NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) for 2 weeks prior to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline.
(a)Heat induced haemolysis (Sakat *et al.*, 2010; Shinde *et al*., 1999)
The reaction mixture (2ml) consisted of 1 ml test sample of different concentrations (100 - 500 µg/ml) and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56ºC for 30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was

taken at 560 nm. The experiment was performed in triplicates for all the test samples. The percentage inhibition was calculated as follows:

Percentage inhibition = (Abs control- Abs sample) × 100/Abs control

(b)Hypotonicity induced haemolysis (Azeem *et al*., 2010)

Different concentration of *Punica granatum*L. flower extracts (100-500µg/ml), reference sample, and control were separately mixed with 1ml of phosphate buffer, 2ml of hypo saline and 0.5ml of HRBC suspension. Diclofenac sodium (100µg/ml) was used as a standard drug . All the assay mixtures were incubated at 37℃ for 30 minutes and centrifuged at 3000rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by a spectrophotometer at 560nm. The percentage hemolysis was estimated by assuming the haemolysis produced in the control as 100%.

Percentage protection = 100- (OD sample/OD control) x 100

IV. Antiproteinase action

The test was performed according to the modified method of **(Oyedepo *et al*., 1995 and Sakat *et al*.,2010) .** The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100 - 500 µg/ml). The mixture was incubated at 37oC for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

V. Anti-lipoxygenase activity (Shinde *et al*., 1999)

Anti-Lipoxygenase activity was studied using Linoleic acid as substrate and lipoxidase as enzyme. Test samples were dissolved in 0.25ml of 2M borate buffer pH 9.0 and added 0.25ml of lipoxidase enzyme solution (20,000U/ml) and incubated for 5 min at 25 ˚C. After which, 1.0ml of lenoleic acid solution (0.6mM) was added, mixed well and absorbance was measured at 234nm. Indomethacin was used as reference standard. The percent inhibition was calculated from the following equation: Percentage inhibition= [{Abs control- Abs sample}/Abs control] x 100

Results of Preliminary Phytochemical Screening Tests

Table 1: Result of Preliminary Phytochemical Screening of *Punica granatum*L.flower extracts.

S.No	Test	Methanolic Extract	Ethyl acetate Extract	Aqueous Extract
1	**Sterols and Triterpenoids**			
a	Liebermann-Burchard test	+	-	+
2	**Glycosides**			
a	Keller-Killiani test	-	+	+
b	Bromine test	-	-	+
3	**Saponins**			
a	Foam test	+	-	-
4	**Alkaloids**			
a	Hager's Test	+	-	+
5	**Test for Flavonoids**			
a	Ferric Chloride test	+	-	+
b	Alkaline Reagent test	+	-	+
c	Lead Acetate Solution test	+	-	+
6	**Tannins**			
a	Gelatin test	+	-	-

7	**Proteins**			
a	Biuret test	-	-	-
8	**Amino Acids**			
a	Ninhydrin test	+	-	+
9	**Carbohydrates**			
a	Benedict's Test	+	-	+

5.3 Results of *In vitro* Anti-inflammatory Activity

The results of different parameters used for determining *In vitro* anti-inflammatory potential of methanolic and aqueous extracts of *Punica granatum*L.flowers are given below:

A) Inhibition of Albumin denaturation

Denaturation of proteins is a well-documented cause of inflammation. Protein denaturation by different solvent plant extracts was studied. *Punica granatum*L.flower extracts were effective in inhibiting heat induced Albumin denaturation **Table 2 .**Maximum percentage of inhibition 71.24% was observed from methanolic extract at a concentration of 500 µg followed by aqueous extract (68.49 %) at the same concentration. Aspirin, a standard anti-inflammatory drug showed maximum inhibition 75.66% at a concentration of 100 µg/ml compared with control.

Table 2: Results of albumin denaturation inhibition activity (%) of methanolic and aqueous extracts obtained from *Punica granatum*L.flowers and aspirin as standard.

S.No.	Type of extract/Std	Conc. µg/ml	Percentage * Inhibition (%I)	IC_{50} (µg/ml)
1	Methanolic	100	36.16±0.509	214
		200	46.05±0.241	
		300	57.50±0.093	
		400	66.48±0.317	
		500	71.24±0.485	
2	Aqueous	100	20.41±0.183	309
		200	38.18±0.317	
		300	53.84±0.158	
		400	63.46±0.317	
		500	68.49±0.485	
3	Standard (Aspirin)	100	75.66±0.398	

***Percentage inhibition is expressed as mean ± SEM of triplicate experiments.*

B) Heat induced haemolysis of erythrocyte

Test extracts (100 -500 µg/ml) inhibited the heat induced hemolysis of RBCs to varying degrees as shown in **Table 3.** Maximum inhibition 72.48 was shown by methanolic extract at a concentration of 500 µg/ml whereas; aqueous extract shows maximum inhibition of 61.32% at the same concentration. Aspirin, standard drug showed maximum inhibition 63.83% at a concentration of 100µg/ml.

Table 3: Results of heat induced haemolysis of erythrocytes (%) of methanolic and aqueous extracts obtained from *Punica granatum*L.flowers and aspirinas standard.

S.No.	Type of extract/Std	Conc. µg/ml	Percentage * Inhibition (%I)	IC_{50} (µg/ml)
1	Methanolic	100	28.96±0.221	240
		200	48.7±0.354	
		300	62.78±0.429	
		400	69.07±0.207	
		500	72.48±0.290	
2	Aqueous	100	22.40±0.214	335
		200	30.73±0.214	
		300	45.86±0.280	
		400	61.32±0.354	
		500	67.87±0.214	
3	Standard (Aspirin)	100	63.83±0.214	

***Percentage inhibition is expressed as mean ± SEM of triplicate experiments.*

C) Hypotonicity Induced Haemolysis

The results showed that methanolic and aqueous extract of *Punica granatum*L.protected the erythrocyte membrane against lysis induced by hypotonic solution as shown in **Table 4**. Diclofenac solution (100 µg/ml) offered a significant protection against the effect of hypotonic solution. At concentration of 500 µg/ml, methanolic extract showed maximum of 72.31% of protection followed by aqueous (64.65%) extract. Diclofenac sodium (100 µg/ml) showed 60.41% inhibition of RBC haemolysis when compared with control.

Table 4: Percent inhibition of hypotonicity induced haemolysis produced by methanolic and aqueous extracts obtained from *Punica granatum*L.flower and diclofenac sodium as standard.

S.No.	Type of extract/Std	Conc. µg/ml	Percentage * Inhibition (%I)	IC_{50} (µg/ml)
1	Methanolic	100	23.84±0.164	302
		200	41.18±0.345	
		300	48.75±0.478	
		400	62.35±0.332	
		500	72.31±0.255	
2	Aqueous	100	18.67±0.439	346

		200	**33.71±0.252**	
		300	**46.92±0.345**	
		400	**58.61±0.332**	
		500	**64.65±0.145**	
3	**Standard (Diclofenac sodium)**	**100**	**60.41±0.439**	

***Percentage inhibition is expressed as mean ± SEM of triplicate experiments.*

D) Proteinase inhibitory action

Extracts obtained from flowers of *Punica granatum*L.exhibited significant antiproteinase activity at different concentrations as shown in **Table 5.** Methanolic extract of *Punica granatum*L. flowers showed maximum inhibition 77.53% at a concentration of 500µg. whereas aqueous showed maximum inhibition of 68.35%. Indomethacin shows maximum inhibition of 64.63% at concentration of 100µg.

Table 5: Antiproteinase activity shown by methanolic and aqueous extracts obtained from *Punica granatum* L.flowers and Indomethacin as standard.

S.NO.	**Type of Extract/Std**	**Conc. µg/ml**	**Percentage * Inhibition (%I)**	**IC_{50} (µg/ml)**
1	**Methanolic**	**100**	**22.78±0.348**	**305**
		200	**34.21±0.214**	
		300	**48.78±0.448**	
		400	**62.96±0.288**	
		500	**77.53±0.138**	
2	**Aqueous**	**100**	**20.36±0.214**	**352**
		200	**32.36±0.280**	
		300	**42.66±0.211**	
		400	**55.23±0.211**	
		500	**68.35±0.277**	
3	**Standard (Indomethacin)**	**100**	**64.63±0.277**	

***Percentage inhibition is expressed as mean ± SEM of triplicate experiments.*

E) Anti-lipoxygenase activity

The establishment of new *in-vitro* test systems has stimulated the screening of plants aiming to find leads for the development of new drugs. **(Gardner *et al.*, 1999).** For this reason, the *in vitro* inhibition of

lipoxygenase constitutes a good model for the screening of plants with anti-inflammatory potential **(Abad *et al*., 1995).** LOXs are sensitive to antioxidants and the most of their action may consist in inhibition of lipid hydro peroxide formation due to scavenging of lipidoxy or lipid peroxy- radical formed in course of enzyme peroxidation. This can limit the availability of lipid hydroproxides substrate necessary for the catalytic cycle of LOX. The methanolic and aqueous extracts of *Punica granatum*L.flowers were checked for anti-lipoxygenase activity as shown in **Table 6.**Both extracts show significant lipoxygenase inhibition with a maximum inhibition of 61% and 71% at 500µg/ml. Indomethacin was used as standard drug, which shows 80% inhibition at 100µg/ml.

Table 6: Antilipoxygenase activity shown by methanolic and aqueous extracts obtained from *Punica granatum*L. flowers and Indomethacin as standard.

S.No.	Type of extract/Std	Conc. µg/ml	Percentage * Inhibition (%I)	IC_{50} (µg/ml)
1	Aqueous	100	16.10±0.249	375
		200	26.24±0.185	
		300	37.56±0.121	
		400	48.74±0.121	
		500	61.10±0.136	
2	Methanolic	100	22.42±0.182	328
		200	31.94±0.118	
		300	47.08±0.182	
		400	59.58±0.118	
		500	71.17±0.118	
3	Standard (Indomethacin)	100	80.20±0.118	

***Percentage inhibition is expressed as mean ± SEM of triplicate experiments*

VI. Discussion

I**nflammation is the body's attempt at self-protection; the aim being to remove harmful stimuli, including damaged cells, irritants, or pathogens - and begin the healing process**.

Antioxidant is a substance that inhibits oxidation, especially one used to counteract the deterioration of stored food products. A substance such as vitamin C or E that removes potentially damaging oxidizing agents in a living organism.

Diabetes mellitus (DM), commonly referred to as diabetes, is a group of metabolic disorders in which there are high blood sugar levels over a prolonged period.

In the present study, flower extracts **(Methanolic, Ethyl acetate Aqueous and 70%)** of *Punica granatum* L.were subjected to Preliminary Phytochemical Screening, anti-inflammatory, anti-oxidant and anti-diabetic studies using *In-vitro*methods.

In the present study, **Preliminary Phytochemical Screening** of flowers of *Punica granatum* L.showed that methanolic and aqueous extracts are rich source of phytochemicals as compared to ethyl acetate extract. Methanolic and aqueous extracts showed positive results for various phytoconstituents, both methanolic and aqueous extracts showed the presence of maximum constituent's viz., sterols and triterpenoids, glycosides, saponins, alkaloids, flavonoids, tannins, proteins, amino acids and carbohydrates. Methanolic extract of *Punica granatum*L.flowers showed negative result for glycosides and proteins. Ethyl acetate showed positive result for glycosides.

The *In-vitro* anti-inflammatory activity of *Punica granatum* L.flower extracts was evaluated by these methods viz., Inhibition of Albumin denaturation, Membrane stabilization Proteinase inhibitory action

and Antilipoxygenase activity. The *In-vitro* anti-inflammatory activity of different extracts of *Punica granatum* L.revealed that the plant has a fair anti-inflammatory potential.

From the result, it was noted that *Punica granatum* L.flower extracts were effective in inhibiting **heat induced albumin denaturation.** Denaturation of proteins is a well-documented cause of inflammation. Maximum percentage of inhibition 71.24% was observed from methanolic extract at a concentration of 500 µg followed by aqueous extract 68.49% at the same concentration.

Aspirin, a standard anti-inflammatory drug showed maximum inhibition 75.66% at a concentration of 100 µg/ml compared with control. The IC_{50} value calculated for methanolic and aqueous extract was found to be 214µg/ml and 309µg/ml respectively.

Membrane stabilization has been used as a method to study the *In-vitro*anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane 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 (**Gandhidasan *et al*., 1991**).

In heat induced haemolysis, the extracts were effective in inhibiting the heat induced haemolysis at different concentrations. Maximum percentage of inhibition 72.48% was observed from methanolic extract at a concentration of 500 µg followed by aqueous extract 67.87% at the same concentration. Aspirin, a standard anti-inflammatory drug showed maximum inhibition 63.83% at a concentration of 100 µg/ml compared with control. The IC_{50} value calculated for methanolic and aqueous extracts was found to be 240µg/ml, and 335µg /ml respectively.

In hypotonicity induced haemolysis, methanolic extract of *Punica granatum* L.flowers at concentration range of 100-500µg/ml protect significantly ($p<0.01$) the erythrocyte membrane against lysis induced by hypotonic solution. Diclofenac sodium (100µg/ml) offered a significant ($p<0.01$) protection against the damaging effect of hypotonic solution. At the concentration of 500µg/ml, methanolic extract of *Punica granatum* L.flowers showed maximum of 72.31% protection as compared to aqueous which shows 64.65% protection at the same concentration whereas, Diclofenac sodium (100µg/ml) showed 60.41% inhibition of RBC haemolysis when compared with control.

In proteinase inhibitory activity, Neutrophils are known to be a rich source of serine proteinase and are localized at lysosomes. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors (**Das and Chatterjee, 1995**).

Methanolic extract exhibited significant antiproteinase activity at different concentrations. It showed maximum inhibition of 77.53% at 500µg/ml while aqueous shows inhibition of 68.35%. Indomethacin showed the maximum inhibition 64.63% at 100µg/ml.

The establishment of new in vitro test systems has stimulated the screening of plants aiming to find leads for the development of new drugs. The plant lipoxygenase pathway is in many respects equivalent to the 'arachidonic acid cascades' in animals. For this reason, the *In-vitro*inhibition of lipoxygenase constitutes a good model for the screening of plants with anti-inflammatory potential. LOXs are sensitive to antioxidants and the most of their action may consist in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipidperoxy-radical formed in course of enzyme peroxidation. This can limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX.

Antilipoxygenase inhibition of methanolic extract by *Punica granatum* L.flowers was found to be 22.42%, 31.94%, 47.08%, 59.58%, and 71.17% at the concentration of 100,200,300,400,500 µg/ml, while aqueous extract shows 16.10%, 26.24%, 37.56%, 48.74%, and 61.10% inhibition at the same concentration. The Standard drug Indomethacin showed an inhibition of 80.20% at a concentration of 100µg/ml. At the concentration of 100 and 200µg/ml, methanolic extract of *Punica granatum*L.not showed significant difference ($p >0.05$) when compared with control. The results obtained from our studies on methanolic extract of *Punica granatum* L.flowers have shown a potential anti-inflammatory activity. The methanolic extract of *Punica granatum* L.flowers inhibited the lipoxygenase enzyme activity. This indicates that methanolic extract of *Punica granatum* L.flowers is more useful in studies of inflammation and in various related physiological studies, aging and diseases such as cancer, neurological disorder etc.

VII. Conclusion

- The current study provided concrete evidence for the anti-inflammatory activity of methanolic and aqueous extracts of flowers of *Punica granatum* L. However, methanolic extract proved to be more effective than the aqueous extract.
- Further, the bio-active compounds can be isolated from these extracts and can be studied for their toxic effects, if they are found safe, they may be used as formulations associated with inflammation and free radical scavenging activity.

- The compound may also be chemically modified so as to enhance its pharmacological activity and suppress toxicological effects (if any), thus it can serve as a lead molecule.

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Maqsood Rashid "Evaluation of Phytochemical Constituents and In vitro Anti-inflammatory Activity of Kashmiri Pomegranate (Punica granatum Linn.) Flower Extract." IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 13.2 (2018): 58-67.