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ANTIINFLAMMATORY AND ANTIOXIDANT ACTIVITY OF POMEGRANATE PEEL EXTRACT

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ABSTRACT
Secretory PLA_2 ($sPLA_2$-IIA) is implicated in inflammatory responses because a high level of this enzyme has been found in inflammatory fluids and cells. PLA_2 enzyme catalyses the breakdown of membrane phospholipid to arachidonic acid and lysophospholipid. Arachidonic acid is in turn converted to proinflammatory mediators and ROS. A single molecule having both antioxidant and PLA_2 inhibition is of great therapeutic importance in designing anti-inflammatory molecules. Pomegranate peel extracted with different solvents with increasing polarity were screened for phenolics, antioxidant activity and $sPLA_2$ inhibition. The acetone extract exhibited high antioxidant activity and $sPLA_2$ inhibition. The concentration of phenolics directly correlated with antioxidant activity and PLA_2 inhibition. P.granatum peel acetone extract also showed dose dependent inhibition of $sPLA_2$ enzyme both by invitro and invivo method. The P.granatum peel acetone extract was further subjected to TLC for separation of components in extract. The extract showed 7-8 bands. Each band was subjected to PLA_2 inhibition and antioxidant activity. PPCII was found to have high PLA_2 inhibition and antioxidant activity. The extract may contain compounds (Phenolics, anthocyanins, flavonoids, tannins, alkaloids) which are both antioxidant and anti-inflammatory in nature and they can be used for clinical development as a new class of anti-inflammatory agents with antioxidant activity.

KEYWORDS: Anti inflammatory, Antioxidant, Free radicals, Phenolics, secretory phospholipase A_2, Punica granatum.

INTRODUCTION
Inflammation is a complex physiological response, resulting from interactions between cells and soluble factors that can arise in any biological tissue in response to infection, ischaemia or injury. Inflammation helps tissue to recover from infection and leads to healing. However, loss of this highly ordered process results in tissue destruction, often caused by an increased infiltration of leukocytes and release of pro-inflammatory mediators at the site of injury [Ward, 1969]. In many inflammatory diseases high level of $sPLA_2$ enzymes (group IIA $sPLA_2$) are detected and found to be responsible for the part of inflammatory reaction [Fuentes et al., 2002; Gilroy et al., 2004]. Injection of purified $sPLA_2$ from synovial fluid and snake venom in to animal joints confirmed the development of an acute inflammatory response with edema, swelling of synovial cells and hyperplasia [Bomalaski et al., 1991; Fawzy et al., 1988; Weichman et al., 1989]. Accordingly, PLA_2 inhibition has become a target for the investigation of novel anti-inflammatory agents. Free radical derivatives of arachidonic acid are generated during catalysis and a free radical chain mechanism has been proposed to explain the conversion of arachidonic acid to PGG_2 [Mason et al., 1980; Helmer et al., 1980]. Free radicals are also generated during the reduction of PGG_2 to PGH_2 [Egan et al., 1976]. Oxidative stress associated activation of PLA_2 has been proposed to be a critical factor in cytotoxicity. A single molecule having both antioxidant and PLA_2 inhibition is of great therapeutic importance in designing anti-inflammatory molecules. Pomegranate peel that exhibited greater antioxidant activity and anti inflammatory activity (PLA_2 inhibition) from previous study was selected for further characterization of the extract.

MATERIALS AND METHODS

Diphenyl picryl hydrazyl radical (DPPH), thiobarbituric acid, butylated hydroxyl anisole, gallic acid were purchased from SRL company. All other chemicals and reagents purchased were of analytical grade. Human pleural fluid was obtained from Princes Krishna Jammanni Tuberculosis (PKTB) and Chest Disease Hospital, Mysore and synovial fluid was obtained from Dr. Hegde's Orthopedic Clinic, Mysore, India. Blood samples were obtained from the healthy volunteers. Lyophilized powder of *Naja naja* and *Vipera russellii* snake venoms were purchased from Hindustan Park, Kolkata, West Bengal, India and Irula Co-operative Society Ltd., Chennai, Tamil Nadu, India, respectively. Albino mice weighing 20-25g were obtained from animal house maintained by University of Mysore, Mysore, India. The animal care and handling were conducted in compliance with the National regulations for animal research. The animal experiments were carried out after review of the protocols by the animal ethical committee of the University of Mysore.

Preparation of fruit extracts

The Punica granatum peel was separated manually from the fruit and shade dried. After drying it was grounded to a fine powder form. The powder obtained was packed in Whatman No.1 filter paper and all the sides were sewed. Now the Pomegranate (P.granatum) peel powder was subjected to soxhlet extraction. The extraction was carried out using various organic solvents of increasing polarity such as hexane, benzene, chloroform, acetone, ethanol, methanol and finally water at 50-60^{o}C, 24 hours for each solvents. All the extracts were dried under vacuum at 40°C. The aqueous extract was lyophilized to obtain powder form. The yield of all extracts was calculated and expressed as % w/w.

Determination of total phenolics by Folin-Ciocalteau assay

The concentration of total phenolics in the different solvent extracts (hexane, benzene, chloroform, acetone, ethanol, methanol and water) of Pomegranate peel was determined by the Folin-Ciocalteau method [Singleton., 1999] with slight modification. All the solvent extracts of P.granatum peel were estimated at 100 µg concentration by their dry weight. The phenolic contents of the different sovent extracts were determined from calibration curve and were expressed in gallic acid equivalents (GAE/100 g).

Antioxidant activity by DPPH method

2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$), also known as α,α-diphenyl-β-picrylhydrazyl, is a free radical used for assessing antioxidant activity. Reduction of DPPH$^{\cdot}$ radical by an antioxidant or by a radical species results in a loss of absorption at 517 nm. Thus the degree of discoloration of the solution indicates the scavenging efficiency of the added substances. Determination of antioxidant activity by the DPPH method [Blios., 1958] was done for all the solvent extracts of P.granatum peel at 100 µg concentration by their dry weight.

Determination of reducing power

The reducing power of all the extracts were determined according to the method of Oyaizu [Yen et al., 1993]. 100 µg concentration of extracts were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1 % potassium ferricyanide and the mixture was incubated at 50 º C for 20 min. At the end of incubation, 2.5 ml of 10 % TCA was added to the mixtures followed by centrifuging at 5000 rpm for 10 min. The supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1 % $FeCl_3$ and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated the reducing power of the samples. The reducing power activity is due to the presence of phenolics (reductones).

Antioxidant activity by TBA method

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a di-adduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm as per Halliwell and Gutteridge [Halliwell et al., 1989]. Normal albino rats of Swiss Wister strain were used for the preparation of liver homogenate. The different solvent extracts of P.granatum peel at 100 µg concentration was mixed with 0.15 M KCl and 0.5 ml of rat liver homogenates. Peroxidation was initiated by adding 100 µl of 0.2 mM $FeCl_3$. After incubation at 37 º C for 30 min, the reaction was stopped by adding 2 ml of ice-cold HCl (0.25 N) containing 4 % TCA and 0.38 %TBA. The reaction mixtures were heated at 80 º C for 60 min. The samples were cooled and centrifuged, and the absorbance of the supernatants was measured at 532 nm. An identical experiment was performed in the absence of the extracts to determine the amount of lipid per oxidation obtained in the presence of inducing agents with out any extract. The % of anti- lipid per oxidative activity (%ALP) is calculated by the following formula: Anti lipid per oxidation (%) =1- sample OD/control ODX100.

Purification of PLA_2 enzymes

$sPLA_2$ from *N. naja* venom was purified to homogeneity as described by Basavarajappa and Gowda [1992]. $sPLA_2$ from V. russellii venom was purified to homogeneity as described by Sathish modified method of Kasturi and Gowda [1989]. $sPLA_2$ from Human pleural fluid (HPF) and human synovial fluid (HSF) was purified by the method of Vishwanath B S et al [1988].

Phospholipase A_2 assay

PLA_2 activity was measured using oleate labeled autoclaved *E.coli* [Patriarca et al., 1972; Vishwanath et al., 1993]. The reaction mixture of 350 µl contained 100 mM Tris-HCl buffer (pH 7.4), 5 mM calcium and 3.18 x 10^9 autoclaved *E. coli* cells (corresponds to 10,000 cpm and 60 nmole lipid phosphorus). The reaction components were mixed in the following order: buffer, calcium, enzyme and water. The reaction was initiated

by adding 30 µl of *E. coli* substrate and incubated at 37 ^{o} C for 60 min. The reaction was terminated by adding 100 µl of 2 N hydrochloric acid and 100µl of fatty acid free BSA (100 gm / L) was added to entrap free fatty acids released. The tubes were vortexed and centrifuged at 20,000 x *g* for 5 min. An aliquot of (140 µl) supernatant containing released ^{14}C-oleic acid was mixed with scintillation cocktail (Ultima Gold, Packard Bioscience, USA) and ^{14}C radiation was measured in Packard Scintillation analyzer. Enzyme activity is expressed as nmole of free fatty acid released min^{-1}, mg^{-1} protein at 37 ^{o}C.

Inhibition of phospholipase A_2 activity

The dried hexane, benzene, chloroform, acetone, ethanol, methanol and water extracts of *P.granatum* peel were dissolved in the range of 1mg/ml and used for PLA_2 inhibition studies. For PLA_2 assay, the amount of protein was chosen such that a 60-70 % hydrolysis of substrate was obtained at 37°C for 60 min. The inhibition of VRV-V-PLA_2 was done with 100 µg concentration of hexane, benzene, chloroform, acetone, ethanol, methanol and water extracts.

Neutralisation of Indirect hemolytic activity

Indirect hemolytic was assayed as described by Bowman and Kalletta [1957]. The substrate for the indirect hemolytic assay was prepared by suspending 1 ml of packed fresh human RBC and 1 ml fresh hen's egg yolk in 8 ml of PBS. 100 µg of all extracts were preincubated with 40 µg of VRV-V-PLA_2 for 30 min at 37 º C. Then to this 1ml of reaction mixture was added and allowed to react for 45 min at 37 º C. Then the reaction was stopped by adding 9 ml of ice cold PBS. The suspension was mixed and centrifuged at 2000 rpm for 20 min. The released hemoglobin was read at 530 nm. Simultaneously one positive control was kept with venom alone and no extract.

Neutralization of edema inducing activity

The procedure of Yamakawa et al.,[1976] as modified by Vishwanath et al., [1987] was followed. Mice weighing 20–25 gms were injected with 5 µg of VRV-V-PLA_2 enzyme alone or mixed with different concentrations of P.granatum peel extract, in a total volume of 20 µl saline into their intra plantar surface of right hind footpads. Respective left footpads received 20 µl of saline or vehicle and served as controls. After 45 min the mice were sacrificed after giving anesthesia (Pentobarbitone, 30 mg / kg, i.p.) and both hind limbs were removed at the ankle joint and weighed individually. The increase in weight due to edema is expressed as percent of sham injected control (117 ± 4 % of uninjected limb) is the ratio of the weight of edematous limb to the weight of normal (sham injected) limb × 100.

RESULTS AND DISCUSSION

As PLA_2 enzyme catalyses the rate limiting step in the conversion of phospholipids of cellular membrane to arachidonic acid metabolite. This arachidonic acid is further oxidatively metabolized by COX and LOX enzymes through a series of free radical intermediates producing various pro-inflammatory lipid mediators and reactive oxygen species (ROS). Antioxidants are useful molecules in regulating these free radicals. However, many antioxidants apart from these beneficial action exhibits strong sPLA_2 enzyme inhibition. Against the generation of these free radicals the organisms have adopted an in built antioxidant defense systems. When endogenous antioxidant concentration becomes low when compared to increase free radicals, it results in oxidative stress. Oxidative stress results in PLA_2 activation. Therefore to neutralize these excess free radicals, exogenous antioxidants are beneficial. Fruits are good sources of antioxidants. Among the various fruit extracts screened for antioxidant activity and PLA_2 inhibition, *P.granatum* fruit exhibited greater antioxidant activity and PLA_2 inhibition. In *P.granatum* fruit, peel showed maximum PLA_2 inhibition and antioxidant activity when compared to its yellow membrane, pulp and seed portion respectively. The peel was selected for further characterization of active molecule involved in antioxidant and PLA_2 inhibition.

The Pomegranate peel was subjected to extraction in soxhlet apparatus with increasing polarity of solvents. The yield of all extracts was calculated and expressed as % w/w as shown in **Table.1**. P.granatum peel extracted with methanol gave maximum yield of extract (20.0 % w/w) followed by ethanol (16.0 % w/w), acetone (15.0 % w/w). The total phenolic content of the acetone extract of P.granatum peel was maximum (98.6 % w/w) followed by the ethanol (66.6 % w/w), water (41.2 % w/w), methanol (38.0 % w/w), benzene (18.0 % w/w), chloroform (12.8 % w/w) and hexane (7.2 % w/w) extracts at 100µg concentration of all extracts as shown in T**able.1**. The antioxidant activity of hexane, benzene, chloroform, acetone, ethanol, methanol and water extracts as measured by their free radical scavenging potentials by the DPPH method are shown in T**able.1** respectively. At 100µg concentration, P.granatum peel acetone extract exhibited maximum (96.0 %) free radical scavenging activity. Ethanol, water, methanol, benzene, chloroform and hexane exhibited 58.0, 32.0, 26.0, 9.0, 6.0 and 3.9 % free radical scavenging activity respectively. The antioxidant activity of soxhlet extracted various solvent P.granatum peel extracts in prevention of lipid peroxidation are shown in **Table.1**. At 100µg concentration, the acetone extract of P.granatum peel shows 89.0 % inhibition of lipid peroxide generation by TBA method. In turn the ethanol, water, methanol, benzene, chloroform and hexane shows 48.0, 26.0, 22.0, 7.0, 5.0 and 4.0 % inhibition of lipid peroxide generation respectively. The reducing power activity of different solvents extracts of P.granatum peel at 100µg concentration using the potassium ferricyanide reduction method is depicted in **Table.1**. At 100µg concentration, the extracts obtained using hexane, benzene, chloroform, acetone, ethanol, methanol and water showed absorbances of 0.01, 0.36, 0.15, 2.960, 2.205, 0.551 and

1.860 respectively at 700nm. Thus, the highest reducing activity was observed in acetone extract of P.granatum peel. The reducing power activity is due to the presence of reductones (phenolics). The extracted samples was subjected to inhibition of PLA_2 activity and indirect hemolytic activity by VRV-V-PLA_2 to correlate the phenolic content and antioxidant activity to that of PLA_2 inhibitory potency (Figure 1 & 2). Hexane, benzene, chloroform, acetone, ethanol, methanol and water extracts of P.granatum peel were screened for VRV-Va-PLA_2 inhibition at 100µg concentration. Acetone extract of P.granatum peel exhibited greater than 95% inhibition as shown in **Figure.1**. The least PLA_2 inhibition was observed in hexane, benzene and chloroform extracts. The neutralization of indirect hemolytic activity of VRV-Va-PLA_2 by hexane, benzene, chloroform, acetone, ethanol, methanol and water extracts of P.granatum peel at 200µg concentration is as shown in **Figure. 2**. The lysis caused by venom PLA_2 is considered as 100 %. Acetone extract of P.granatum peel exhibited 80 % of inhibition of indirect hemolytic activity exhibited by VRV-Va-PLA_2. This is followed by ethanol, water and methanol extracts respectively. Samples extracted with non polar solvents failed to inhibit PLA_2 activity. The acetone extract of P.granatum peel that showed maximum PLA_2 inhibition and antioxidant activity was chosen for further studies. The acetone extract of P.granatum peel was dissolved in minimum amount of DMSO and later volume was made up to a desired concentration with PBS. Dose dependent inhibition of all $sPLA_2$ enzymes with acetone extract of P.granatum peel was carried out. The P.granatum peel extract on TLC separation gave 7-8 bands. When all the bands were checked for PLA_2 inhibition and antioxidant activity the second band named as PPCII was found to have maximum PLA_2 inhibition and antioxidant activity at 5µg concentration. Dose dependent inhibition was also carried out for PPCII to determine the IC_{50} concentration. Dose dependent inhibition of purified $sPLA_2$ enzymes from HSF, HPF (Group IIA), VRV-Va-PLA_2 from V.russellii (group IIA) and NN-XIa-PLA_2 from N.naja (Group IA) by acetone extract of P.granatum peel was carried out in rage of 0-100µg concentration. **Figure.3** shows that human inflammatory and snake venom group II $sPLA_2$ enzymes were inhibited up to 90 % at 50µg concentration of extracts. On the other hand, the pomegranate peel acetone extract showed less potency towards group I, NN-XIa-PLA_2 with 80 % inhibition at 60µg concentration. The linear regression analysis of linear portion of concentration dependent inhibition curves were used to calculate IC_{50} values for all $sPLA_2$ enzymes by acetone extract of P.granatum peel. The IC_{50} values were calculated for all the four $sPLA_2$ enzymes by the extract as shown in **Table-2**. The result of IC_{50} values suggests that inflammatory $sPLA_2$ enzymes are preferentially inhibited over snake venom $sPLA_2$ enzymes. Dose dependent free radical scavenging activity by DPPH method was seen in acetone extract of P.granatum peel as shown in **Figure.4**. The concentration of extract ranged from 0-100µg level. The IC_{50} value, which is the amount of extract needed to scavenge 50 % of DPPH radical for the acetone extract of P.granatum peel, was found to be 5µg. Dose dependent inhibition of lipid peroxide formation by acetone extract of P.granatum peel is as shown in **Figure.5.** The IC_{50} concentration is found to be 18µg for 50 % inhibition of lipid peroxide formation. The reducing power activity of pomegranate peel acetone extract at different concentration is as shown in **Figure.6.** Dose dependent neutralization of indirect hemolytic activity of VRV-Va-PLA_2 by acetone extract of P.granatum peel is shown in **Figure.7**. The concentration of extract ranged from 1:01 to 1:06. At 1:06 (venom**:** extract) concentration of extract there was complete neutralization of lysis of RBC cells by venom PLA_2. Since In vitro $sPLA_2$ activity and In situ hemolytic activities of $sPLA_2$ enzymes were inhibited effectively, the effect of acetone extract of P.granatum peel on edema inducing activity of VRV-Va-PLA_2 was studied. Acetone extract of pomegranate peel neutralized the VRV-Va-PLA_2 induced edema in dose dependent manner as shown in **Figure.8**. The edema was completely neutralized at 100µg concentration of extract.

DISCUSSION

The free radicals produced in eicosanoid metabolism may escape and act on variety of organelle membrane lipids, protein, nucleic acids and sugars there by in activating their function and finally resulting in cell death. $sPLA_2$-IIA plays a pivotal role in the propagation and amplification of inflammation. The anti-inflammatory activity of glucocorticoid appears to down-regulate PLA_2-IIA expression, and this evidence suggests that inhibition of PLA_2-IIA represents a target for the treatment of inflammatory disease [Vishwanath et al 1974]. Scavenging of these free radicals along with the inhibition of eicosanoid production is advantageous in the treatment of chronic anti-inflammatory diseases. The yield is less than 1% with extraction carried out using non-polar solvents such as hexane, benzene and chloroform. Ethanol and methanol samples also exhibit good amount of phenolics and antioxidant activity, whereas material extracted with non polar solvents like hexane, benzene and chloroform showed least content of phenolics and antioxidant activities. The concentration of total phenolics directly correlates the antioxidant activity of extracts. Free radicals are released during the oxidative metabolism of arachidonic acid. These free radicals inturn bring about PLA_2 activation and lipid peroxidation there by enhancing the inflammatory process. Thus the removal of these free radicals by antioxidants can stop the progression of PLA_2 mediated arachidonate oxidative metabolism and there by suppresses the release of pro-inflammatory lipid mediators which results in chronic inflammation. The data obtained reveal that the *P.granatum* peel acetone extract is a free radical inhibitor and also a primary antioxidant that react with free radicals. The free radical scavenging activity is directly proportional to the amount of total phenolics in *P.granatum* peel acetone extract.

Active research has been driven in recent years on fruits and vegetables due to their biologically beneficial effects emanating from antioxidant activities of phenolic phytochemicals. The data presented here indicated that the marked antioxidant activity of *P.granatum* peel extract seems to be due to the presence of polyphenols, which may act in similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction. A compound has been isolated from acetone extract of *P.granatum* peel by preparative TLC method. TLC of acetone extract of *P.granatum* peel exhibited 8-9 bands shown in Figure 9. When all the bands were checked for PLA_2 inhibition and antioxidant activity the second band (PPCII) showed highest activity when compared to others as shown in Figure 10. Therefore the compound from the second band was eluted using methanol and centrifuged and the solvent was evaporated to dryness for further analyses.

CONCLUSION

In conclusion, the present data suggests that *P.granatum* peel acetone extract contains compounds (Phenolics, anthocyanins, flavonoids, tannins, alkaloids) which are both antioxidant and anti-inflammatory in nature and they can be used for clinical development as a new class of anti-inflammatory agents with antioxidant activity.

ACKNOWLEDGEMENT

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Table-1: Soxhlet extracted Punica granatum peel*

Extracts	Yield (% w/w)	Total Phenolics (% w/w)	Antioxidant activity		
			DPPH (% activity)	TBA (% activity)	Reducing power (A_{700nm})
Hexane	0.4	7.2	3.9	4.0	0.01
Benzene	0.2	18	9.0	7.0	0.036
Chloroform	0.05	12.8	6.0	5.0	0.015
Acetone	15.0	98.6	96.0	89.0	2.960
Ethanol	16.0	66.6	58	48.0	2.205
Methanol	20.0	38.0	26.0	22.0	0.551
Water	9.3	41.2	32.0	26.0	1.860

*Yield (%w/w), phenolics (%w/w), antioxidant activity by DPPH and TBA method and reducing power activity of soxhlet extracted Punica granatum peel with increasing polarity of organic solvents at 50-60 ^oC for 24 hr each.

Table 2: IC_{50} values* of Punica granatum peel acetone extract (µg/350µl) against different $sPLA_2$ enzymes tested.

Source of PLA_2	Pomegranate peel acetone extract
HSF-PLA_2	6
HPF-PLA_2	12
VRV-Va-PLA_2	18
NN-XIa-PLA_2	20

*=IC_{50} value is defined as the amount of extract (µg) required to inhibit the enzyme activity by 50% in the given reaction mixture of 350µl. The linear regression analysis of the linear portion of the dose dependent inhibition curve of each enzyme was used for the calculation of IC_{50}.

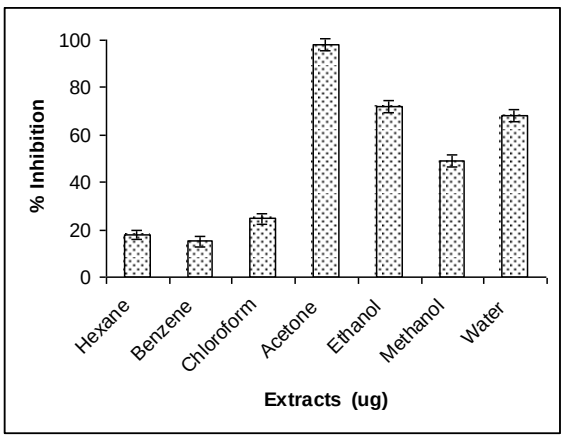

Figure 1: Inhibition of VRV-Va-PLA_2 by Hexane, Benzene, Chloroform, Acetone, Ethanol, Methanol and Water extracts of Pomegranate peel. The data represents mean ± SD (n=3).

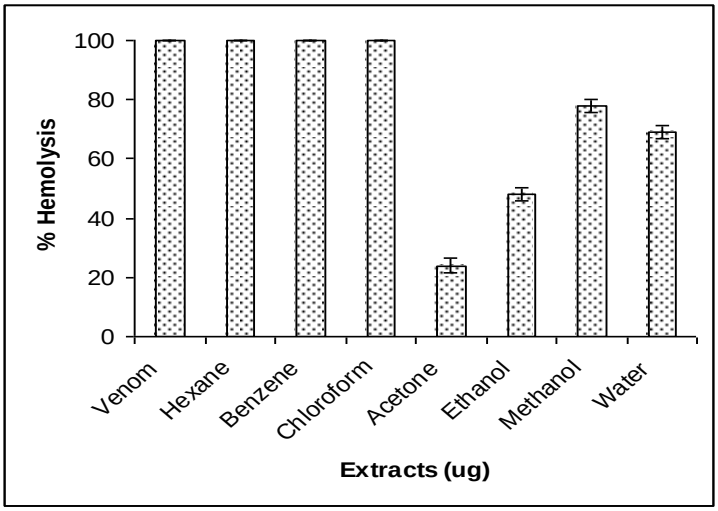

Figure 2: Neutralisation of indirect hemolytic activity of VRV-Va-PLA$_2$ by Hexane, Benzene, Chloroform, Acetone, Ethanol, Methanol and Water extracts of Pomegranate peel at 200 µg concentration. The data represents mean ± SD (n=3).

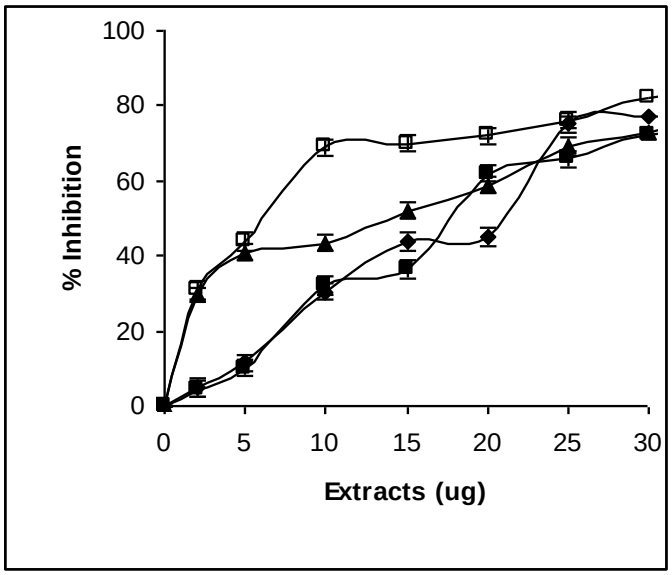

Figure 3: Dose dependent inhibition of various sPLA$_2$ enzymes by acetone extract of pomegranate peel. The data represents mean ± SD (n=3).

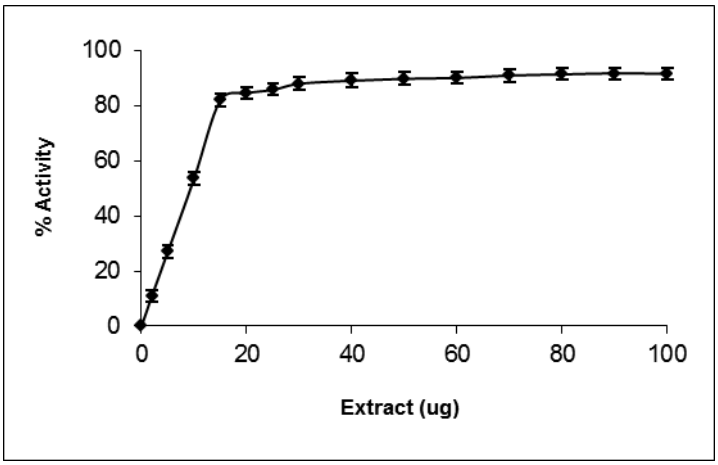

Figure 4: Dose dependent radical scavenging activity of acetone extracted pomegranate Peel by DPPH method.

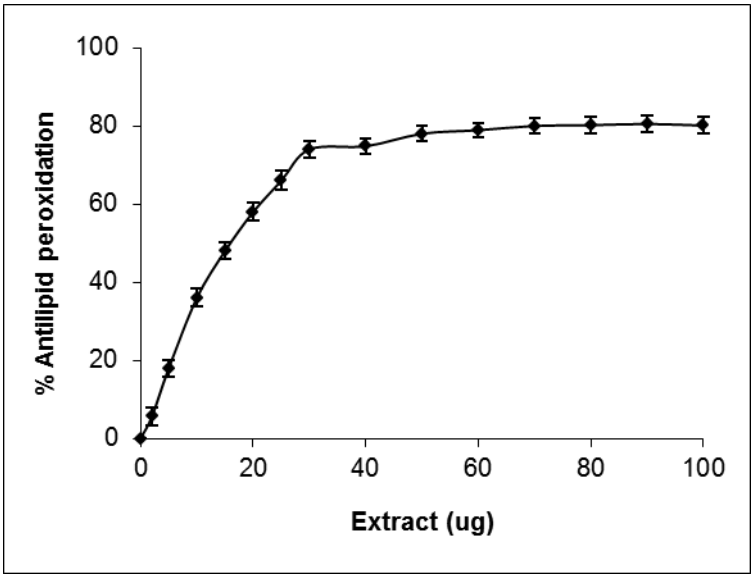

Figure 5: Dose dependent inhibition of lipid peroxidation by Pomegranate Peel acetone extract.

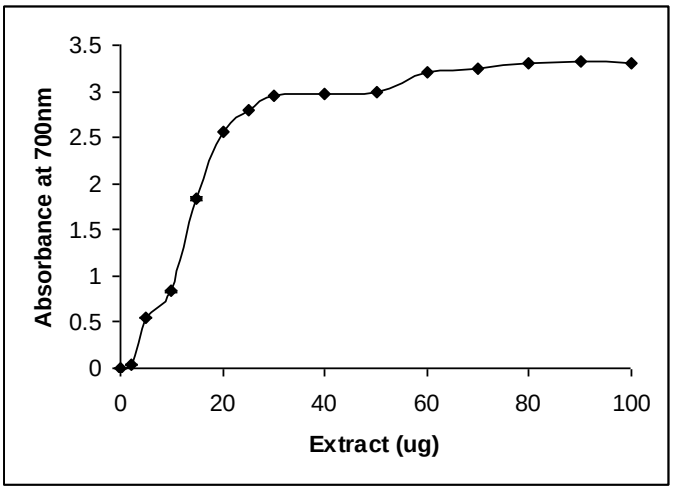

Figure 6: Dose dependent reducing power activity of pomegranate peel acetone extract.

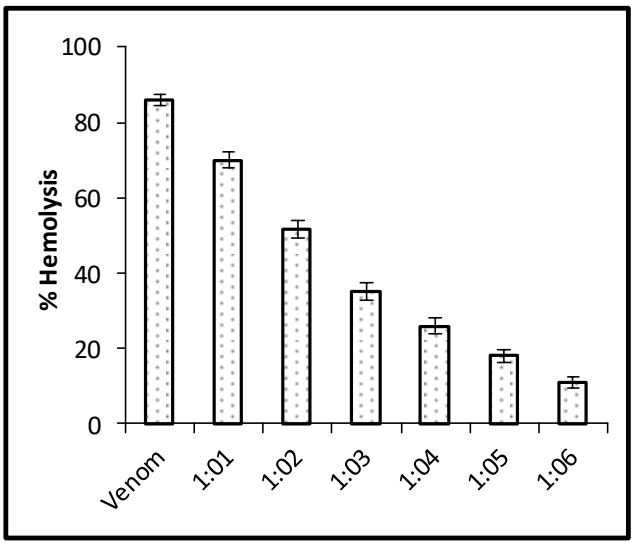

Figure 7: Dose dependent inhibition of indirect hemolytic activity of VRV-Va-PLA$_2$ by Pomegranate Peel acetone extract. Each bar represents mean± SD(n=3).

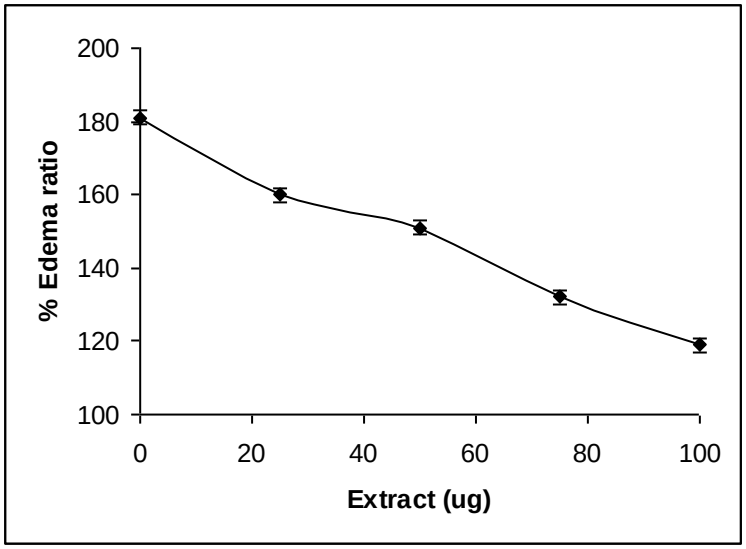

Fig. 8: Dose dependent inhibition of edema inducing activity of VRV-Va-PLA$_2$ by Pomegranate Peel acetone extract. Data represents mean± SD (n=3).

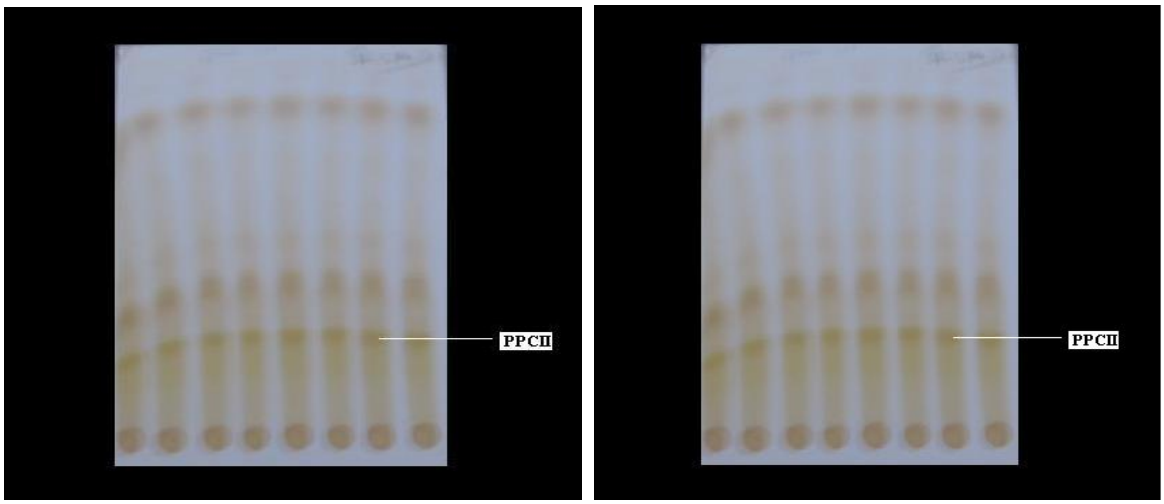

Fig.9: TLC of acetone extract of P.granatum peel.

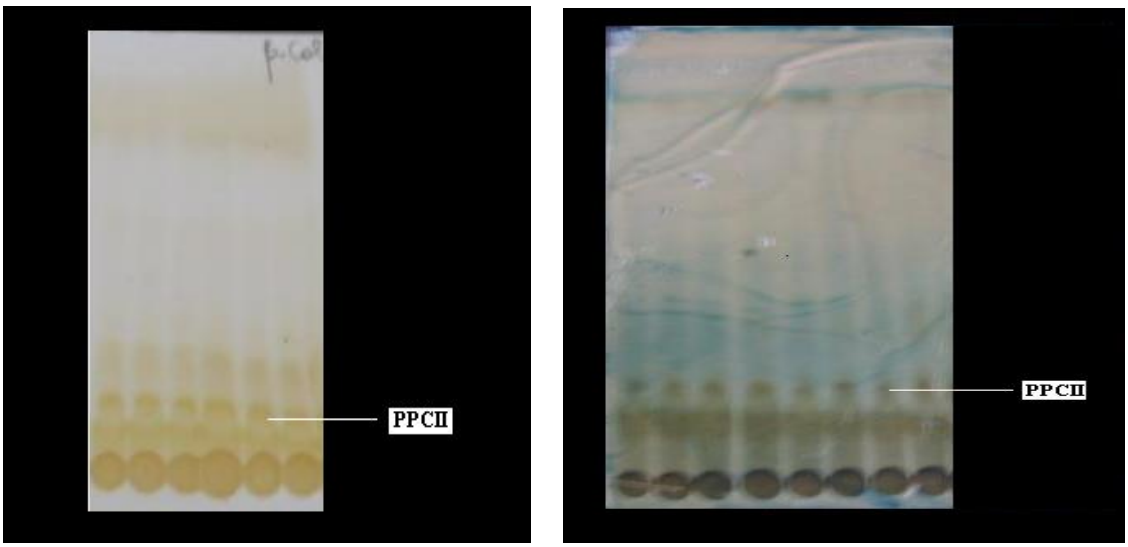

Fig. 10: TLC of acetone extract of P. granatum peel with two different sprays. Spray A with β-Carotene Linoleate solution shows antioxidant activity and Spray B with ferric chloride and potassium ferricyanide solution shows the presence of phenolic compounds in second band (PPCII).

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