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Effects of *Punica granatum* on milk-induced leucocytosis and eosinophilia in mice

[Efecto de *Punica granatum* en ratas con leucocitosis y eosinofilia inducida por leche]

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Abstract
Punica granatum Linn. (Punicaceae) commonly known as Pomegranate is a dark greenish large deciduous shrub or small tree, about 5-10 m height. Flower buds are traditionally used in the treatment of asthma and allergy. The aim of this study was to validate the traditional antiallergic property using milk-induced leucocytosis and milk-induced eosinophilia in rats. Flower buds of the plant were extracted successively using various solvents to obtain the respective extracts. These extracts were administered to mice at the dose of 50 and 100 mg/kg, orally. Only the ethanol extract showed significant reduction in leukocyte and eosinophil count, these results are a validation of the use of the extract of polar compounds of *P. granatum* flower buds as an antiallergic agent.

Keywords: *Punica granatum*; Punicaceae; flower bud; leukocytosis; eosinophilia.

Resumen
Punica granatum Linn. (Punicaceae) comúnmente conocida como granada es un pequeño árbol o arbusto grande de hoja caduca, de unos 50-10 m de altura y de color verde oscuro. Los botones florales se utilizan tradicionalmente en el tratamiento del asma y la alergia. El objetivo de este estudio fue validar la propiedad tradicional antialérgica utilizando ratas con leucocitosis y eosinofilia inducida por leche. Los botones florales de la planta se extrajeron sucesivamente con varios solventes para obtener los extractos respectivos. Estos extractos se administraron a los ratones a dosis de 50 y 100 mg / kg, por vía oral. Sólo el extracto obtenido con etanol mostró una reducción significativa en el recuento de leucocitos y eosinófilos, estos resultados son una validación del uso del extracto de compuestos polares de los botones florales de *P. granatum* como un agente antialérgico.

Palabras Clave: *Punica granatum*; Punicaceae; botones florales; leucocitosis, eosinofilia.

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INTRODUCTION

Punica granatum Linn. (Punicaceae) is a dark greenish, large deciduous shrub or small tree cultivated all over in India. Commonly it is called as *Pomegranate* in English and *Anar* or *Dalim* in Hindi. The various parts root, bark, leaves, fruit and flower buds are used in traditional medicine for the treatment of asthma, diarrhea, dysentery, tuberculosis, antidotes for snakebite and as anthelmintic and astringent (Nadkarni, 1982). The plant has antioxidant (Elfalleh *et al*., 2009), antimicrobial (Al-Zoreky, 2009), antiplasmodial (Dell'Agli *et al*., 2009), antidiabetic (McFarlin *et al*., 2009), Hepatoprotective (Celik *et al*., 2009), anticancer (Khan, 2009) and antihistaminic (Barwal et al., 2009) activities. A total of 18 compounds were found in pomegranate aroma profiles, including monoterpenes, aldehydes, alcohols, monoterpenoids and linear hydrocarbons. The most abundant compounds were trans-2-hexenal, 3-carene, α-terpinene and α-terpineol (Calín-Sánchez *et al*., 2011). Unsaponifiable matter from *P. granatum* seed oils consist of squalene, policosanol, β-sitosterol, cycloartenol, β- and δ-tocopherol (Caligiani *et al*., 2010). Punicalagin isolated from crude hydro alcoholic extract prepared from the fruit peel of *P. granatum* showed strong activity against *Candida albicans* and *C. parapsilosis* (Endo *et al*., 2010). Two new beta-sitosterol esters have been isolated from the flowers of *P. granatum* as stigmast-5-en-3beta-ol-3beta-dodecanoate (beta-sitosterol laurate) and stigmast-5-en-3beta-ol-3beta-tetradecanoate (beta-sitosterol myristate) along with the known compounds as n-tricosane, n-heptacosanyl n-hexanoate, olean-5,12-dien-3beta-ol-28-oic acid, and olean-12-en-3beta-ol-28-oic acid (Bagri *et al*., 2009). Punicic acid found in *P. granatum* is an omega-5 fatty acid capable of inhibiting breast cancer proliferation (*Grossmann et al*., 2010). Gallic acid, methyl gallate, ellagic acid, (+) catechin, isoquerecitrin, D-mannitol, ursolic acid, Oleanolic acid, beta-Sitosterol and Daucosterol are isolated from *P. granatum* (Rena *et al*., 2009). Pomegranate tannins significantly inhibited ulcerative formation (Lai *et al*., 2009). A new dimeric gallic acid glycoside named Humarain was isolated from stem bark of *P. granatum* (Tantray *et al*., 2009). Present work was undertaken to check antiasthmatic potential of the plant.

MATERIALS AND METHODS

Plant Material

Fresh flower buds of *P. granatum* were collected from Ahmednagar district of Maharashtra in November 2007 and authenticated by Mr. S.C. Majumdar, Botanical Survey of India, Pune, where a sample specimen (Voucher number: BSBP1) has been deposited.

Extraction

Dried and coarsely powdered flower buds of *P. granatum* were subjected to successive solvent extraction in Soxhlet extractor using petroleum ether, chloroform and ethanol as solvent and the marc left was refluxed with water. All the extracts were vacuum dried to produce PEE (0.7275% w/w), CLE (2.05% w/w), ETE (56% w/w) and AQE (16% w/w) respectively.

Animals

Male albino mice (Swiss strain) weighing 22-25 g were housed under standard laboratory conditions, in a group of six each. The animal had free access to food and water. The animal ethical committee of the institute approved all protocols of the study.

Drugs and Chemicals

Following chemicals were used for the study.
Chemicals: Petroleum ether ($60\text{-}80^0$c) AR, chloroform AR, ethanol AR and tween 80 (PCL India).

Milk-induced Leucocytosis

The procedure was followed as described by Brekhman *et al*., (1969), Bhargava and Singh (1981) and Taur *et al*., (2007). Mice were divided into six groups (n=6). Blood samples were collected from retro-orbital plexus. Total leukocyte count was done in each group before drug administration and 24 hrs after milk injection (boiled and cooled). Blood was sucked in WBC pipette up to mark and further diluted with WBC diluting fluid. Pipette was shaken for few seconds and kept aside for 5 min. Neubaur's chamber was charged with above fluid and total leukocyte count was done. Group I was treated with vehicle (10 % tween 80 in distilled water). Group II received vehicle and milk (4 ml/kg, sc), where the animals belonging to other groups received PEE, CLE, ETE and AQE (50 and 100 mg/kg, po, each). After 1 hr of drug treatment each animal was injected with milk. Difference in Total leucocytes count before and 24 hr after the drug administration was calculated.

Milk-induced Eosinophilia

The procedure was followed as described by Brekhman *et al*., (1969), Bhargava and Singh (1981) and Taur *et al*., (2007). Mice were divided into six groups (n=6). Blood samples were collected from retro-orbital plexus. Eosinophil count was done in each group before drug administration and 24 hrs after milk injection (boiled and cooled). Blood was sucked in WBC pipette up to mark and further diluted with eosin solution. The eosin solution facilitates destruction of all corpuscles except eosinophil. Pipette was shaken for few seconds and kept aside for 5 min. Neubaur's chamber was charged with above fluid and eosinophil count was done. Group I was treated with vehicle (10% tween80 in distilled water). Group II received vehicle and milk (4 ml/kg, sc), where the animals belonging to other groups were treated with PEE, CLE, ETE and AQE (50 and 100 mg/kg, po, each), respectively and after 1 hr of drug treatment each animal was injected with milk (4 ml/kg, sc). Difference in total eosinophil count before and after 24 hr of drug administration was calculated.

Statistical Significance

The data was analyzed for statistical significance using one-way ANOVA followed by Dunnett's test. $P<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Milk-induced Leucocytosis

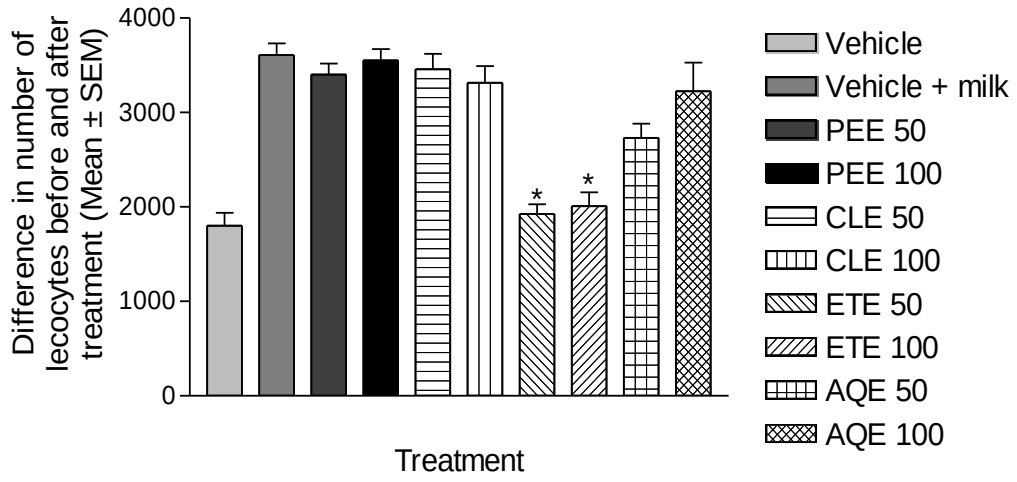
Amongst mice pretreated with various extracts of *P. granatum* flower buds, only ethanol extract showed significant reduction in leukocyte count induced by milk, whereas other extracts failed to reduce leukocyte count significantly (Figure 1). Physical and chemical stressors such as trauma, polluted air exposure, radiation etc. has been reported to concurrently produce immunodeficiency and oxidative stress. Suppression of immunity takes place due to exposure to polluted air and leads to respiratory diseases. Reactive nitrogen and oxygen species damages airways and play a role in pathophysiology of asthma. So, a drug having antistress activity induces a state of non-specific increased resistance (SNIR) against a variety of stress (Joharapurkar *et al*., 2003). After parental administration of milk, there is increase in total leukocytes count, and this stressful condition can be made normalized by administration of an antistress or adaptogenic drug. Furthermore, leukocytes during asthmatic inflammation release the inflammatory mediators like cytokines, histamine and major basic protein, which promote the ongoing inflammation (Brekhman *et al*., 1969). Thus ethanol extract showed protective effect against milk-induced leucocytosis. White blood cells (leukocytes) move toward and adhere to the vessel walls. Such white blood cells as neutrophils pass into the tissue and form an exudate with the fluid that had escaped from the tissue. As a result the walls of the vessels lose their impermeability to protein, which escapes along with water from tissue cells. This loss of fluid sometimes leads to sluggish blood flow and coagulation. Fibrinogen, a plasma protein, also escapes to the tissue and is acted on by thrombin to form fibrin. Strands of fibrin eventually cover the injured area, walling the inflamed area off and preventing possible spread of infection. Gamma globulin, a plasma substance that contains antibodies, also enters the tissue and accelerates the engulfment of bacteria by cells known as phagocytes. White blood cells called granulocytes are capable of phagocytosis; of these, the basophils and the tissue mast cells are the major sources of inflammation mediators. Other granulocytes, such as neutrophils, also function through the release of lysozome, an enzyme that destroys bacteria, and eosinophils act to control allergic reactions (http://www.shvoong.com).

Milk-induced Eosinophilia

Amongst mice pretreated with various extracts of *P. granatum* flower buds, ethanol extract showed significant reduction in eosinophil count induced by milk, whereas other extracts did not reduced eosinophil count significantly (Figure 2). The type-I hypersensitivity reaction leads to the development of edema, vascular dilation and eosinophilic infiltration (Justice et al., 2003). In late phase, especially in the development of allergic asthma, eosinophil plays a role as inflammatory cells as it secretes mediators which results in epithelial shedding, bronchoconstriction and promotion of inflammation in respiratory tract (Brigden, 1999). So abnormal increase in peripheral eosinophil to more than 4% of total leukocyte count (eosinophilia) is associated with respiratory disorder, often allergic in nature together with pulmonary infiltrates (Ehright *et al*., 1989). Ethanol extract significantly reduced eosinophils, thus it is useful as antiallergic and may be useful in the treatment of asthmatic condition. Eosinophils are thought to mediate inflammatory and cytotoxic events associated with allergic disorders, including bronchial asthma, rhinitis and urticaria (Gleich *et al*., 1993, Kroegel *et al*., 1994). The eosinophilic response has been identified as a key alteration in the pathogenesis of asthma and other allergic diseases. A close-

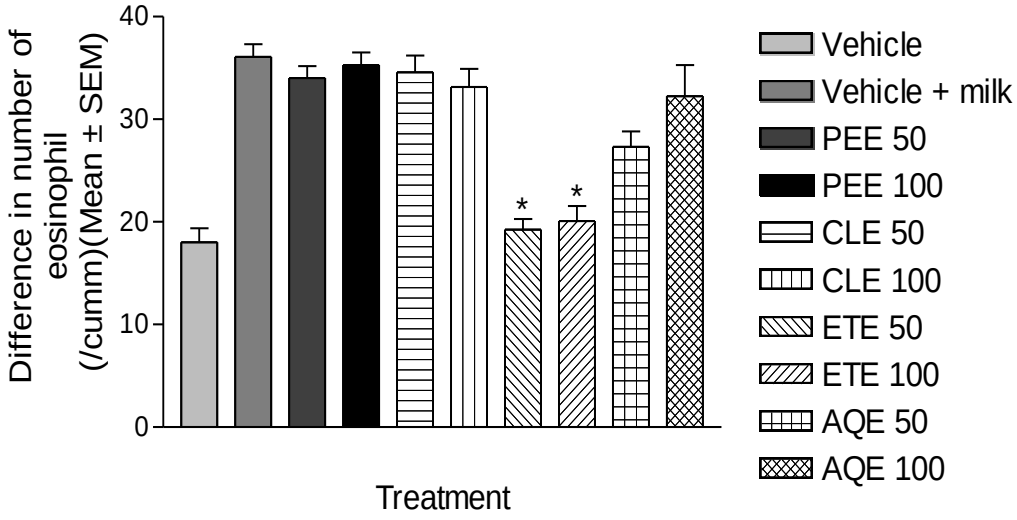
correlation between disease severity and eosinophilia, and the eosinophil ability to provide toxic and pro-inflammatory agents are the major elements supporting the interpretation that there is indeed a causal relationship between these phenomena (Bandeira-Melo, 1997). The eosinophil is well recognized as a central effector cell in the inflamed asthmatic airway. Eosinophils release toxic basic proteins and lipid mediators such as cysteinyl-leukotrienes that cause bronchial epithelial damage and airflow obstruction. Eosinophil-selective cytokines and chemokines including interleukin (IL)-5, eotaxin and RANTES may represent targets for novel asthma therapies (Sampson, 2000).

Figure 1. Effect of various extracts of flower buds of *P. granatum* on milk-induced leucocytosis in mice.



Data is presented as mean ± S.E.M. * < 0.05 compared with vehicle + milk group.

Figure 2. Effect of various extracts of flower buds of *P. granatum* on milk-induced eosinophilia in mice.



Data is presented as mean ± S.E.M. * < 0.05 compared with vehicle + milk group.

CONCLUSIONS

Ethanol extract of *P. granatum* flower bud reduced milk-induced leukocytosis and eosinophilia. Increased level of leukocytes and eosinophil is the condition of allergy. Thus we may conclude that ethanol extract of *P. granatum* is having antiallergic effect. As ethanol extract inhibited leukocytes and eosinophils more significantly than other extracts it can be concluded that the antiallergic constituent is present in ethanol extract. Further research is necessary to isolate that antiallergic component.

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