ROLE OF MALVA PARVIFLORA FLOWER EXTRACT ON ALLERGEN-INDUCED EOSINOPHILIA IN MICE

ABSTRACT:
The present study was undertaken to investigate the effect of water extract of the flower malva plant (Malva parviflora) as an anti-inflammatory and anti-allergic on allergen-induced eosinophilia by inducing three models of inflammation. The models were asthma, peritonitis and pleurisy. The results of the present study indicate that the intranasal challenge (i.n.), intraperitoneal (i.p.) injection and intrathoracic (i.t.) injection to mice sensitized with ovalbumin chicken (OVA) induced eosinophil accumulation in the bronchoalveolar lavage (BAL), in the peritoneal cavity and in the pleural cavity, respectively. The asthma model was chosen to determine the specified dose, the appropriate time and the route of administration of the extract. The results of the present investigation showed that daily oral administration (50 mg/kg p.o.) of the extract on day 0 of sensitization to the day of challenge, exactly one hour before challenge was more effective than i.p. and subcutaneous s.c. administration. Treatment with flower’s extract in actively sensitized mice challenged by OVA either i.n., i.p., or i.t. showed a reduction in the number of both leukocytes and eosinophils. However, serum IgE level was reduced in case of asthma and pleurisy model but no reduction in the antibody production in the peritonitis. The present data suggested that pretreatment with malva flower extract inhibits the cell migration in the asthma, peritonitis and pleurisy model. This inhibition extends to the systemic immunoglobulin response IgE against OVA particularly in the asthma and pleurisy model.

INTRODUCTION:
The participation of eosinophils in allergic reactions is suggested by the presence of specific receptors for anaphylactic immunoglobulin, adhesion molecules and pro-inflammatory mediators at their surface (Capron, 1992). Eosinophils are prominent inflammatory cells involved in allergic disorders, which are recruited in elevated numbers into the airways, peritoneal cavity (Amorim et al., 1993) and the pleural cavity of several species, including guinea-pigs, mice and rats, following antigen challenge (Lellouch-Tubiana et al., 1988; Gulbenkian et al., 1990; Lima et al., 1991; Okudara et al., 1991). The ability of activated eosinophils to release eosinophil-derived neurotoxin has associated these cells with epithelial damage and tissue injury (Gleich, 1990).

In addition, the number of eosinophils in the airways correlates with the severity of the late phase asthma (Bousquet et al., 1990) suggesting that their presence in inflamed tissues contributes to the perpetuation and the amplification of the disease. However, the mechanisms responsible for the attraction and the localisation of eosinophils at the site of allergic reactions remains, to be elucidated fully.

Previous studies indicate that some cytokines promote eosinophil chemotaxis in various species, including mice. These cytokines are Interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF), which are involved in monocyte-macrophage and eosinophil proliferation (Lopez et al., 1986, 1987& 1988) and IL-5 which acts specifically on the eosinophil lineage (Lopez et al., 1988). IL-5 is primarily produced by activated Th2 lymphocytes, even though mast cells (Plaut et al., 1989) and eosinophils (Desreumaux et al., 1992) are also a source for this cytokine.

The flowers of malva, Malva parviflora family malvaceae are rich in mucilage, a complex mixture of polysaccharides that form soothing gelatinous fibers when water is added. The flower tea was traditionally used internally for soothing sore throats, laryngitis and tonsillitis, coughs, dryness of lungs and digestive upsets. Mucilage as a good source of soluble fibre is particularly recommended...
for soothing gastric disease (Farina et al., 1995, Duke, 1997).

The flower’s liquid extract also displays anti-inflammatory activity where it contains high concentration of colorful purple flavonoids called anthocyanins (Wichtl, 1994).

This study has focused on the effects of antigen challenge in sensitized mice in terms of cell migration, and cell infiltration in the bronchoalveolar lavage, peritoneal cavity and thoracic cavity of sensitized mice following antigen administration using an extract of malva flower as an anti-inflammatory and anti-allergic.

**MATERIALS AND METHODS:**

Balb/C mice, crystalline ovalbumin chicken OVA, aluminum hydroxide (alum), ketamine, xylazine, eosin, methylene blue, Bovine serum albumin (BSA), saline, phosphate buffered saline (PBS), malva flower.

**Malva flower extract (MFE):**

Dried powdered malva flower was extracted using boiling water, and strained after 10 minutes, then the extract was collected, according to Farina et al. (1995).

**Dose:**

In the present study, the malva flower dose used was 50 mg/kg body weight dissolved in 0.4 ml distilled water according to Wichtl (1994). The dose was administered either intraperitoneal (i.p.), pharyngo-oral (p.o.) and subcutaneous (s.c.) form the first day of sensitization (immunization) to the challenge day.

Balb/C mice weighing 25-35g were used in this study. The animals were obtained from Theodor Bilharz Research Institute, Al-Giza-Egypt. the experimental animals were housed in cages at temperature of 20-20°C and were fed standard food and water ad-libitum. The animals were grouped in each experiment as control and/or treated group. Each group contained five animals.

**Asthma-model:**

**Allergen immunization/challenge protocol:**

Mice received an i.p. injection of 0.2 ml saline containing (100 µg) of OVA complexed with aluminum hydroxide (0.4 mg), according to Klaus et al., (1998) on day 0 and 14. Fourteen days after the second intraperitoneal immunization, mice were anesthetized with 0.2 ml i.p. of Ketamine (0.44 mg/ml) xylazine (6.3 mg/ml) in normal saline before receiving an intranasal (i.n.) dose of 100 µg OVA in 0.05 ml normal saline.

**Detection of different cell types in the bronchoalveolar lavage fluid:**

Forty-eight hour post intranasal OVA challenge, the mice were sacrificed. The trachea cannulated, and bronchoalveolar lavage (BAL) performed by flushing lung airways 3 times with 1ml PBS. Bronchoalveolar lavage (BAL) fluid cells from a 0.05 ml aliquot of the pooled sample were counted using a hemocytometer and the remaining fluid centrifuged at 4°C for 10 min at 200 g. after resuspension of the cell pellet in 20µl of 10% bovine serum albumin BSA, BAL cell smears were made on glass slides.

To stain eosinophils, dried slides were stained with Discombe’s diluting fluid (0.05% aqueous eosin and 5% [vol/vol] acetone in distilled water) for 5-8 min, rinsed with water for 0.5 min., and counterstained with 0.07% methylene blue for 2 min. as described by Basten et al. (1970).

**Determination of eosinophil number in lavage fluids:**

To determine the number of eosinophils in lavage fluids; lavage cells were applied to glass slides after centrifugation and stained with Discombe’s diluting fluid, percentage of eosinophils was determined by counting in eight power fields (magnification, 40X; total area, 0.5 mm²) per area selected randomly under a low power of magnification (4X), and dividing this number by the total number of cells per high power field, to obtain the absolute number of eosinophils in the lavage, this percentage was multiplied by the total number of cells recovered in the lavage fluids, as described by Gonzalo et al. (1996).

**Peritonitis-model:**

**Animals and sensitization protocol:**

Male Balb/C mice aged 8 weeks weighing approximately 25-35 g were actively sensitized by a subcutaneous (s.c.) injection of 0.4 ml 0.9% W/V NaCl saline containing 100 µg ovalbumin adsorbed in 1.6 mg aluminum hydroxide. Seven days later the animal received the same dose of ovalbumin in the presence of Al (OH)₃ and were used 7 days thereafter, according to Andersson and Brattssand (1982)

**Antigen induced peritonitis:**

Peritonitis was induced by the intraperitoneal (i.p.) injection of 0.4 ml of a solution containing 10 µg ovalbumin diluted in sterile saline (10µg/ cavity) at 48h after antigen challenge, animals were sacrificed by an overdose of ether and the peritoneal cavity was opened and washed with 3 ml of heparinised saline (10 U/ml). 48 hours later, total leukocytes present in the peritoneal lavages were counted; differential cell counts were performed after centrifugation and staining with discombe’s fluid as described above.

**Allergic pleurisy-model:**

**Antigen-induced pleurisy:**

Allergic pleurisy was induced in ovalbumin-sensitized mice by intrathoracic injection of 0.1 ml saline containing ovalbumin...
(12 µg/ cavity) 14 days after sensitization. Animals were sacrificed 48 hours later, and the thoracic cavity was rinsed with 1 ml of sterile phosphate buffered saline (PBS) containing heparin (10U/ml), according to Stenvan et al. (1996).

The pleural washes were collected and total leukocyte counts were obtained after dilution of the pleural fluid in Turk solution (2% acetic acid) differential cell counts were performed after centrifugation and staining with discombe’s fluid as described above.

**Measurement of serum level IgE:**

Detection of IgE by enzyme linked immunosorbent assay ELISA according to Ledermann et al. (1991). The serum of OVA-immunized mice was collected before antigen challenge and after the mice received the last dose of malva flower extract.

**Statistical analysis:**

Student’s t-test was used to determine the significance of differences between control and treated groups. Results are reported as mean ± and standard deviation (SD) according to Winer (1971).

**RESULTS:**

**Asthma-model:**

**Effect of dose dependent of malva flower extract on cell migration in bronchoalveolar lavage (BAL):**

Different doses 50, 20 and 10 mg/kg from the extract were administered on day 0 of immunization to day 28. Bronchoalveolar lavage (BAL) was collected 48h post challenge. The data in (Table 1) showed that 50mg/kg produced the highest decreasing in both leucocyte (2.74 × 10^6 ± 0.8) and eosinophil number (1.56 × 10^5 ± 0.36). The doses 30 and 10mg/kg produced no significant decrease of leucocyte number (5.26 × 10^6 ± 0.73 and 5.58 × 10^6 ± 1.29) or eosinophil number (4.12 × 10^5 ± 0.62 and 4.34 × 10^5 ± 1.29), respectively. Compared to control group the leukocyte number was (6.60 × 10^6 ± 1.08) and the eosinophil number was (1.56 × 10^5 ± 0.83), respectively.

**Table 1. Total leukocyte migration in BAL (asthma-model) (dose-dependent of malva flower extract).**

<table>
<thead>
<tr>
<th>Leukocytes × 10^6/ml BAL</th>
<th>Control (mg/kg)</th>
<th>Treated 50 mg/kg</th>
<th>Treated 30 mg/kg</th>
<th>Treated 10 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>6.60 ± 1.08</td>
<td>2.74 ± 0.80***</td>
<td>5.26 ± 0.73</td>
<td>5.58 ± 1.29</td>
</tr>
</tbody>
</table>

**Eosinophils × 10^5/ml BAL**

| Mean ± SD               | 5.15 ± 0.83    | 5.56 ± 0.42***  | 4.34 ± 0.62     | 4.34 ± 1.29     |

*** Significant at P < 0.001

**Effect of administration route of extract on cell migration in BAL:**

OVA-immunized mice were received 50mg/kg malva flower extract by a different route of administration; intraperitoneal, subcutaneous, and oral. Extract was daily administered on day 0 of immunization to day 28. BAL was collected after 48h post challenge. The data (Table 2) showed that oral administration produced the highest inhibition in both leucocyte number (2.64 × 10^6 ± 0.51) and eosinophil number (1.84 × 10^5 ± 0.32). Also subcutaneous administration recorded markedly inhibition in both leucocyte (3.56 × 10^5 ± 0.61) and eosinophil number (2.52 × 10^5 ± 0.28). No significant change was observed in intraperitoneal administration in leucocyte and eosinophil number (5.90 × 10^6 ± 0.92), respectively compared to control group without extract the leucocyte and eosinophil number were (6.52 × 10^6 ± 0.71 and 5.24 × 10^5 ± 0.36), respectively.

**Table 2. Effect of route administration of malva flower extract on leukocyte and eosinophil number (asthma-model).**

<table>
<thead>
<tr>
<th>Leukocytes × 10^6/ml</th>
<th>Control (i.p.)</th>
<th>Treated (p.o.)</th>
<th>Treated (s.c.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>5.94 ± 0.55***</td>
<td>3.19 ± 0.54***</td>
<td>3.00 ± 0.79***</td>
</tr>
</tbody>
</table>

*** Significant at P < 0.001; Intraperitoneal (i.p.); Pharyngo-oral (p.o.); Subcutaneous (s.c.)

**Effect of malva flower extract on cell migration and serum IgE level in the three models of inflammation:**

**Asthma-model:**

**Effect of MFE on cell migration in bronchoalveolar lavage:**

Bronchoalveolar lavage was obtained 48 h post intranasal OVA challenge. The data in table 4 and figures 1&2 showed that oral administration of (50 mg/kg/day) malva flower extract produced significant decrease of leukocyte number (2.64 × 10^6 ± 0.51) consequently eosinophils number (1.84×10^5 ± 0.32) was reduced in the treated
group compared to control group. The leukocyte number was $(6.52 \times 10^6 \pm 0.71)$ and the eosinophils number was $(5.24 \times 10^5 \pm 0.36)$.

### Table 4. Effect of malva flower extract on cell migration and serum IgE level in the three models of inflammation.

<table>
<thead>
<tr>
<th>Models of inflammation</th>
<th>Leukocyte no. × 10^6/ml</th>
<th>Eosinophil no. × 10^5/ml</th>
<th>Serum IgE level g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.52 ± 0.71</td>
<td>5.24 ± 0.36</td>
<td>10.00 ± 1.58</td>
</tr>
<tr>
<td>Treated</td>
<td>2.64 ± 0.51***</td>
<td>1.84 ± 0.32***</td>
<td>4.00 ± 1.22***</td>
</tr>
<tr>
<td>Asthma</td>
<td>5.72 ± 1.37</td>
<td>6.02 ± 0.72</td>
<td>2.16 ± 0.21</td>
</tr>
<tr>
<td>Treated</td>
<td>3.66 ± 0.56</td>
<td>2.90 ± 0.74***</td>
<td>0.21 ± 0.14</td>
</tr>
<tr>
<td>Peritonitis</td>
<td>5.68 ± 0.45</td>
<td>0.85 ± 0.24</td>
<td>1.84 ± 0.37</td>
</tr>
<tr>
<td>Treated</td>
<td>2.62 ± 0.94***</td>
<td>0.13 ± 0.11***</td>
<td>0.48 ± 0.11***</td>
</tr>
<tr>
<td>Pleurisy</td>
<td>0.45 ± 0.94</td>
<td>0.13 ± 0.11***</td>
<td>0.48 ± 0.11***</td>
</tr>
</tbody>
</table>

Treated mice: received orally 50mg/kg p.o. of malva flower extract. * Significant at $P \leq 0.05$, *** Significant at $P < 0.001$ as compared to control group.

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**Effect of MFE on serum IgE level:**

Sensitized mice received orally 50 mg/kg/ day malva extract from the first day of immunization. On day 28 after the mice had received the last dose of extract (one hour before challenge), the serum was taken to measure the antibody production. Data in table 4 and figure 3 showed significant difference between the control (10 ± 1.58) and treated (4 ± 1.22) group with malva extract in the serum level IgE.

**Peritonitis- model:**

**Effect of MFE on cell migration in the peritoneal cavity:**

Peritoneal washing was obtained 48 h after the i.p. challenge of 10 µg/ cavity in mice that had received s.c. OVA and alum. Malva flower extract (50mg/kg) was orally administered at the first day of sensitization to day 14 (one hour before challenge). Data in table 4 and figures 1&2 showed the significant decrease in the number of leukocytes $(3.66 \times 10^6 \pm 0.56)$. Consequently the number of eosinophils $(2.90 \times 10^5 \pm 0.74)$ was reduced compared to OVA immunized challenged mice without extract (control group) the number of leukocytes was $(5.72 \times 10^6 \pm 1.37)$ and eosinophil number was $(6.02 \times 10^5 \pm 0.72)$.

**Effect of MFE on serum IgE level:**

Immunized mice were orally received 50 mg/kg malva extract from the day 0 of immunization to day 14 and before i.p. challenge, the serum was taken to measure the antibody production Ig E. Results (table 4, figure 3) showed no decrease in the antibody IgE production. Consequently there is no significant change between control $(2.16 \pm 0.21)$ and treated group $(2.10 \pm 0.14)$.

**Pleurisy-model:**

**Effect of MFE on cell migration in the pleural cavity:**

Pleural washing was obtained 48 h after the intrathoracic i.t. challenge of 12 µg OVA/ cavity in mice that had received i.p. OVA and alum on day 0 and day 14. Malva extract (50 mg/kg) was orally administered at day O of immunization to day 28 (one hour before challenge), gave the result (Table 4, Figs 1&2) which showed that the leukocytes $(2.62 \times 10^6 \pm 0.94)$ in the pleural washing was markedly decreased. Consequently eosinophils number $(0.24 \times 10^5 \pm 0.11)$ was reduced in the treated group (with malva extract) compared to control group. The leukocyte number was $(5.68 \times 10^6 \pm 0.45)$ and the eosinophils number was $(0.85 \times 10^5 \pm 0.13)$.

**Effect of MFE on serum IgE level:**

Serum was taken after the last dose of malva flower extract and before i.t. challenge. The data (Table 4, Fig. 3) showed a significant difference between treated group $(0.37 \pm 0.11)$ and control group $(1.84 \pm 0.48)$. 
DISCUSSION:

Many plant species have been used as an anti-inflammatory for treatment of asthma and peritonitis (Rogerio et al., 2006 & 2008; Simone et al., 2007). In the present study the flower of Malva parviflora has been investigated for use as anti-allergic and anti-inflammatory.

The malva flower extract has anti-inflammatory activity and contains high concentrations of colorful purple of flavonoids called anthocyanins. Also, the leaves and flowers of malva contain mucilage (Schmidgall et al., 2000) made up to high molecular weight acidic polysaccharides. These polysaccharides are composed mainly of glucuronic acid, galacturonic acid, rhamanose and galactose, the flower tea was traditionally used internally for laryngitis, gastritis, breathing disorder, bronchitis (Calssen and Blaschek, 1998).

The exact dose of malva flower extract (MFE), the appropriate time, and the route of administration were determined in case of asthma model, the data showed that the oral administration of (50mg/kg) from MFE on day 0 of sensitization to the day of challenge was effective than i.p and s.c. administration, and the best time for final dose was at one hour before challenge. These obtained results were generalized on the other two models of inflammation (peritonitis and pleurisy).

Eosinophil numbers are augmented in several disease such as pollen, sensitive rhinitis (Bentley et al., 1992), asthma (Azzawi et al., 1992), parasite infection (Secor et al., 1990) and rheumatoid arthritis (Venge, 1990).

The cellular infiltrate that characterizes asthma consists of mononuclear cells and eosinophils. The ability to control leukocyte infiltration into the lungs is viewed as the key to regulating disease severity (Lukacs, 2001). Asthma is generally regarded as a T-cell mediated disease. Allergens cause to differentiation of naïve T-cells into Th2 cells, which then secrete cytokines such as interleukin-4 (IL-4), IL-5 and IL-13 (Ramshaw et al., 2001). IL-4, which is pivotal in the pathogenesis of allergic disorders, act on B cells to facilitate IgE production (Haas et al., 1999, Ramshaw et al., 2001). Increased IgE production in response to common environmental antigens is the hallmark of allergic disease such as bronchial asthma (Hamelmann et al., 1999). IL-4 also induces the rolling and adhesion of circulating eosinophils to endothelial cells. Therefore, inhibitors of IL-4 signaling pathway have been suggested as therapeutic targets (Barnes, 2000; Romagnani, 2002).

The malva flower extract used in asthma and pleurisy models has the property that reduces free IgE, (membrane-bound IgE B cells) and decreases IgE production by these cells. Furthermore, this extract fails to bind to IgE on FcεRI on mast cells and basophils. Based on these properties, the extract does not induce mast cell, basophil activation and degranulation and the flower extract may have an anti-allergic effect on allergic asthma and pleurisy by reducing IgE secretion from B cells and, in consequence, inhibiting the cell migration in BAL and pleural cavity.

Lipid mediators such as platelet activating factor (PAF) or leukotriene (LT) B₄ are potent chemotactic agents for eosinophils from different species (Ford –Hutchinson et al., 1980; Lellouch-Tubiana et al., 1988; Coëffier et al., 1991; Martins et al., 1991). Furthermore, it has been suggested that cytokines released by activated T-lymphocytes, such as interleukin-5 (IL-5), IL-3 or granulocyte-macrophage colony stimulating factor (GM-CSF) may play a role in eosinophil accumulation at the site of the allergic reactions (Owen et al., 1987; Rothenberg et al., 1988; Sanderson, 1992). This phenomenon may depend on the ability of those cytokines to induce eosinophil proliferation from their bone marrow precursors and to enhance their survival. Thus drugs are inhibiting the pro-inflammatory activities of lipid mediators on one hand and those interfering with the activation of T-lymphocytes, on the other, are potentially useful in the treatment of allergic disorders.

On the other hand, in peritonitis model, although the administration of malva extract reduced the cell accumulation in the mouse peritoneal cavity, no inhibition of serum level IgE was detected. It is unknown whether antigen-induced eosinophil accumulation in the peritoneal cavity is dependent on IgE or whether it is independent. The insignificant changes may be through degranulation and activation of the mast cells. This result in agreement with (Ford-Hutchinson et al., 1980) who demonstrated that, the mechanisms responsible for the attraction and localisation of eosinophils at the site of allergic reactions depend on another lipid mediator, such as platelet activating factor (PAF). The concept that IL-5 may be involved in in vivo eosinophil recruitment has been already supported by Kaneto et al. (1991) that showed that pretreatment of sensitized mice with a specific antibody against IL-5 decreased the antigen-induced eosinophil influx in the peritoneal cavity. Kyan-Aung et al. (1992) demonstrated that IL-1 induced adhesion of eosinophils, but not neutrophils, to endothelial cells.

Another possibility for the inhibitory effect of malva flower extract on peritoneal cell migration may result from its ability to prevent the formation of eosinophilotactic cytokines responsible for eosinophil accumulation triggered by antigen challenge.
and its ability to act on the expression of adhesion molecules on endothelial cells (Kenato et al., 1991).

It is concluded that, malva flower extract may be applicable to bronchial allergic asthma, peritonitis and pleurisy to reduce inflammation by inhibiting cellular infiltration in the BAL, peritoneal, and pleural cavity by reducing IgE in asthma and pleurisy models. The anti-inflammatory effect of malva flower extract may result from its effect on IgE production and some inflammatory mediator, much additional study is required to determine the platelets activating factor and IL-5.

REFERENCES:


دور زهور نبات الخبيرة على مولد الحساسية المحت لجذüm الخلايا الإيزينوفيلية في الفئران

في هذا البحث تم دراسة تأثير مستخلص زهور نبات الخبيرة على مولد الحساسية المحت لجذüm الخلايا الإيزينوفيلية، وذلك على ثلاثة أنظمة محدثة للألفان بمثابة مولدات للالتهابات تحضير طريقة OVA (مولد الحساسية) عن طريق النقاط بحرين: جردعة أولى، وجردعة ثانية كانت الأنظمة كالآتي: - نظام الألوية الطبيعي لاستثمار جذعم إضافية من مولد الحساسية عن طريقة النافل للسيف القصري المحذوفة. - نظام الألفان البروتيني وهو عبارة عن حق جذعم إضافية من مولد الحساسية للفئران. وللشفط البروتيني للسيف القصري المحذوف. - النظام الأخير هو نظام النافل الطبي للسيف القصري المحذوف، وتم دراسة تأثير نبات الخبيرة على جذعم الخلايا للفئران والشفط البروتيني والسيف القصري المحذوف، وذلك بعد حدوث التهاب في الفئران البروتيني والسيف القصري المحذوف، وكذلك بعد خروج محتويات الخلايا الصلبة مثل العامل المجمع للصفائح الدموية (PAF)، أو أنواع أخرى من السيتوبين. 

المحقق:

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