Original Article

In Vivo Pharmacological Testing of Herbal Drugs for Anti-Allergic and Anti-Asthmatic Properties

Naiyer Shahzad[1,2,3], Abdullah R. Alzahrani[1,2], Ibrahim Abdel Aziz Ibrahim[1,2], Kriti Soni[4], Imran Shahid[1], Safaa Mohammed Alsanosi[1], Alaa Falemban[1,2], Ibrahim Mufadhi M. Alanazi[1,2], Ghazi A. Bamagous[1], Saeed Saeed Al-Ghamdi[1,2], Amal M. Mahfoz[5]

[1]Department of Pharmacology and Toxicology, Faculty of Medicine, Umm Al-Qura University, Holy Makkah, Saudi Arabia, [2]Saudi Toxicology Society, Faculty of Medicine, Umm Al-Qura University, Holy Makkah, Saudi Arabia, [3]Department of Pharmacology, Singhania Hospital and Research Centre, Singhania University, Jhunjhunu, Rajasthan, India, [4]Formulation Development, Dabur Research Foundation, Ghaziabad, Uttar Pradesh, India, [5]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Modern University for Technology and Information, Cairo, Egypt

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ABSTRACT

Background: Asthma is a chronic inflammatory disease characterized by reversible airway obstruction, hyperresponsiveness, and remodeling. Asthma prevalence has increased significantly globally over the last decade, and it remains incurable to this date. **Aims and Objectives:** The present study evaluated some of the antiasthmatic medicinal plants to assess their mode of action. **Materials and Method:** Animal models for milk-induced leukocytosis, milk-induced eosinophilia, mast cell degranulation, clonidine-induced catalepsy, and active paw anaphylaxis were used to assess the pharmacological effects of Ammi visnaga, Medicago sativa, and Urtica dioica. **Results:** Mice pretreated with diazepam, methanolic extract of *M. sativa,* and *U. dioica* exhibited significant ($P < 0.05$) inhibition in milk-induced leukocytosis. However, only *M. sativa* showed statistically significant ($P < 0.05$) results. All plants showed a statistically significant ($P < 0.05$) tendency to decrease milk-induced eosinophilia. Methanolic extracts of all plants significantly ($P < 0.05$) protected mast cells against degranulation by clonidine. *A. visnaga* and *U. dioica* significantly ($P < 0.05$) protected mice against clonidine-induced catalepsy. An acute treatment by *M. sativa* potentiated the catalepsy, while it significantly inhibited the catalepsy ($P < 0.05$) upon chronic treatment. In the allergic inflammation model, methanolic extracts of all plants under study decreased paw thickness in a statistically significant manner ($P < 0.05$). **Conclusion:** All the three plants in this study demonstrated anti-inflammatory and antihistaminic effects, as well as decreased paw thickness, validate anti-allergic properties. *A. visnaga* showed a mast cell-stabilizing effect. *A. visnaga* and *U. dioica* inhibited the histamine-mediated clonidine-induced catalepsy from mast cells which proves the antihistaminic activity of these plants.

KEYWORDS: *Ammi visnaga (khell), antihistaminic, eosinophil, leukocyte, Medicago sativa (alfalfa), Urtica dioica (nettle)*

INTRODUCTION

Asthma is an obstructive lung disease that occurs due to inflammation and affects millions of people of all age groups.[1,2] The cardinal symptoms are cough with wheezing, shortness of breath, and/or chest tightness, which worsen at night and/or in the early morning.[3,4] Bronchial asthma is characterized by reversible airflow obstruction, persistent airway hyperactivity, and airway remodeling.[5-8] Inflammatory cell infiltration is one of the immunohistopathologic characteristics of asthma (in sudden-onset).[9,10] Although asthma has been identified for many centuries, it still occupies a prominent place on the list of incurable diseases.

Folk medicine/alternative medicine is knowledge,

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Address for correspondence: Dr. Naiyer Shahzad, Department of Pharmacology, Singhania Hospital and Research Centre, Singhania University, Pacheri Bari, Jhunjhunu – 333 515, Rajasthan, India.
E-mail: shahzadnaiyer@gmail.com

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skills, and practice based on the traditional medicine system. It is used to maintain/improve one's health or to treat physical and/or mental illnesses. Alternative medicine is well-known in India (Ayurvedic Medicine), China (Traditional Chinese Medicine), and Greece–Arab countries (Graeco-Arabic or Unani Medicine). Islamic Medicine/Tibb Nabawi/or Prophetic Medicine also occupies a vast portion of alternative medicine worldwide.[11,12] Folk medicine provides useful leads in improving the quality of life in asthmatic patients. Although folk medicine does not play any important role in emergency or crisis management, it provides an effective alternative for maintenance or long-term therapy and, thus, has the potential to consequently reduce the frequency of emergency cases in the future.[13] A large number of plants that can relieve asthma and/or alleviate the symptoms have been mentioned in various texts such as *Charaka-Samhita*, *Susurta,* and *Bhavprakash.*

Pharmacology has played an important role in the development of new drugs from naturally occurring chemicals/or substances using a scientific and logical approach. The early allopathic medicines for asthma were derived from plants such as *Atropa belladonna, Justicia procumbens*, *Datura stramonium*, *Digitalis purpurea*, *Euphorbia pilulifera*, and *Rauvolfia serpentina*.[14-16] The gold-standard treatment for asthma is inhalers of beta-(2)-agonist and corticosteroids. In the last 40 years of research, leukotriene antagonists have been introduced in antiasthmatic drugs, which are less effective than existing treatments and corticosteroids, whereas highly effective therapies are not acceptable because of carrying risks of side effects. Therefore, a new approach to the further improvement of treatment for asthma is a challenge. *Medicago sativa* (alfalfa), *Urtica dioica* (nettle), and *Ammi visnaga* (khell) are the common traditional medicines in Saudi Arabia. These plants have been used in folk medicine to treat and/or prevent asthma and they have antihistaminic, anti-allergic, anti-inflammatory, immunomodulatory, and smooth muscle relaxant properties.[17] However, the clinical uses of these drugs in asthma remain unclear. Therefore, we established the protective effect of these drugs on animal models of allergy and asthma in the present study.

Materials and Methods

Collection and authentication of plant material

Fresh and healthy *M. sativa* (alfalfa) leaves were collected from farms in the Al-Qassim region, and *U. dioica* (nettle) leaves were collected from the mountains in the Taif region. The aerial part of *A. visnaga* (khell) was procured from local herbal drug vendors in Makkah. These crude materials of plants were identified and authenticated by Associate Professor (Dr.) Perwez Alam, Department of Pharmacognosy and Phytochemistry, King Saud University, Saudi Arabia.

Preparation of extract

Ammi visnaga

Approximately half a kilogram of the aerial part of *A. visnaga* was ground into coarse powder. Ground specimens were taken with a 1:1 methanol–water mixture for Soxhlet extraction. The extraction procedure was continued for 72 h. When all soluble constituents were dissolved in the solvent, extracts were filtered and evaporated in a rotary evaporator (Buchi, Switzerland) at a temperature and pressure of 50°C ± 5°C and 175 mbar, respectively to yield dry masses. *A. visnaga* extract (AVE) thus obtained was lyophilized to get dry powder (~12.3% w/w). The final product was preserved in polyethylene sachets and stored at 8°C for the next experimental use.

Medicago sativa

Almost half a kilogram of the aerial part of *M. sativa* was mechanically crushed into coarse powder. The powdered drug samples were taken with a 1:1 methanol–water mixture for Soxhlet extraction. The extraction was continued for 72 h until all soluble constituents dissolved in the solvent. The soluble extracts were filtered and evaporated in a rotary evaporator (Buchi, Switzerland) at a temperature of 50°C ± 5°C and pressure of 175 mbar to yield semi-solid masses. *M. sativa* extract (MSE) thus obtained was lyophilized to yield a dry powder (~ 15.3% w/w), collected, and stored at 4°C until further use.

Urtica dioica

Thoroughly washed leaves were dried under shade. The crushed leaves were extracted continuously for 48 h at 60°C–70°C with distilled water. The resultant *U. dioica* extract (UDE) was filtered under a reduced temperature and pressure and concentrated in a rotatory evaporator (Buchi, Switzerland). The obtained semisolid residue was then lyophilized to get a powder (~ 11.7% w/w).

AVE, MSE, and UDE were dissolved in PEG 400 and water (*q. s*) was added to get the solution of desired strength.

Animal care and housing

Male albino Swiss mice (*Mus musculus*) (25 ± 5 g) were procured from Umm Al-Qura University animal house facility. Animals were divided into groups of five and

housed in polyvinyl cages to acclimate to the standard temperature (23°C ± 2°C) and humidity (55% ±5%) of the light/dark cycle (12 h: 12 h)/day. Animals were given standard food pellets (Rana Food Company) with an excess water supply. Orbital venous blood was collected using light ether anesthesia from all groups of animals to obtain baseline data prior to drug administration. All the animals were then subjected to different treatment plans [Figure 1]. All the experimental works were done in strict compliance with Biomedical Animal Ethics Committee regulations.

Methods used for evaluating anti-asthmatic effect

Milk-induced leukocytosis

Mice were divided into five groups of five animals each (n = 25). Milk was used to induce leukocytosis. One group served as control and was treated with vehicle and milk. Other groups were treated with AVE, MSE, UDE (200 mg/kg *p. o.* each), and diazepam (1 mg/kg *i. p.*), respectively; 30 min later, pasteurized milk (4 mL/kg *s. c.*) was injected into each animal.[18] The total leukocyte difference before and after 24 h of drug administration was calculated. Diazepam was used as a reference standard.[19]

Milk-induced eosinophilia

For the measurement of eosinophils, the milk-induced leukocytosis model was used, the only difference being measurement of eosinophil count instead of total leukocyte count (n = 25). Periorbital venous blood was collected in a white blood cell pipette diluted with eosin solution. The diluent facilitates the destruction of all corpuscles except eosinophils. In this model, diphenhydramine (1 mg/kg *i. p.*) was used as a reference standard. A difference in the eosinophil count before and after 24 h of milk injection was noted.[18]

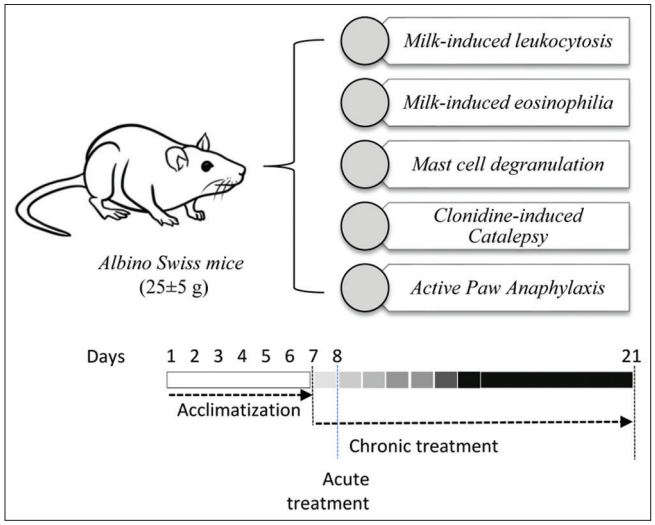

Figure 1: Albino Swiss mice were housed in polyvinyl cages. They were acclimatized for seven days in standard temperature (23°C ± 2°C) and humidity (55% ±5%) condition in a 12/12 h light/dark cycle. On the 8th day, the treatment was started and animals were subjected to different treatments. Extracts were administered acutely as a single dose for 1 day and chronic treatment for 14 days

Mast cell degranulation

A 3-day drug treatment schedule was followed in five groups of mice with five animals in each (n = 25). One group received vehicle (0.1 mL *i. p.*) and served as control and one group was treated with disodium cromoglycate (DSCG) (10 µg/mL) as a reference standard. The remaining three groups were given AVE, MSE, and UDE (200 mg/kg *p. o.*). On the 4th day of treatment, 0.9% normal saline (4 mL/kg *i. p.*) was injected into each animal. After 5 min of injection, peritoneal fluid was collected by gentle massage and transferred into siliconized test tubes containing 7–10 mL RPMI-1640 buffer medium (pH 7.2–7.4). This solution was then centrifuged at 400–500 rpm for 15 min. A pellet of mast cells was washed with the same buffer medium twice by centrifugation, discarding the supernatant. These cells were challenged with clonidine (50 µg), incubated at 37°C in a water bath for 10 min, followed by staining with 1% toluidine blue, and observed under a microscope (×10). A total of 100 cells were counted from different visual areas. The percentage of protection against degranulation was calculated.[20]

Clonidine-induced catalepsy

Indirect antihistaminic activity of the extract was determined by a bar test in clonidine-induced catalepsy.[21] Mice were divided into four groups each with five animals (n = 20). Clonidine (1 mg/kg, *s. c.*) was injected after 30 min into mice that had previously been treated with the vehicle (0.1 mL *i. p.*), AVE, MSE, UDE (200 mg/kg *p. o.*, each), and diphenhydramine (1 mg *i. p.*). The doses were selected on the basis of previous studies. The forepaws of mice were placed on a horizontal plate 1 cm in diameter and 3 cm above the table surface. The time required to remove the paws from the bar was noted for each animal. The duration of catalepsy was measured at 0, 15, 30, 60, 90, 120, 150, and 180 min intervals.

Active paw anaphylaxis

Mice were divided into five groups each with five animals (n = 25) mice were sensitized by injecting 0.25 mcg of egg albumin adsorbed on 6 mg of aluminum hydroxide gel subcutaneously. On day 12 of sensitization, the animals were challenged with 10 mcg of egg albumin in saline in the subplantar region of the hind paw. The contralateral paw received an equal volume of saline. The paw thickness was measured using a micrometer 15 min after the challenge. The difference in paw thickness reflected the edema due to antigen–antibody

reaction.[22] AVE, MSE, and UDE (200 mg/kg *p. o.*) and dexamethasone (0.27 mg/kg *s. c.*) were administered 30 min prior to the antigen challenge. Dexamethasone was used as a reference standard.[22]

Statistical analysis

All the experimental data were reported as mean ± SEM Student's *t*-test or one-way analysis of variance followed by Dunnett's test determined the statistical significance between the groups by Graph Pad Prism 5 software. P <0.05 was considered to be statistically significant.

Results

Effect of *Medicago sativa extract*, *Ammi visnaga extract*, and *Urtica dioica extract* on milk-induced leukocytosis in mice

The leukocyte count in the vehicle-treated mice was 2000 ± 223. A significant (P < 0.05) decrease in leukocyte count was observed in the MSE-treated and diazepam-treated groups where diazepam was used as a reference standard. AVE exposure exhibited a nonsignificant increase, whereas in UDE-treated animals, a highly significant (P < 0.01) increase in leucocyte count was reported [Figure 2].

Effect of *Ammi visnaga extract, Medicago sativa extract*, and *Urtica dioica extract* on milk-induced eosinophilia in mice

The eosinophil count difference in vehicle-treated mice 24 h before and after the subcutaneous injection of milk was 8.33 ± 4.81. This difference in total eosinophil count was a significant (P < 0.05) increase. The subcutaneous injection of milk numerically increased the eosinophil count. Pretreatment with AVE, MSE, and UDE decreased the eosinophil counts significantly (P < 0.05), while the diphenhydramine (used as reference standard) group expressed a highly significant (P < 0.01) decrease [Figure 3].

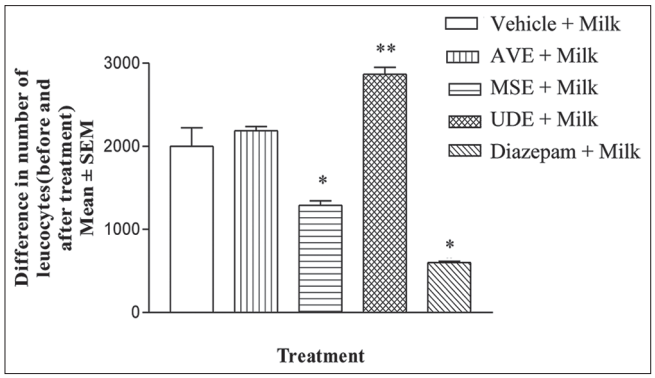

Figure 2: Effect of *Ammi visnaga* extract, *Medicago sativa* extract, and *Urtica dioica* extract on milk-induced leukocytosis in mice. All the values were expressed as mean ± SEM. n = 5, $F_{(3, 16)}$ = 60.78, * P < 0.05, ** P < 0.01 compared to vehicle-treated group (one-way analysis of variance followed by Dunnett's test)

Effect of *Ammi visnaga extract, Medicago sativa extract*, and *Urtica dioica* extract on clonidine-induced mast cell degranulation in mice

Clonidine-induced degranulation of mast cells in mice was inhibited *in vitro* by 10 µg/ml of DSCG, a mast cell stabilizer. DSCG offered highly significant (P < 0.01) 85.22% ± 4.56% protection when compared with vehicle-treated group. All the drugs were administered for 14 days. Pretreatment with AVE, MSE, and UDE produced significant (P < 0.05) mast cell protection [Figure 4].

Effect of *Ammi visnaga extract, Medicago sativa extract*, and *Urtica dioica* extract on egg albumin-induced active paw anaphylaxis

Pretreatment with dexamethasone (0.27 mg/kg *s. c.*) and AVE, MSE, and UDE (200 mg/kg *p. o.* each) decreased the subplantar region paw thickness of right hind paw significantly (P < 0.05) in egg albumin-challenged mice. The numerical presentation of paw thickness for vehicle, AVE, MSE, UDE, and dexamethasone-treated groups was 0.82 ± 0.02 mm, 0.40 ± 0.03 mm, 0.42 ± 0.03 mm, 0.35 ± 0.12 mm, 0.28 ± 0.03 mm, respectively [Figure 5].

Effect of *Ammi visnaga extract* and *Urtica dioica extract* on clonidine-induced catalepsy in mice

Animals treated with clonidine (1 mg/kg *s. c.*) exhibited catalepsy. The maximum duration of catalepsy was observed at 30 min and 60 min for 172.0 ± 10.25 s and 238.40 ± 7.19 s, respectively, and then gradually decreased to 58.40 ± 10.66 s at 180 min. A single dose of AVE and UDE inhibited clonidine-induced catalepsy significantly (P < 0.05) compared to the clonidine-treated group. The catalepsy was inhibited throughout the duration of the experiment, *i. e*. up to 180 min post administration. Vehicles, AVE, and UDE *per se* did not exhibit catalepsy [Figure 6].

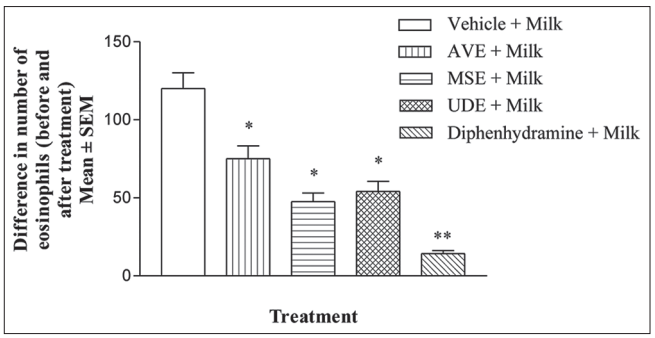

Figure 3: Effect of *Ammi visnaga* extract, *Medicago sativa* extract, and *Urtica dioica* extract on milk-induced eosinophilia in mice. The values were presented as mean ± SEM. n = 5, in each group, $F_{(3, 16)}$ =17.35, * P < 0.05, ** P < 0.01 compared to vehicle-treated group (one-way analysis of variance followed by Dunnett's test)

Effect of *Medicago sativa extract* on clonidine-induced catalepsy in mice

Animals subjected to clonidine (1 mg/kg *s. c.*) exhibited catalepsy. The duration of catalepsy at 30 min was 172.0 ± 10.25 s and the maximum duration of catalepsy observed was 238.40 ± 7.19 s at 60 min; then, it gradually decreased and a duration of 58.40 ± 10.66 s was seen at 180 min. A single dose of MSE (acute treatment) did not inhibit clonidine-induced catalepsy, whereas chronic treatment for 14 days significantly ($P < 0.05$) inhibited catalepsy between 30–90 min compared to the control group treated with clonidine. Diphenhydramine was used as a reference standard which significantly inhibited catalepsy ($P < 0.05$). Vehicle and MSE *per se* did not exhibit catalepsy [Figure 7].

Discussion

Asthma is an inflammatory airway disease that arises in response to allergens, where many cell types play an important role in its etiology. Eosinophils, mast cells, and T-lymphocytes are the most significant cells.[23] The decreased airway hyperresponsiveness is somehow associated with inflammation of a variety of stimuli.[24,25] In the early phase, allergen-specific immunoglobulin E cells are activated.[26] There is a brisk mast cell activation of the airway,[27] which, in turn, rapidly releases proinflammatory mediators such as eicosanoids[28] and histamine[29] and accelerates airway smooth muscle contraction as well as mucus secretion.

Histamine produced tracheobronchial muscle contraction in experimental animals and humans.[30] Eyre and Chand (1978) classified and discussed the biological distribution of histamine receptor subtypes and species variation has been reported with respect to the distribution of histamine receptors.[31,32] Various substances (drugs/chemicals) have been studied for the pharmacological effect on goat trachea.[33] Spasmogens such as barium chloride (0.1–51.2 mcg), acetylcholine (0.1–12.8 µg), and histamine (0.1–102.4 mg) expressed dose-dependent contraction on goat tracheal chain preparation,[34] and mepyramine maleate reported for its antihistaminic effect (H_1-antagonism). Inhibitors of histamine-induced contractions, anti-inflammatory agents, and agents stabilizing mast cells are commonly used in asthma management. In the present study, *U. dioica* showed marked antihistaminic

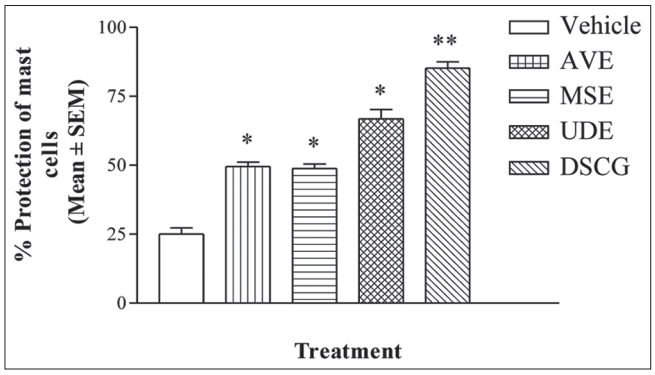

Figure 4: Effect of *Ammi visnaga* extract, *Medicago sativa* extract, and *Urtica dioica* extract on clonidine-induced mast cell degranulation in mice. The values were presented as mean ± SEM. $n = 5$, $F_{(4, 20)} = 91.80$, * $P < 0.05$, ** $P < 0.01$ compared to vehicle-treated group (one-way analysis of variance followed by Dunnett's test)

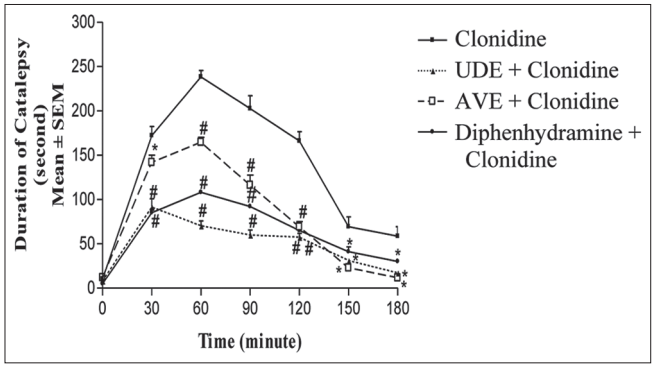

Figure 6: Effect of *Urtica dioica* extract and *Ammi visnaga* extract on clonidine-induced catalepsy in mice. The values were presented as mean ± SEM. $n = 5$, * $P < 0.05$, # $P < 0.01$ compared to clonidine-treated group (one-way analysis of variance followed by Dunnett's test)

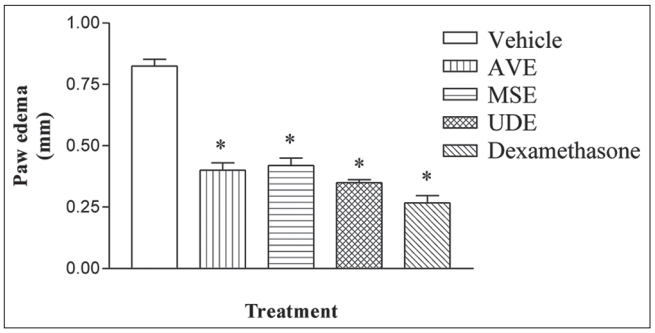

Figure 5: Effect of *Ammi visnaga* extract, *Medicago sativa* extract, and *Urtica dioica* extract on egg albumin-induced active paw anaphylaxis in mice. The values were presented as mean ± SEM. $n = 5$, in each group, * $P < 0.05$, $F_{(5, 24)} = 65.59$, compared to vehicle-treated group (one-way analysis of variance followed by Dunnett's test)

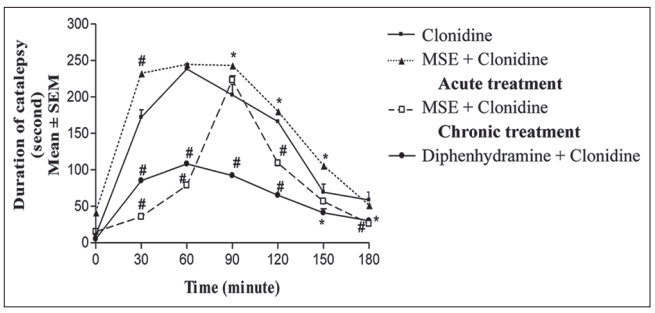

Figure 7: Effect of *Medicago sativa* extract on clonidine-induced catalepsy in mice. The values are presented as mean ± SEM. $n = 5$, * $P < 0.05$, # $P < 0.01$ compared to clonidine treated group (one-way analysis of variance followed by Dunnett's test)

activity compared to *A. visnaga* and *M. sativa* diphenhydramine as a reference standard.

Many pharmacologically active substances contributed to the development of the animal model of catalepsy. Inhibition of dopaminergic D_2-receptors by neuroleptic agents in substantia nigra induces catalepsy.[8] Anticholinergic activity of antidepressants is suggested due to enhanced dopaminergic activity or inability of central cholinergic release in the perphenazine-induced catalepsy model of neuroleptic agents. The above pharmacological agents also correlated the brain histamine content with the different stages of catalepsy.[33,35] According to the findings of the present study, clonidine-induced catalepsy in mice is mediated by histamine release from mast cells, which is inhibited by *A. visnaga* and *U. dioica*. A single dose of *M. sativa* potentiated the catalepsy, while significant inhibition of catalepsy was observed with repeated dose (chronic treatment for 14 days) administration. This observation indicates the release of histamine followed by its depletion, as reported by Dhanalakshmi *et al*.[36]

Active paw anaphylaxis is a medical emergency, in which exposure causes sensitization, followed by re-exposure of antigen–antibody (*Ag*/*Ab*) reaction. Drugs having a steroid-like effect will inhibit *Ag*/*Ab* reaction and COX inhibitors will act on PG synthesis and cause inhibition of inflammation in the late phase. As *A. visnaga* contains steroidal lactones, it significantly inhibited the allergic reaction and subplantar region hind paw edema in albumin (10 mcg)-challenged mice. *M. sativa* and *U. dioica* also significantly decreased the paw thickness. The resultant therapies should impinge on the underlying development of these diseases rather than providing symptomatic relief or palliative treatment alone.[37]

Conclusion

On the basis of the above findings and discussion, we have reached the conclusion that the plants which have antiasthmatic effects show various modes of action. Plants used in the present study like *A. visnaga* showed anti-inflammatory and mast cell-stabilizing effects. All three plants used in the study decreased paw thickness proving their anti-allergic property and also showed anti-inflammatory and antihistaminic effects. *M. sativa* decreased eosinophilia. *A. visnaga* and *U. dioica* inhibited the clonidine-induced catalepsy which is mediated by histamine release from mast cells, which, in turn, proves the antihistaminic activity of these plants. Since asthma involves several biochemical substances, a herbal formulation containing plants antagonizing the effect of various biochemical substances would be beneficial for the patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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