

Nirma Univ J Pharm Sci; 2019, 6(2) 73-100 © 2014, Nirma University, Ahmedabad, Gujarat, India ISSN 2348-4012



ARTICLE

BIOACTIVITY GUIDED ISOLATION OF URSOLIC ACID FROM CLERODENDRUM SERRATUM ROOTS AND EVALUATION OF ITS EFFICACY AGAINST ASTHMA IN GUINEA PIGS.

Niyati Acharya

Department of Pharmacognosy, Institute of Pharmacy, Nirma University Ahmedabad, Gujarat- 382481, India.

Abstract

Present study investigated anti-asthmatic activities of ursolic acid isolated from ethyl acetate fraction of crude extract of C. serratum roots along with its anti-inflammatory potential. Methanol extract (70%) was fractionated in to ethylacetate and the fraction was subjected to Gas chromatography-mass spectrum (GC-MS) in order to identify possible phytoconstituents. Column chromatography of the ethyl acetate fraction of C. serratum roots yielded ursolic acid, which was characterized by its FT-IR, mass and NMR spectral data. The effect of ursolic acid on guinea pig was assessed using inflammation of the airway caused by histamine and ovalbumin, inflammatory cell count in blood and broncho-alveolarlavage fluid, quantitative cytokine determination (IL-4, IL-5 and TNF- α) in serum and BALF, and histopathology of lung tissue. The results indicated that ursolic acid has the potential to prevent inflammatory mediators into the local lung and trachea tissues and may be useful in the management of asthma.

Key words: Asthma, Clerodendrum serratum, Cytokines, Inflammation, Ursolic Acid

1. Introduction

Asthma, a chronic inflammatory disease of the airways, characterized by infiltration of T-lymphocytes and eosinophil, overproduction of mucus and airway hyperresponsiveness. Inflammatory mediators have a great role in the pathogenesis of chronic airway disease and facilitate the recruitment, activation, and trafficking of inflammatory cells in the airways. Inflammatory cells contribute to the generation of Th2 cytokines, chemokines, Tumour necrosis factor (TNF)- α and histamine (Liu et al., 1991), levels of which are increased in asthmatic lungs (Williams and Galli, 2000). Th2 cytokines participate in asthma pathogenesis by stimulating Bcells and induce the infiltration of eosinophils and other inflammatory cells into the airways (Hamelmann et al., 1999; Zhang et al., 1999). TNF- α , is usually associated with Th1 responses (Bazzoni and Beutler, 1996; Costa et al., 1993; Gordon and Galli, 1991 Meiler et al., 2006). Recent asthma research is focused on chronic inflammation and remodelling of the airways. Many medicinal plants are used in traditional system of medicine as an alternative to treat inflammatory diseases and have been known to provide relief from the symptoms, comparable to that obtained by allopathic medicines. Several extract/fractions along with plant derived secondary metabolites have been successfully reported to interfere directly or indirectly in the pathophysiology of inflammation. In this perspective, the crude 70% methanol extract and fractions (ethyl

acetate and *n*-butanol) of roots of Clerodendrum serratum had been evaluated in our previous study for anti-asthmatic, anti-inflammatory and antioxidant to establish the traditional claims of roots. Our previous study concluded that ethyl acetate fraction of C. serratum roots (EFCSR) had potent anti-asthmatic activity that included mast cell stabilizing, bronchodilatory activities and anti-histaminic activity with inherent antioxidant and anti-inflammatory activity. The subsequent phytochemical analysis revealed the presence of polyphenols, steroids and terpenoids in EFCSR which might be responsible for the observed effects. However, the underlying mechanism and actual biologically active ingredients with antiasthmatic potential was not known. Some previous reports on flavonoids (Rogerio et al., 2007) and terpenoids (Lee et al., 2010) have shown possible efficacy against inflammatory disorders and found to significantly inhibit asthmatic responses via inhibition of chemical mediator release in bronchoalveolar lavage fluids (BALF). The increasing interest to elucidate the role of saponins and/or polyphenols for asthmatic potential has prompted scientific research into the separation and characterization of the active components. In the present investigation, EFCSR was subjected to bioassay-guided fractionation leading to the isolation of a triterpenoid. ursolic acid (UA). which was characterized and evaluated for antiasthmatic potential in animal models. The isolated compound was characterized by from its physical properties and FT-IR, EI-MS, ¹³C-NMR and ¹H-NMRspectral

data. A pilot study was conducted to determine the efficacy of UA against histamine-induced bronchospasm in guinea pigs. The protective effect of UA (15 mg/kg) was investigated for anti-asthmatic activity using ovalbumin (OVA) induced airway inflammation in guinea pigs. Effects on asthmatic responses were investigated by measuring the leukocytes and differential cell count (in blood and BALF), cytokines (in serum and BALF), histamine (in BALF) and accompanying histopathological changes in lung tissues.

2. Material and methods

2.1 Drug and chemicals

Ursolic acid, histamine and ovalbumin (OVA) were purchased from Sigma Aldrich Chemical Co. (India). Ketotifen fumarate was gifted by Torrent Research Centre, Ahmedabad, India. Dexamethasone was purchased from Zydus Research Pvt. Ltd., Ahmedabad, India.Aluminium hydroxide gel and Formaldehyde solutions were obtained from S. D. Fine Chemicals, Mumbai, India. Perchloric acid was purchased from Ranbaxy Fine Chemicals Ltd., New Delhi, India. Ketamine wasobtained from Triveni Interchem Pvt. Ltd., Gujarat, India. Xylazine was obtained from Festiva Pharma, Gujarat, India. Kits for IL-4, IL-5 and TNF- α were bought from Pro LabMarketing Pvt. Ltd., New Delhi, India. All other chemicals and solvents used were of standard analytical grades.

2.2 Plant collection, authentication and identification

The roots of *Clerodendrum serratum* Linn. were collected from Government Ayurvedic Udhyan, Gandhinagar, Gujarat (India) in the month of August, 2011. Herbarium specimen (10EXTPHDP49CS11) was deposited in the Department of Pharmacognosy, Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, India. The roots were washed and dried under sun light for 15 days. The dried material was then subjected to pulverization and the powdered sample was passed through 60# sieve. Standardization of powdered sample was carried out by the usual authentication parameters as described in the standard herbal pharmacopoeia methods.

2.3 Preparation of extract and fractions:

Powdered roots of C. serratum (1kg) were extracted thrice with 70% methanol (5 L v/v) in soxhlet extractor at the boiling temperature for 24 h, followed by filtration. Combined filtrates were concentrated and evaporated to dryness to yield methanolic extract of C. serratum i.e. MECSR (yield: 12.9% w/w). The dried extract was suspended in 500 ml of water and subsequently fractionated with ethyl acetate $(3 \times 250 \text{ ml})$ by refluxing for 2 h followed by evaporation to dryness under reduced pressure. It was then designated as Ethyl acetatefraction of C. serratum (EFCSR, yield: 2.42% w/w) and was stored in labelled bottles at 4°C for further use.

Stock solution (1 mg/ml) of the fractionEFCSR was prepared by dissolving 100 mg of dried fraction in 100 ml of

methanol. Aliquots from these stock solutions were further diluted with methanol as per the concentrations required and used for quantitative estimation of phytoconstituents, anti-inflammatory and anti-asthmatic studies etc.

2.4 Determination of chemical composition of EFCSR fraction

Gas chromatography-Mass Spectrum (GC-MS) analysis of EFCSR was performed on a GCMS-QP2010 (Shimadzu) instrument fitted with Elite-1 fused silica capillary column (30 mm x 0.25 mm inner diameter x 0.25 µm film thickness) composed of 5% Diphenyl and 95% Dimethyl poly siloxane. Electron ionization system with ionizing energy of 70 eV was used for detection. Helium (99.99%) was used as carrier gas at a constant flow rate of 1.0 mL/min and an injection volume of 2 µl was employed (Split ratio of 10:1). The oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. An electron ionization (EI) Mass spectrum was acquired over a mass range 10 to 400 Da at a scan interval of 0.5 seconds. Total GC running time was 45 minutes. The identification and characterization of chemical compounds in EFCSR was based on GC retention time. The mass spectra were computer matched with those of standards available in mass spectrum libraries (NIST, 1998).

2.5 Isolation of Ursolic Acid (UA):

Preparation of the column: Glass column (60cm x 4.5cm) was packed by wet filling.

The slurry of adsorbent (silica gel; 60-120 mesh) was prepared by mixing the adsorbent in the methanol and used as stationary phase. It was then poured into glass column and allowed to settle. The air entrapped was removed by stirring with glass rod. Excess of solvent was run off.

Preparation of sample and loading: EFCSR (5g) was dissolved in a minimum volume of methanol, adsorbed on silica gel (60-120 mesh), dried and applied on a column with 10 cm internal diameter and 50 cm length.

Selection of mobile phase and separation of the phytoconstituents: The combinations of solvents optimized for TLC separation were used as mobile phase for column chromatography. Alteration in the composition of the eluting solvent was achieved by adding the second solvent of more polarity gradually to a reservoir of the first. The EFCSR (5 gm) was subjected to silica gel column chromatography with a gradient of increasing polarity from total nhexane to total ethyl acetate followed by total chloroform to total methanol as mobile phase. Mobile phase was passed with constant flow rate (5ml/min). Fractions (about 135) of 50 ml each were collected (table 1). The separation of phytoconstituents was monitored by thin layer chromatography (TLC) developed with the different solvent systems mentioned for each fractions using vanillin sulphuric acid as the detecting agent (Wagner et al., 1996). Fractions with similar TLC pattern were pooled together and evaporated to dryness under reduced pressure. Those fractions which were eluted

in considerable quantities and in pure form were characterized after recrystallization from methanol.

Fractions (weight)	Eluting solvents and Detection composition (% v/v)		Remarks
1 to 6 (100 mg)	<i>n</i> -hexane (100 %)	<i>n</i> -hexane: ethyl acetate (9:0.5)	It contained one UV- active spot (254 nm) with the R_f value of 0.77.
7 to 9 (150 mg)	<i>n</i> -hexane/ethyl acetate (9:1)	<i>n</i> -hexane: ethyl acetate (8:2)	Showed two violet spots at the R_f value of 0.45 and 0.50 on derivatized with vanillin sulphuric (VS) acid reagent and subsequently heating at 110 °C
10 to 15 (250 mg)	<i>n</i> -hexane/ethyl acetate (8:2)	toluene-ethyl acetate-formic acid (8:2:0.1)	Single band with R_f value of 0.52 on derivatization with VS reagent <i>From the non-derivatized TLC</i> <i>plates, the single band with</i> R_f <i>value of 0.52 was scraped,</i> <i>recrystallized for further</i> <i>purification from methanol and</i> <i>characterized. This was denoted</i> <i>as Compound I</i>
16 to 21 (220 mg)	<i>n</i> -hexane/ethyl acetate (7:3)	toluene-ethyl acetate-formic acid (8:2:0.1)	Single band with R_f value of 0.58 on derivatization with VS reagent <i>From the non-derivatized TLC</i> <i>plates, the single band with</i> R_f <i>value of 0.58 was scraped,</i> <i>recrystallized for further</i> <i>purification from methanol and</i> <i>characterized. This was denoted</i> <i>as Compound II</i>
22 to 27 (200 mg)	<i>n</i> -hexane/ethyl acetate (6:4)	<i>n</i> -hexane: ethyl acetate (8:2)	It contained one UV active spot with Rf of 0.29 and two blue colored spots 0.13 and 0.17 on derivatization with VS reagent
37 to 48 (180 mg)	$\begin{array}{c} n-\text{hexane/ethyl acetate} \\ (5:5) \end{array} \qquad \begin{array}{c} n-\text{hexane: ethyl} \\ acetate (6:3) \end{array}$		One blue colored spot at Rf of 0.29 on derivatization with VS reagent

Table 1	Separtion	of phytoconstituents	from ethyl acetate	fraction of C.	serratum roots
---------	-----------	----------------------	--------------------	----------------	----------------

49 to 54 (200 mg)	<i>n</i> -hexane/ethyl acetate (3:7)	<i>n</i> -hexane: ethyl acetate (2:6)	Three violet colored spots at Rf of 0.23, 0.28, 0.37 on derivatization with VS reagent	
55 to 63 (220 mg)	Ethyl acetate (100%)	<i>n</i> -hexane: ethyl acetate (1:4)	Five violet colored spots at R_f of 0.22, 0.27, 0.48, 0.56 and 0.63 on derivatization with VS reagent	
64-69 (200 mg)	Chloroform (100 %)	Chloroform: methanol(4:1)	Two blue colored spots at Rf of 0.66 and 0.61 on derivatization with VS reagent	
70-84 (250 mg)	Chloroform/methanol (9:1)	chloroform/ methanol(4:1)	One UV active spot at Rf of 0.65 and one blue colored spot at Rf of 0.54 on derivatization with VS reagent	
85-100 (340 mg)	Chloroform/methanol (8:2)	chloroform- methanol-formic acid (4:2:0.1)	The single band with R_f value of 0.53 on derivatization with VS reagent From the non-derivatized TLC plates, the single band with R_f value of 0.53 was scraped, recrystallized for further purification from methanol and characterized. This was denoted asCompound III	
100-115 (280 mg)	Chloroform/methanol (5:5)	Chloroform: methanol(3:2)	Two blue colored spots at Rf of 0.60 and 0.64 on derivatization with VS reagent	
115-135 (420 mg)	Methanol (100 %)	-	For washing	

The *sub fraction 1* was further separated by silica gel column chromatography and eluted with increasing amount of toluene to ethyl acetate (90:10 to 70:30 v/v) as eluent. Total three subtractions (subtractions 1A to 1C) were collected by monitoring the elution process with TLC using vanillin sulphuric acid as spray reagent. The *sub fraction 1C* eluted using toluene/ethyl acetate (85:15 v/v) was purified by preparative TLC using toluene-ethyl acetate-formic acid (8:2:0.1) mobile phase and recrystallized for further purification from methanol, as a pure pale yellow amorphous powder (24 mg).

2.6 Characterization of UA:

Isolated compound was subjected to different characterization techniques. The IR spectra were obtained using JASCO FT-IR 5300 (Japan) in KBr disc and absorption peaks in terms of wave numbers (cm-1) were noted for isolates (in methanol). EIMS (electron impact mass spectrum) in positive mode, were recorded on Shimadzu LCMS 2010A (Japan) instrument. NMR spectra were recorded on Brucker (200MHz) supercon multi nuclei probe spectrophotometer (West Germany) at 400 MHz (1H) and 100 MHz (13C). Chemical shifts were recorded as δ value (ppm) using TMS (tetra methyl silane) as internal standard. The spectra were observed on pyridine-d5.

2.7 Animals and drug preparation/administration

The male Dunkin-Hartley guinea pigs (350-500 g) were housed in an airconditioned area with a light-dark period of 12 hours (temperature:22±2 ° C, humidity: 50±5 percent). Feed was given to animals with an ad-libitum free access to water by means of a regular pellet diet (certified Amrut Rotent Food, Pune, India). All studies were conducted in compliance with the protocol accepted by the Institutional Animal Ethics Committee (IAEC) in full adherence with ethic guidelines. All experiments were carried out with strict adherence to ethical guidelines and were conducted according to the protocol approved by the Institutional Animal Ethics Committee (IAEC) (Approval no: IAEC/DPS/SU/1415) and according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Ursolic acid was suspended with 0.5% sodium carboxyl methyl cellulose before oral administration. The animal dose selection of UA (Lee et al., 2010) was done on the basis of literature reports with no toxicity observed in preliminary experiment.

2.8 Bronchodilatory Effect of UA treatment on guinea pigs

The Broncho-dilatory activity of UA was evaluated in guinea pigs by exposing them to 0.5% w/v of histamine hydrochloride. Briefly, guinea pigs (250-350 g body weight) of either sex were selected and randomly divided into 05 groups each containing six animals.

Group 1 (vehicle control): 0.5% CMC-Na (1 ml/kg b.w.);

Group 2 (positive control): ketotifen fumarate (1 mg/kg b.w., *p.o.*) treated;

Groups 3-5: 7.5, 15 and 30 mg/kg b.w., *p.o.* of UA treated, respectively;

The single dose treatment was given one and half hour before challenged with histamine aerosol. Later the animals were exposed to an aerosol of 0.5% w/v of histamine hydrochloride. The time to onset of pre-convulsion dyspnoea (PCD) during challenge with histamine aerosol was noted for each animal (Sheth et al., 1972).

2.9 Effect of UA treatment on ovalbumin (OVA)-induced airway inflammation in guinea pigs

Experimental groups and drug treatment: Animals were divided into seven groups (n=6/group).

Group I received distilled water, nonsensitized controls (2.5 ml/kg b.w.);

Group II, disease control group, sensitized to ovalbumin and supplemented with respective vehicle

Group III, the positive control group, ovalbumin and DXM was provided (5 mg/kg b.w.);

Group IV was sensitized to ovalbumin and provided UA (15 mg/kg b.w.);

All animals (except group 1) were sensitized and challenged using subcutaneous injection of 100 μ g of OVA (which was adsorbed onto 100 mg of aluminium hydroxide in saline) on day 0 as the first sensitization. Two weeks later, an antigen was enhanced using the same dosage (i.e., on day 14). The daily oral doses of test and reference drugs or vehicle were conducted on day 18 and persisted until day 29.

During days 18-29, 2.5 h after receiving the appropriate drug or vehicle treatment, the animals were challenged with 0.5% w/v of aerosolized OVA for 10 min. For the challenge, conscious animals were placed into a plastic circular chamber connected to Nebulizer (Omron Medical Company Ltd., Japan). Animals from the non-sensitized group, group-I have been subjected to the same treatment of aerosolised saline (Duan et al., 2003).

2.10 Broncho alveolar lavage fluid (BALF) and serum preparation:

At 24 h after the last OVA challenge (on day 30), blood sample (3 ml) was collected from each animal under light ether anaesthesia. Then each sample was divided into two parts. A non-heparinized tube for serum separation was used for the first aliquot (2.5 ml); the isolated serum was stored at -80°C until quantitative determination of cytokines (IL-4, IL-5 and TNF- α). The second portion (0.5 ml) was kept for leukocyte count in heparinised tube. Each sample was centrifuged at $500 \times g$ for 10 min at 4°C and the cells in the pellet were washed and processed for further studies using 0.5 ml of saline. And stored for additional studies.

Broncho alveolarlavage fluid (BALF) was collected from each animal at the end of the experiment after collecting blood samples. The overdose of ketamine (30 mg / kg b.w.) and of xylazine (20 mg/ kg b.w.) in all the guinea pigs. Into the trachea a polypropylene cannula (24 G) was inserted to the lungs, then the normal saline solution (10 ml) was administered by a 10 ml syringe (0.9 percent w / v) at 37 °C and after five minutes. recovered The recovered lavage fluid (5 ml) was centrifuged at 500×g for 10 min at 4°C; for the determination of cytokine, the

corresponding supernatant has been extracted and preserved at -80 ° C. The cells in the pellet have been salinised in 0.5ml and processed in cell counts.

2.11 Inflammatory cell count in blood and BALF:

The total and differential cell counts blood and BALF were manually with a haemocytometer performed after staining with Giemsa solution. A minimum of 200 cells were counted using a compound microscope and identified as macrophages, eosinophil's, neutrophils, or lymphocytes based on normal morphologic criteria (Sanjar et al, 1990).

2.12 Cytokines in serum and BALF:

In each sample of recovered serum (400 μ l) and BALF (4.5 ml), the concentrations of TNF- α , IL-4 and IL-5 were calculated using the procedure of the manufacturer's immunosorbent assay (ELISA) kits. All plates have been analyzed in an automated reader(Robonik India Pvt. Ltd., India).

2.13 Lung tissue histopathology:

Immediately after the Broncho alveolar fluid sample, lung tissue lobes were separately dissected for each animal One lobe was used in non-lavagely determined histamine, and the other for tissue histology. Tissues were washed with normal saline (5 ml) and then put in 10% v / v of formaldehyde solution for one week for the lung tissue histopathology. After fixation, tissue were blocked in paraffin wax, sectioned at 5 µm thickness and

stained with haematoxylin and eosin dye to assess morphology. Tissues were subsequently mounted and cover slipped for microscopic examination.

2.14 Histamine assay on lavage lung tissue:

Lung tissue $(200\pm20 \text{ mg})$ homogenate was prepared in normal saline, 2.5 ml of per chloric acid (0.4 N) was added followed by mixing and centrifugation (4000×g, 7min at 4°C), resulting supernatant was taken to a test tube having 0.25 ml of 5 N NaOH, 0.75 g NaCl and 5ml n-butanol. The mixture was vortexed for 5 min to partitUrsion histamine into the butanol and after centrifugation; the aqueous phase was discarded by aspiration. In order to remove residual amounts of histidine extracted to the *n*-butanol, the organic phase was further shaken with 2.5 ml salt saturated 0.1 N NaOH solution to remove any residual histamine. The mixture was recentrifuged and the *n*-butanol layer was transferred to a test tube containing 2 ml of 0.1 N HCl and 5 ml n-heptane. After shaking for 5 minutes the tube was centrifuged and the organic phase was removed by aspiration. A 2 ml aliquot of the aqueous phase was transferred to a test tube and 0.4 ml of 1 N NaOH was added followed by 0.1 ml of o-phthalaldehyde reagent. After 4 minutes, 0.2 ml of 3 N HCl was added. The solution was then transferred to a cuvette and the fluorescence (excitation wavelength: 356 nm and emission wavelength: 450 nm) was measured with a spectrofluorimeter (SL-174, Elico, India) (Shore et al., 1959).

Statistical analysis

Results are reported as mean \pm S.E.M. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by post hoc Tukey's test; differences were considered statistically significant at P<0.05. All statistical analyses were performed using the Graph Pad software.

3. Results

3.1 Determination of chemical composition of EFCSR of C.

The GC-MS analysis of EFCSR enabled the presence of eighteen components (table 2) identified on the basis of retention time (RT), fragmentation patterns and data comparison with NIST mass spectral library. These compounds mainly comprised of acids, hydrocarbons, esters and alcohols. *n*-Hexadecanoic acid (19.03%) was identified as a major constituent followed by diethyl phthalate (16.44%), all trans-squalene (10.72%), dodecamethylpentasiloxane (7.53%). acetic acid hydroxy- ethyl ester (6.47%) etc. along with other major and minor



Figure 1 GC-MS analysis of EFCSR of C. serratum roots

Retention time	Area %	Compound Name	
0.025	4.92	n-Dodecanal	
0.103	6.47	Acetic acid hydroxy- ethyl ester	
0.479	5.93	Acetic acid	
17.782	7.53	Dodecamethylpentasiloxane	
19.483	2.09	<i>n</i> -Dodecanoic acid	
19.921	16.44	Diethyl Phthalate	
21.730	4.11	Tetradecanoic acid	
23.805	19.03	<i>n</i> -Hexadecanoic acid	
25.484	3.14	Oleic acid	
25.673	1.70	n-Octadecanoic acid	
28.936	3.34	1,2-Benzenedicarboxylic acid dioctyl ester	
29.230	1.74	<i>n</i> -Hexacosane	
29.970	3.34	n-Octacosane	
30.683	1.92	<i>n</i> -Nonacosane	
30.968	10.72	All trans-Squalene	
31.372	2.34	<i>n</i> -Tetracosane	
32.685	2.34	<i>n</i> -Tritriacontane	
34.460	2.91	Stigmast-5-en-3-ol	

 Table 2 GC-MS analysis of EFCSR of C. serratum roots

Ethyl acetate fraction of successive 70% methanol extract of C. serratum roots was subjected to silica gel column chromatography to yield compounds I,later confirmed as ursolic acid,from gradient of n-hexane/ethyl acetate eluent. The characterization of isolated compounds was performed using different spectral

techniques (IR, MS, 1H-NMR and 13C-NMR) to reveal their identity. Compound I was identified as ursolic acid (Lin et al., 1987; Tundis et al., 2002) by direct comparison of the spectra with those reported in literature.

Compound I (Ursolic Acid)

TheCompound I (Ursolic Acid)was isolated as pale yellow amorphous powder

(24 mg), subjected to further identification and characterization studies (table 3; Figure 2-6).

State:	Pale yellow amorphous powder			
Chemical test:	Lieberman Burchardt test			
FT-IR (KBr) (cm ⁻¹):	3437, 2944, 2877, 2650, 1698, 1463, 1387, 1364, 1322, 1303, 1237, 1207, 1188, 1106, 1093, 1008, 949, 825, 815, 679, 655, 539			
EI-MS (<i>m</i> / <i>z</i>):	457 [M]+(calc. for C ₃₀ H ₄₈ O ₃), 439, 411, 421, 391, 369, 355, 343, 331, 307, 279, 261, 247, 231, 217, 203, 191, 177, 163, 151, 137, 118, 102, 88, 82, 56			
¹ H NMR (Pyridine, 400 MHz, ppm):	δ H 3.44 (1H, t, J = 8.8 Hz, H-3), 5.46 (1H, br s, J = 3.6 Hz, H-12), 2.09 (1H, dt, J = 4.0, 13.1 Hz, H-16α), 2.30 (1H, dt, J = 4.8, 13.4 Hz, H-15β), 2.61 (1H, br d, J = 11.2 Hz, H-18), 1.19 (1H, m, Ha-22), 1.22 (3H, s, Me-23), 1.02 (3H, s, Me-24), 0.87 (3H, s, Me-25), 1.00 (3H, s, Me-26), 1.21 (3H, s, Me-27), 0.98 (3H, d, J = 6.8 Hz, Me-29), 0.93 (3H, d, J = 5.8 Hz, Me-30)			
¹³ C NMR (Pyridine, 100 MHz, ppm):	$ \begin{split} \delta & \subset 39.0 \ (\text{C-1}), 28.0 \ (\text{C-2}), 78.1 \ (\text{C-3}), 39.3 \ (\text{C-4}), 55.8 \ (\text{C-5}), 18.7 \\ (\text{C-6}), 34.2 \ (\text{C-7}), 39.9 \ (\text{C-8}), 48.0 \ (\text{C-9}), 37.2 \ (\text{C-10}), 23.6 \ (\text{C-11}), \\ 125.5 \ (\text{C-12}), 139.2 \ (\text{C-13}), 42.2 \ (\text{C-14}), 28.6 \ (\text{C-15}), 24.8 \ (\text{C-16}), \\ 48.0 \ (\text{C-17}), 53.5 \ (\text{C-18}), 39.4 \ (\text{C-19}), 39.3 \ (\text{C-20}), 31.0 \ (\text{C-21}), \\ 37.4 \ (\text{C-22}), 28.7 \ (\text{C-23}), 16.5 \ (\text{C-24}), 15.6 \ (\text{C-25}), 17.5 \ (\text{C-26}), \\ 23.8 \ (\text{C-27}), 180.0 \ (\text{C-28}), 17.4 \ (\text{C-29}), 21.4 \ (\text{C-30}) \end{split} $			







Figure 3 Mass spectrum of isolated Ursolic Acid



Figure 4 ¹H-NMR spectrum of isolated Ursolic Acid



Figure 5¹³C-NMR spectrum of isolated Ursolic Acid



Figure 6 Structure of ursolic acid

Effect of UA treatment in guinea pigs on histamine-induced bronchospasm

3.2 Effect of UA treatment on histamineinduced bronchospasm in guinea pigs

The dose dependent and statistically significant (p<0.001 to p<0.05) bronchodilation potential was observed with increase in preconvulsion time on pretreatment with UA isolated from EFCSR in guinea pigs on exposure of 0.5 % w/v of histamine hydrochloride aerosol (table 4). From this study, it was notable that UA post treatment at doses of 15 and 30 mg/kg significantly prolonged the preconvulsion time compared to positive control (ketotifen fumarate, 1 mg/kg) animals. Hence, a lower dose (15 mg/kg) was selected for further studies.

Treatment	Dose (mg/kg b.w., p.o.)	% Increase in PCD time	
CMC-Na (Control)	1ml/kg	4.18±0.42	
Ketotifen fumarate	1	60.55±1.40***	
UA	7.5	$28.45 \pm 0.63^*$	
	15	$35.24{\pm}0.80^{*}$	
	30	47.15±0.31**	

Table 4 Effect of UA treatment in guinea pigs on histamine-inducing bronchospasm

n=6, ***P<0.001, **P<0.01, *P<0.05 compared with data of CMC-Na (control)

Effect of UA treatments on ovalbumin (OVA)-induced airway inflammation in guinea pigs

3.3 Effect of UA treatments on body weight: *Each animal from the OVA control and experimental groups showed no significant body weight differences compared to the non-sensitized controls during the study period* (Figure 7). Effects upon appetite/ water consumption or outward appearance (i.e. fur coat, eyes) was not observed.



Figure 7 Effect of treatments on body weight of guinea pigs. Data are represented as mean \pm SEM, n=6 guinea pigs per group. NC: Non-sensitized control, OVA-C: disease control sensitized with ovalbumin, OVA+DXM: diseased treated with dexamethasone (5 mg/kg), OVA+UA: diseased treated with ursolic acid (15 mg/kg)

3.4 Effect of UA treatment on total cells in blood and BALF:

To determine the protective effects on airway inflammation, the total cell numbers in blood and BALF were examined in disease control and experimental groups treated with UA (table 5).

The total number of leukocytes in blood samples of the disease control animals were significantly increased (p<0.01) compared to the non-sensitized controls.

Dexamethasone (p<0.001), UA (p<0.05) treatment significantly inhibited the recruitment of leukocytes into blood as compared with disease control animals.

The study of BALF showed that compared to non-sensitized controls the number of total cells in OVA control (p<0.001) guinea pigs has been significantly increased. Dexamethasone (p<0.001) and UA (p<0.01) treatment significantly decreased the total number of cells in contrast with diseased control animals.

Treatment	Total cellular count (x10 ⁵ cells/ml)				
	Blood	BALF			
NC	8.22±0.11	8.51±0.15			
OVA-C	21.96±0.16**	17.64±0.26***			
OVA + DXM	12.92±0.17 ^{###}	10.74±0.14 ^{###}			
OVA + UA	$17.64 \pm 0.20^{\#}$	14.28±0.12 ^{##}			

n=6, Significantly different from non-sensitized control ***p<0.001, **p<0.01; significantly different from OVA-control #p< 0.05, ##p<0.01, ###p< 0.001

3.5 Effect of UA treatments on differential leukocytes in blood and BALF:

As shown in table 6, OVA (disease control) sensitized animals showed significant influx of leukocytes, eosinophil's and other inflammatory cells into blood and BALF compared to the non-sensitized control group animals. However, the numbers of circulating eosinophils, neutrophils, lymphocytes and monocytes were decreased significantly in dexamethasone and UA treated animals, respectively as compared to those numbers in OVAcontrol guinea pigs in blood (table 6). OVA sensitized guinea pigs treated with test samples displayed similar suppressive effects on differential cell count into BALF. The reference drug, dexamethasone (p<0.001) significantly decreased eosinophil's, neutrophils, lymphocytes and monocytes count upon asthma induction.

Table 6 Effect of UA treatments on differential cells count in blood and BALF (x 10⁵cells/ml)

Treatments	Eosin	ophil	Neutrophils		Lymphocytes		Monocytes	
	Blood	BALF	Blood	BALF	Blood	BALF	Blood	BALF
NC	0.54±	0.40±	0.220±	0.240±	1.20±	1.00±	0.64±	0.40±
	0.007	0.005	0.005	0.015	0.046	0.060	0.010	0.010
OVA-C	0.93±	0.83±	0.360±	0.390±	3.56±	3.38±	1.13±	0.83±
	0.012**	0.010***	0.009**	0.018***	0.045***	0.026***	0.027**	0.020***
OVA + DXM	0.60±	0.42±	0.230±	0.250±	2.96±	2.79±	0.72±	0.62±
	0.010###	0.011###	0.007###	0.009###	0.010###	0.012###	0.015###	0.018###
OVA + UA	0.72±	0.53±	0.251±	0.285±	3.31±	3.28±	0.83±	0.71±
	$0.009^{\#}$	0.016##	$0.007^{\#}$	0.019##	0.028##	0.031##	$0.017^{\#}$	0.012#

n=6, significantly different from non-sensitized control ^{***}p<0.001, ^{**}p<0.01, ^{*}p<0.05; significantly different from OVA-control [#]p< 0.05, ^{##}p<0.01, ^{###}p< 0.001

3.6 Effect of treatments on cytokines level in serum and BALF:

The level of serum IL-4, IL-5 and TNF- α in OVA-sensitized guinea pigs were significantly (p<0.001) greater than nonsensitized control. Dexamethasone treatment (p<0.001) caused significant reduction in the levels of IL-4, IL-5 and TNF- α as compared to the OVA-control. Treatment with other test samples also showed reduction in cytokines level analyzed in serum (Table 7). To establish the mechanisms underlying the inhibitory activity of isolated compounds (OA) in airway inflammation, the levels of cytokines (IL-4, IL-5 and TNF- α) in BALF were also examined. The levels of IL-4, IL-5 and TNF- α in BALF were significantly increased in OVAsensitized guinea pigs, compared with those in non-sensitized control animals.

Treatment	IL-4		IL-5		TNF-a	
	Serum	BALF	Serum	BALF	Serum	BALF
NC	51.41±	20.13±	17.20±	26.61±	21.32±	18.22±
	0.54	0.95	0.67	1.01	0.80	0.89
OVA-C	87.36±	53.42±	47.34±	59.34±	56.48±	46.31±
	1.32***	1.07***	0.42***	1.21***	1.14***	1.29***
OVA + DXM	54.80±	34.86±	30.18±	33.12±	27.21±	24.40±
	1.55###	0.94 ^{###}	0.88 ^{###}	1.36###	1.27###	1.22###
OVA + UA	60.45±	40.67±	35.26±	40.86±	39.37±	33.87±
	1.04##	$0.45^{\#}$	$0.48^{\#}$	1.03#	1.89#	$0.78^{\#}$

 Table 7 - Effect of UA treatments on cytokines level in serum and BALF

n=6, significantly different from non-sensitized control $^{***}p < 0.001$. Significantly different from OVA-control $^{\#\#}p < 0.001$; $^{\#\#}p < 0.01$; $^{\#}p < 0.05$

3.7 Effect of UA treatment on histopathological changes in lung tissue:

The airway inflammation effect of UA was illustrated by lung histopathological examination. Massive inflammatory cells infiltration was detected in the peribronchovascular lung areas of ovalbumin challenged guinea pig compared to non-sensitized control guinea exhibiting pig, severe airwav inflammation. Treatment with dexamethasone (5 mg/kg), UA showed a significant reduction of inflammatory cells (Figure 8).



Figure 8 Effect of UAtreatment on histopathological changes in lung tissue represents haematoxylin and eosin stained lung tissue. A: normal lung histology (non-sensitized control), B: disease control (sensitized with ovalbumin), C: lung tissue section from OVA+DXM (5 mg/kg) treated animal, E: lung tissue section from OVA+UA (15 mg/kg) treated animal

3.8 Effect of treatments on histamine level in lung tissue:

Histamine content in lung tissues of the disease control guinea pigs was significantly higher (p<0.01) than that of

the non-sensitized animals (table 8). Treatment with UA (p<0.05) normalized the elevated histamine level into lung tissues. However, their effects were lower than those of DXM (5 mg/kg).

Treatment	Concentration (mg/gm. of tissue)
NC	4.3±0.02
OVA-C	7.9±0.10 ^{***}
OVA + DXM	5.1±0.05 ^{###}
OVA + UA	6.1±0.03 [#]

Table 8 Effect of UA treatments on histamine level in lung tissue

4. Discussion

The present study was to evaluate the potential of EFCSR in asthma and to determine the chemical composition using GC-MS analysis. The EFCSR could be assigned to the presence of polyphenols including flavonoids and terpenoids, which further supports the results of phytochemical studies performed in earlier study. Based on the results of phytochemical studies performed for the EFCSR, the isolation of bioactivesresponsible for the antiasthmatic potential were planned to separate them using column chromatography technique.

Column chromatography was performed and ursolic acid was isolated. The ursolic acid was obtained as pale yellow amorphous powder and gave positive Liebermann burchard test indicating its triterpenoid nature. The FT-IR spectrum showed characteristic absorption band at 3436 cm⁻¹ and 3331cm⁴ indicating the stretching of hydroxyl (-OH) group mainly from phenolic groups. A characteristic peak at 1697cm¹ appeared in compound due to carbonyl carbon (C=O).

The mass spectrum of the ursolic acidshowed base peak at m/z 456 and m/z 439 which is characteristic for ursane type of triterpenes of α - or β - amyrin series with 12-13 double bond

in agreement with the molecular formula $C_{10}H_{10}O_{1}$. Ursane type of triperpenes contain α -amyrin skeleton while oleane type of triterpenes show its C_{w} isomer, β -amyrin skeleton. Apart from the molecular ion peak, the EI-MS showed other prominent fragment ions at m/z 248, 203, 177, 133, 95 and 69. which were characteristic for $\Delta 12$ amyrin skeleton (Awan et al., 2013). The prominent fragments ion peak at m/z 248 and 203 are characteristics of cleavage of B and C rings. Furthermore, the mass fragmentation at m/z 248 also indicated the location of hydroxyl group in ring A instead of in ring B, C, D or E and the ion fragment at m/z 203 indicated the presence of a carboxylic group in the ring D.

The 1H NMR spectrum of ursolic acid displayed signals for five methyl groups of H-22 to H-27 (δ 1.19, 1.22, 1.02, 0.87, 1.00, 1.21 ppm) together with signals corresponding to methyl protons of H-29 (δ 0.98 ppm, 3H, d, J = 6.8 Hz) and H-30 (δ 0.93 ppm, 3H, d, J = 5.8 Hz). The signal at δ 2.61 ppm attributed to H-18 (1H, d, J = 11.7 Hz) while H-3 signal observed at δ 3.44 (1H, t, J = 8.8 Hz). At δ 5.46 ppm was observed a large triplet attributed to H-12. NMR spectral data of ursolic acid showed signals corresponding to a triterpenoid skeleton of ursane type. The 1H NMR spectrum displayed signals for five methyl groups (δ 0.73, 0.76, 0.87, 0.93 and 1.04) together with signals corresponding to methyl protons of H-30 (δ 0.82, 3H, d, 3J ¹/₄ 6.4 Hz) and H-29 (δ 0.91, 3H, d, 3J ¹/₄ 6.0 Hz). The signal at δ 2.14 was attributed to H-18 (1H, d, 3J 1/4 11.3 Hz) while H-3a signal was observed at δ 3.20 (1H, did). Finally, at δ 5.40 was observed a large triplet attributed to H-12. The 13C-NMR spectrum of isolated ursolic acid showed 30 signals, consisting of seven quaternary carbons, seven methines, nine methylenes and seven methyls. The most downfield signal resonated at $\delta 180.0$ is attributed to the carboxylic acid (C-28). The appearance of signals at δ 125.5 (C-12) and 139.2 (C-13) indicated the presence of a double bond in urs-12ene triterpenoid. Attribution of all resonances displayed on the 1H-NMR and 13C-NMR spectrum of isolated ursolic acid was accomplished by direct comparison with literature data (Ghosh et al., 2014).

The effects of EFCSR-isolated UA (15 mg / kg) have been observed in guinea pigs using asthma model caused by ovalbumin (OVA) to identify the mechanism of action as it shows similar effects such as human allergic asthma (Smith and Broadley, 2007). OVA challenge induces anaphylactiv reactions due to histamine,

leukotrienes. prostaglandins, thromboxane A2 and platelet activating factor release. (Ogunbiyi and Eyre, 1985). Dexamethasone was used as cell migration inhibitor to the sites of inflammation (van der Velden, 1998). This study demonstrates that oral administration of UA (15 mg/kg), suppressed OVA-induced airwav inflammation. accompanied bv reduction of cytokine and histamine levels.

histamine From the induced bronchospasm study, it was notable that UA post treatment at all doses prolonged the pre convulsion time compared to positive control indicating the possible bronchodilatory activity. In this study, no animals showed any significant difference in body weight experimental period during the compared to the non-sensitized control animals, suggesting treatment did not interfere with the normal growth. Disease control group exhibited irritability, sneezing and hyperrhinorrhoea, indicated the severity of disease

Asthma is a major form of inflammatory airway disease After exposure to a specific trigger, namely eosinophil, inflammatory cells (Azzawi et al., 1990), neutrophils (Sur et al, 1993), lymphocytes (Robinson et al.,

1993). macrophages/ monocytes (Bentley et al, 1992) and mast cells infiltrate the airways (Fan and Mustafa, 2006).The main feature of allergic asthma is the eosinophilic inflammation of the airways with an increase in activated and degranulated asthma (Bousquet et al., 1990). Correlation between the eosinophil activation status in the airways and hyperrespondence bronchial development has been linked guinea pigs (Pretolani et al, 1994), non-human primates (Gundel et al., 1992) and humans (Wardlaw et al., 1988). In patients with allergic Asthma neutrophils are prominent inflammatory cells in the airways(Conese et al., 2003) and PGs, TXA2, LB4 and PAF capable is considered to be an important part of pathogenesis (Anticevich et al., 1996). Sputum analysis of patients with asthma and late-phase allergens, increasing numbers of lymphocytes expressing mRNA for IL-4 and IL-5 have been identified where they are associated with increased numbers of eosinophils (Till et al., 1995; Ying et al., 1997). Alveolar macrophages express the low-affinity receptor for IgE-FccRII (Melewicz et al., 1982) and expression appears to be increased in asthmatic subjects compared to healthy persons (Williams et al., 1992). Inflammatory mediators (MacDermot

et al., 1984) can induce endothelial cell activation, cell recruitment, and prolonged survival of eosinophil (Gosset et al, 1992). In the present study, the disease control animals showed significant increase in total and differential cellular counts in blood and Broncho alveolarlavage fluid in (BALF) directly correlate with increases in the level of cellular infiltration. Treatment with reference and test drugs alleviates broncho alveolar inflammation via decreasing the infiltration of differential cells blood inflammatory in (particularly eosinophil's and neutrophils) as well as in BALF. Thus, the results of the study suggested the possible usefulness of test drugs to control the activation of inflammatory process underlying exacerbation of allergic asthma.

Asthma treatment is now developed through a number of specific cytokine and chemical inhibitors, cytokines play a critical role in the facilitating and propagating of inflammation in asthmatic airways (Chungm and Bames, 1999). In particular, TNF- α and Th2-type cytokine interleukin (IL)-4and IL-5 (Martin et al., 2004) play central roles in initiating and sustaining an asthmatic response by regulating the recruitment and/or activation of airways mast cells and eosinophil's. IL-

4 is a critical factor for the regulation of T cell commitment to the CD4-Th2 phenotype and plays an essential role in immunoglobulin (IgE) switching in β cells, mucus hyper-secretion, and eosinophil infiltration into lung tissue. Thus, IL-4 appears to be important in early stages of Th2 cell the development. By contrast. IL-5regulates the growth, differentiation, and activation of eosinophil's and provides an essential signal for the recruitment of this leukocyte to the lung during allergic inflammation. IL-4 and IL-5 may also regulate eosinophil's trafficking by activating adhesion systems at the vascular endothelium. Activated eosinophils induce the release of pro-inflammatory mediators (Hogan et al., 1997). TNF- α may be an important mediator in the initiation of chronic inflammation, by activating the secretion of cytokines from a variety of cells in the airways (Hughes et al., 1995). The immune mediating activation of TNF- α can have a significant amplifying effect (Kips et al, 1992). There is evidence that asthmatic airways have increased expression of TNF- α (Bradding et al., 1994). In the present study, we have measured the level of elevated inflammatory cell markers IL-4, IL-5 and TNF- α in serum as well as in BALF. The results of our study suggest that, cytokines IL-4, IL-5 and TNF- α

were increased predominantly in disease control (OVA-sensitized) animals indicating stronger anaphylactic sensitization leading to severe inflammation. Treatment with DXM and UA suppressed the level of these mediators significantly as compared to OVA-controls.

During allergic reactions the local release of histamine was recognized for many years as an important step for antigen hypersensitivity. Bartosch et al. (1932) first reported the release of histamine from the pig lung during anaphylaxis. Acute histamine release following an allergic or not allergic result attack may in bronchoconstriction. which is modulated by selective H1-receptor antagonists (Holgate and Finnerty, 1989). Further, histamine has been shown to activate eosinophils (Raible et al., 1994). In the present study, the fluorometric analysis of histamine content in lung tissues was carried out.

The existence of flavonoids and terpenoids in plants were reported to be responsible for anti-asthmatic properties along with antioxidant, anticarcinogenic, anti-allergic and antiinflammatory effects (Tanaka and Takahashi, 2013). These compounds contribute to their anti-asthmatic potential by inhibiting the release of

chemical mediators such as histamine and other preformed granule associated mediators by inhibiting the activation of basophils and mast cells, synthesis of Th2 type cytokines, such as IL-4 and IL-13 and CD-40 ligand expression by high-affinity immunoglobulin E (IgE) receptor-expressing cells, such as mast cells and basophils (Park, et al., 2010). Various flavonoids and terpenoids such as quercetin, apigenin, and betulinic acid have shown beneficial effects in asthmatic animal models. In support, our observation also confirmed a plausible molecular basis for the antiasthmatic effect of UA isolated from C. serratum roots. In spite of the results presented in this study, it is necessary to explore the exact mechanism of the isolated compound.

5. Conclusion

From this study it can be inferred that UA isolated from C. serratum roots can have an anti-asthmatic potential due to of inflammatory inhibition cell infiltrations, release / synthesis of Tcell derved cytokines;IL-4,IL-5 and TNF- α . In addition, UA inhibited the releasing in the local lung tissue and trachea of mediators such as histamine, which was verified by historical pathology. These findings suggest that the C. serratum roots may be a valuable therapy for asthma management; however, a well-designed clinical trial is warranted, which includes persistent, mild or moderate asthmatic patients.

6. References

- Anticevich SZ, Hughes JM, Black JL, Armour CL. Induction of hyper responsiveness in human airway tissue by neutrophils-mechanism of action. Clin Exp Allergy., 1996, 26(5):549-56.
- Awan, Z.I., Rehman, H., Minhas, F.A., Awan, A.A., 2013. Antiplasmodial activity of compounds isolated from Viburnum nervosum. International Journal of Pharmaceutical Science Invention. 2(5):19-24.
- Azzawi, M, Bradley, B, Jeffeiy, PK, Frew, AJ, Wardlaw, AJ., Knowles, G, et al., 1990. Identification of activated T lymphocytes and eosinophil's in bronchial biopsies in stable atopic asthma. Am Rev Respir Dis. 142 (6): 1407-1413.
- Bartosch, H., Feldberg, W., Nagel, E., 1932. Das Freiwerden eines histaminahnlichen stoffes bei der anaphylaxie der Meerschwein chens. Pflungers Arch Ges Physiol. 230(1):129-153.
- Bazzoni, F., Beutler, B., 1996. The tumor necrosis factor ligand and receptor families. N. Engl. J. Med. 334:1717-1725.

- Bentley, A.M., Menz, G., Storz, C., 6. Robinson, D.S., Bradley, B., Jeffery, P.K., et al., 1992. Identification of T macrophages, lymphocytes, and activated eosinophil's in the bronchial in mucosa intrinsic asthma. symptoms relationship to and bronchial responsiveness. Am Rev Respir Dis. 146(2):500-506.
- Bousquet, J., Chanez, P., Lacoste, J.Y., Bameon, G., Ghavanian, N., Enander, I., et al., 1990. Easinophillic inflammation in asthma. N Engl J Med. 323(15):1033-1039.
- Bradding, P., Roberts, J.A., Britten, K.M., Montefort, S., Djukanovic, R., Mueller, R., et al., 1994. Interleukins-4, -5 and -6, and tumor necrosis factor-α in normal and asthmatic airways: Evidence for the human mast cell as a source of these cytokines Am J Respir Cell Mol Biol. 10(5):471-480.
- Chungm, K.F., Barnes, P.J., 1999. Cytokines in asthma. Thorax. 54(9):825-857.
- Conese, M., Copreni, E., Di Gioia, S., De Rinaldis, P., Fumarulo, R., 2003. Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. J Cyst Fibros. 2(3):129-35.
- Costa, J.J., Matossian, K., Resnick, M.B., Beil, W.J., Wong, D.T., Gordon, J.R., Dvorak, A.M., Weller, P.F., Galli, S.J., 1993. Human eosinophil's can

express the cytokines tumor necrosis factor-alpha and macrophage inflammatory protein-1 alpha. J. Clin. Invest. 91:2673–2684.

- Duan, W., Kua, I.C., Selvarajan, S., Chua, K.Y., Bay, B.H., Wong, W.S., 2003. Anti-inflammatory effects of genistein, a tyrosine kinase inhibitor, on guinea pig model of asthma. Am J Respir Crit Care Med. 167(2):185-192.
- Fan, M., Mustafa, S.J., 2006. Role of adenosine m airway inflammation in an allergic mouse model of asthma. Int Immunopharmacol. 6(1):36-45.
- 14. Ghosh, S., Bishayee, K., Khuda-Bukhsh, A.R., 2014. Oleanolic acid isolated from ethanolic extract of Phytolacca decandra induces apoptosis in A375 skin melanoma cells: drug-DNA interaction and signaling cascade. Journal of Integrative Medicine. 12(2):102-114.
- 15. Gordon, J.R., Galli, S.J., 1991. Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. J. Exp. Med. 174:103–107.
- Gosset, P., Tsicopoulos, A., Wallaert, B., Joseph, M., Capron, A., Tonnel, A.B., 1992. Tumor necrosis factor-α

and interleukin-6 production by human mononuclear phagocytes from allergic asthmatics after IgEdependent stimulation. Am Rev Respir Dis. 146(3):768-774.

- Gundel, R.H., Wegner, C.D., Letts, L.G., 1992. Antigen-induced acute and late-phase responses in primates. Am Rev Respir Dis. 146(2):369-373
- Hamelmann, E., Tadeda, K., Oshiba, A., Gelfand, E.W., 1999. Role of IgE in the development of allergic airway inflammation and airway hyperresponsiveness in a murine model. Allergy 54:297-305.
- Hogan, S.P., Mould, A., Kikutani, H., Ramsay, A.J., Foster, P.S., 1997. Aeroallergen-induced Eosinophilic Inflammation, Lung Damage, and Airways Hyperreactivity in Mice Can Occur Independently of IL-4 and Allergen-specific Immunoglobulins The American Society for Clinical Investigation. 99(6):1329–1339.
- 20. Holgate, S.T., Finnerty, J.P., 1989. Anti-histammines in asthma. J Allergy Clin Immunol. 83(2):537-547.
- Hughes, J.M., Stringer, R.S., Black, J.L., Armour, C.L., 1995. The effects of tumor necrosis factor-a on mediator release from human lung. Pulm Pharmacol. 8(1):31-36.
- 22. Kips, J.C., Tavernier, J., Pauwels, R.A., 1992. Tumor necrosis factor causes bronchial hyper-responsiveness

in rats. Am Rev Respir Dis. 145(21):332-336.

- Lee, J.Y., Moon, H., Kim, C.J., 2010. Effects of hydroxy pentacyclic triterpene acids from Forsythia viridissima on asthmatic responses to ovalbumin challenge in conscious guinea pigs. Biol. Pharm. Bull. 33(2):230-237.
- Lin, C.N., Chung, M.I., Gan, K.H., Chiang, J.R., 1987. Xanthones from formosan Gentianaceous plants. Phytochemistry. 26:2381-2384.
- 25. Liu, M.C., Hubbard, W.C., Proud, D., Stealey, B.A., Galli, S.J., Kagey-A., Sobotka. Bleeker, E.R., Lichtenstein, L.M., 1991. Immediate and late inflammatory responses to ragweed antigen challenge of the peripheral airways in allergic asthmatics: Cellular, mediator and permeability changes. Am. Rev. Respir. Dis. 144:51-58
- MacDermot, J., Kelsey, C.R., Waddell, K.A., Richmond, R., Knight, R.K., Cole, P.J., et al., 1984. Synthesis of leukotriene B4, and prostanoids by human alveolar macrophages, analysis by gas chromatography/mass spectrometry. Prostaglandins. 27(2):163-167
- Martin, J.G., Suzuki, M., Ramos-Barbon, D., Isogai, S., 2004. T cell cytokines: animal models. Paediatr Respir Rev. 5 (Suppl A):S47-S51.

- Meiler, F., Zimmermann, M., Blaser, K., Akdis, C.A., Akdis, M., 2006. Tcell subsets in the pathogenesis of human asthma. Curr. Allergy Asthma Rep. 6:91–96.
- Melewicz, F.M., Kline, L.E., Cohen, A.B., Spiegelberg, H.L., 1982. Characterization of Fc receptors for IgE on human alveolar macrophages. Clir. Exp Immunol. 49(2)364-370.
- Ogunbiyi, P.O., Eyre, P., 1985. Pharmacological studies of pulmonary anaphylaxis in-vitro. Agents Action. 17(2):158-173.
- Park, H.S., Kim, S.R., Kim J.O., Lee Y.C., 2010. The roles of phytochemicals in bronchial asthma. Molecules.15:6810-6834
- 32. Pretolani, M., Ruffle, C., Joseph, L.D., Lobb, R.R., Vargaftig, B.B., 1994. Antibody to very late activation antigen 4 prevents antigen-induced bronchial hyperreactivity and cellular infiltration in the guinea pig airways. J Exp Med .180(3):795-805.
- Raible, D.G., Lenahan, T., Fayvilevich, Y., Kosinski, R., Schulman, E.S., 1994. Pharmacologic characterization of a novel histamine receptor on human eosinophil's. Am J Respir Crrt Care Med. 49(6):1506-1511.

- 34. Robinson, D., Hamid, Q., Bentley, A., Ying, S., Kay, A.B., Durham, S.R., 1993. Activation of CD4+ T cells, increased Th2-type cytokine mRNA expression, and eosinophil recruitment in Broncho alveolar lavage after allergen inhalation challenge in patients with atopic asthma. J Allergy Clin Immunol. 92(2):313-324.
- Rogerio, A.P., Kanashiro, A.C., Fontanari, da Silva, E.V.G., Lucisano-Valim, Y.M., Soares, E.G., Faccioli L.H., 2007. Anti-inflammatory activity of quercetin and isoquercitrin in experimental murine allergic asthma. Inflamm. res. 56:402–408.
- 36. Sanjar, S., Aoki, S., Boubekeur, K., Chapman, I.D., Smith, D., Kings, M.A., Morley, J., 1990. Eosinophil accumulation in pulmonary airways of guinea-pigs induced by exposure to an aerosol of platelet activating factor: effect of anti-asthma drugs. Br. J. Pharmacol. 99:267-272.
- Sheth, U.K., Dadkar, N.K., Kamat, N.G., 1972. Selected topics in experimental pharmacology. vol-5, Kothari book depot, Bombay, pp. 63.
- Shore, P.A., Burkhalter, A., Cohn, V.H., 1959. A method for the fluorometric assay of histamine in tissues. J Pharmacol Exp Ther. 127:182-186.
- 39. Smith, N., Broadley, K.J., 2007. Optimisation of the sensitization conditions for an ovalbumin challenge

model of asthma. Int Immunopharmacol. 7(2):183-190.

- 40. Sur, S., Crotty, T.B., Kephart, G.M., 1993. Sudden onset fatal asthma: A distinct clinical entity with few eosinophil's and relatively more neutrophils in the airway submucosa? Am Rev Respir Dis. 148(3):713-719.
- Tanaka T., Takahashi R., 2013. Flavonoids and Asthma. Nutrients. 5:2128-2143.
- 42. Till, S., Li, B., Durham, S., Humbert, M., Assoufi, B., Huston, D., et al., 1995. Secretion of the eosinophilactive cytokines interleukm-5, granulocyte/macrophage colonystimulating factor and mterleukin-3 by Broncho alveolarlavage CD4+ and CDS+ T cell lines in atopic asthmatics, and atopic and non-atopic controls. Eur J Immunol. 25(10):2727-2731.
- Tundis, R., Deguin, B., Menichini, F., Tillequin, F., 2002. Iridoids from Putoria calabrica. Biochem. Syst. Ecol. 30: 689-691.
- Van der Velden, V.H., 1998. Glucocorticoids: mechanism of action and anti-inflammatory potential in asthma. Mediators Inflamm.7:229–37.
- Wagner, H., Baldt, S., Zgainski, E.M., 1996. Plant drug analysis. Springer: Berlin. pp. 12-15.

- Wardlaw, A.J., Dunnette, S., Gleich, G.J., Collins, J.V., Kay, A.B., 1988. Eosinophils and mast cells in Broncho alveolar lavage in subjects with mild asthma: Relationship to bronchial hyperreactivity. Am Rev Respir Dis. 137(1):62-69.
- Williams, C.M., Galli, S.J., 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. J. Exp. Med. 192:455–462.
- Williams, J., Johnson, S., Mascali, J.J., Smith, H., Rosenwasser, L.J., Borish, L., 1992. Regulation of low affinity IgE receptor (CD23) expression on mononuclear phagocytes in normal and asthmatic subjects. J Immunol. 149(8):2823-2829.
- 49. Ying, S., Humbert, M., Barkans, J., Corrigan, C.J., Pfister, R., Menz, G., 1997. Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T-cells, eosinophil's, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. J Immunol. 158(7):3539-3544.
- Zhang, D.H., Yang, L., Cohn, L., Parkyn, L., Homer, R., Ray, P., Ray, A., 1999. Inhibition of allergic inflammation in a murine model of asthma by expression of a dominantnegative mutant of GATA-3. Immunity 11:473–482.