Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(10):907-914

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Validation of ameliorative efficacy of *Azima tetracantha* leaf extracts on asthma through mast cell stabilization assay

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ABSTRACT

Life style modification and increase in pollution have contributed to airway inflammation in asthma. Mast cells by degranulation releases histamine and play a key role in the pathogenesis of type I allergy and asthma. Leaves of Azima tetracantha is used in traditional medicine for the treatment of cold and cough. Study aim was to scientifically validate the potential of Azima tetracantha leaf extracts to inhibit peritoneal mast cell degranulation induced by compound 48/80. The bioactive constituents in the petroleum ether, ethyl acetate and methanol leaf extracts of Azima tetracantha were authenticated by HPTLC finger printing. The extracts with phenols, flavonoids and tannins as its phytoconstituents significantly inhibited mast cell degranulation in a dose dependent manner. The petroleum ether extract at 250mg/Kg, 500 mg/Kg and 1000mg/Kg showed 23±1.62%, 43±3.09% and 49±2.54% o f intact mast cells while ethyl acetate extract showed 32±2.04%, 49±2.89% and 66±3.47% respectively. Met hanol extract comparatively produced a better mast cell protecting effect by showing 40±2.76%, 52±2.65%and 7 1±3.76% at the dose range of 250mg/Kg, 500 mg/Kg and 1000mg/Kg. The mast cell stabilizing effect shown by the extracts of Azima tetracantha confirms its candidature as anti-allergic plant in treatment of asthma.

Key words: *Azima tetracantha*, HPTLC, mast cell degranulation assay, asthma, allergy

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INTRODUCTION

Among the chronic allergic disorders of the airways, asthma is highly prevalent. Globally 400 million people will be crippled by this respiratory disorder by 2025 [1] and in India around 15 million people beyond the age of 15 are suffering from asthma as reported by Indian Council of Medical research [2]. Effector cells and cellular elements of the immune system play an important role in the pathogenesis. Mast cells are the prominent central effector cells in the airways, gastrointestinal tract and skin contributing for the induction of inflammation in Ig-E mediated allergic conditions [3]. Mast cells posses the high affinity receptor (FcεRI) for Ig E. Cross linking of IgE - FcεRI by multivalent antigen primes the exocytosis of mast cells releasing the preformed proinflammatory mediators such as histamine, tumor necrosis factor, serotonin, kinins and proteases from the secretory granules. Chemokines, cytokines and lipid mediators are produced as late phase response reaction. Liberation of these inflammatory mediators in the air passages of the lungs causes airway obstruction, inflammation and bronchial hyper responsiveness leading to of wheezing, breathlessness, chest tightness and coughing.

Among the various anti-asthmatic drugs like corticosteroids, α2 agonists, and leucotriene antagonists, mast cell stabilizers like sodium chromoglycate and kitotifen are widely used in the treatment of asthma. Long term usage of these synthetic drugs are not advisable due to side effects like disturbance of the adrenal system, depression of central nervous system, cardiac abnormalities, muscle tremor etc., [4]. Plant derived products were found to inhibit

the degranulation of mast cells in an effective and safe manner. Phytochemicals as mast cell stabilizers further inhibit the allergic complications due to the release of inflammatory mediators. Thus an extract from a plant source which could prevent the degranulation of mast cells can serve as an effective candidate in the treatment of asthma.

Azima tetracantha, a rambling shrub (*Salvadoraceae* family) is found in Tropical Africa to India, Sri Lanka, Madagascar, Comoro Islands and Philippinesis is largely known for its medicinal uses in the Indian System of Medicine. The leaves are used to treat several inflammatory conditions including cough and asthma [5] and Kirtikar *et al*. [6]. Although many biological effects of *Azima tetracantha* have been reported, no study was conducted to reveal its response to mast cell granulation effect. In this study, we investigated the membrane stabilization effect of successive petroleum ether, ethyl acetate and methanol leaf extracts of *Azima tetracantha* on mast cell using compound 48/80 which induces degranulation.

EXPERIMENTAL SECTION

Chemicals

Compound 48/80 (condensation product of N-methyl-P-methoxy Phenethylamine with formaldehyde) was purchased from Sigma – Aldrich Co. (St. Louis, MO, USA). All other chemicals, solvents and reagents used were of analytical grade and purchased through the authorized dealers.

Preparation of *Azima tetracantha* extracts

Leaves of *Azima tetracantha* Lam. were collected from Foothills of Sirumalai, Dindugal and it was identified and authenticated (Voucher Specimen no.34) by Dr. T. Sekar, Associate Professor, Post Graduate and Research Department of Botany, Pachaiyappa's College, Chennai. The leaves free of adulterants were air dried and coarsely powdered. The powder was extracted in soxlet extractor successively with petroleum ether, ethyl acetate and methanol. The dried extracts were dissolved in the appropriate solvents for further analysis.

High-performance thin layer chromatography (HPTLC) finger printing and

HPTLC finger printing studies were carried out according to the method described by Wagner *et al*. [7]. The apparatus set up has a Camag HPTLC SYSTEM comprising a CAMAG Automatic TLC sampler 4, a Camag TLC Scanner 3, a Camag twin-trough chamber (20X10 cm) and a Syringe (25 µl). Chromatographic estimations were performed under the following conditions: stationary phase – precoated silica gel 60 F254 aluminium sheets (20 X 10 cm); suitable mobile phase – (n- hexane : ethyl acetate : formic acid : acetic acid (60:40:2.5:2.5) solvent mixture) ; chamber saturation time – 45 min; temperature – 25 ± 2º C ; migration distance – 50 mm. The following application parameters were used: spray gas used was Nitrogen and sample solvent type was ethanol. The detection wavelength was 408 nm and slit dimensions were 4.00 x 0.30 mm. The following spotting parameters were used: band width 15 mm and the space between two bands 11 mm.

Sample analysis

An aliquot of extract were spotted on precoated TLC plates, using a automatic spotter under a nitrogen stream. The plate was developed for up to 50 mm at constant temperature using a mixture of n- hexane : ethyl acetate : formic acid : acetic acid (60:40:2.5:2.5) as the mobile phase in a Camag twin- trough chamber previously saturated with mobile phase. The plate was removed from the chamber and dried in oven at 60º C for 5 min. Photometric measurements were performed at 408 nm with Camag TLC scanner 3 using CATS 4 software incorporating the track optimization option.

Preliminary phytochemical investigation

The phytochemical results confirms the presence of alkaloids, phenolic compounds, reducing sugars, flavonoids, tannins and glycosides in the successive petroleum ether, ethyl acetate and methanol extracts in variable quantities [8].

Animals

Studies were carried out using male wistar rats 200– 250 g obtained from the Centre for Toxicology and Developmental Research, Sri Ramachandra University, Chennai, India. They were housed in standard cages, allowed free access to standard dry pellet diet (Hindustan Lever Ltd., Bangalore, India) and water ad libitum. The rats were acclimatized to laboratory condition for 10 days before commencement of experiment. This study was

conducted according to the guidelines approved by Institutional Animal Ethics Committee (IAEC-XVI/SRU/116/2009) of Sri Ramachandra University.

Mast cell stabilizing activity, *in vitro*

Mast cell degranulation assay utilizes Compound 48/80 a potent mast cell degranulating / depleting agent as decribed by Norton [9]. Four overnight-fasted male wistar rats (200-250 g) were sacrificed with an overdose of anesthetic ether. The abdomen was cut open to expose the intestine. Pieces of mesentery with connecting lobes of fat and blood vessels were rapidly dissected out and small pieces of the mesentery were cut and placed in beakers for 30 ± 1 min containing ringer locke solution (NaCl - 154 mM, KCl - 5.6 mM, Calcium chloride ($CaCl_2$) - 2.2 mM, Sodium bicarbonate ($NaHCO_3$) - 6mM and dextrose - 5.5 mM in 500 ml of distilled water). Saline, Petroleum ether, ethyl acetate and methanol extracts (250, 500, 1000 µg/ml) of *Azima tetracantha* were added to petridishes containing few ml of ringer locke solution and few pieces of mesentry tissue and incubated for 20 min at room temperature. The tissues were exposed to Compound 48/80 (0.8 µg/ml in Ringer locke solution) to promote mast cell degranulation and the tissues were incubated further for 20 min. The pieces of mesentery were then removed and stained with O-Toluidine blue for about 2.5 min and the tissue was then washed in acetone and then xylene (2 changes each) and placed on clean slides. Excess fatty layers and adhering small intestine tissues were carefully removed. The stained mesentery pieces were viewed through a digital light microscope (M/S. Motic, Korea) at 10x magnification and 100 mast cells were counted. The number of intact and fragmented or disrupted mast cells was noted. The results were expressed as percentages of fragmented or disrupted mast cells and the intact mast cells.

Statistical analysis

All data are reported as mean ± standard error of mean. Statistical analysis was done using SPSS 12.0 for windows package. The statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

RESULTS AND DISCUSSION

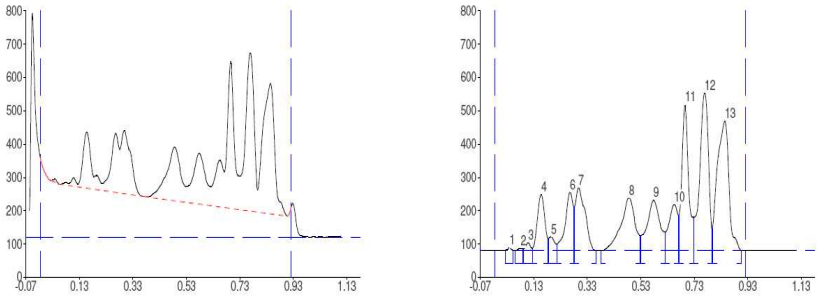
Asthma, a disease to breathe with an open mouth was due to the obstruction in outflow of the air from the lungs caused by inflammation. Mast cells play a predominant role in allergic reactions. On stimulation they release potent inflammatory mediators such as histamine, proteases, chemotactic factors, cytokines, metabolites of arachidonic acid like leukotriene C4 (LTC4) and prostaglandin D2 (PGD2), tumour necrosis factor , IL-4, IL-5 and IL-6 [10]. These substances causes constriction of bronchi, swelling of mucous membrane, increased mucous secretions resulting in difficulty in breathing with wheezing sound [11]. Plants products which could manipulate the activities of mast cells can find a better place as antiasthmatic drugs. In order to validate the efficacy of *Azima tetracantha* for its use in cold and cough, the effect of *Azima tetracantha* leaf extracts on the mast cell degranulation was investigated.

High-performance thin layer chromatography (HPTLC) finger printing

Quality, consistency and stability of herbal extracts or products can be assessed by Chromatographic finger-printing of phytoconstituents. *Portulaca oleracea* [12], *Pandanus odoratissimus* [13] and *Albizia amara* [14] are some herbs which are authenticated by HPTLC finger printing techniques. In the present investigation, the HPTLC chromatographic pattern of the petroleum ether extract of *Azima tetracantha* showed 13 peaks at Rf values 0.05, 0.09, 0.13, 0.18, 0.22, 0.28, 0.36, 0.53, 0.62, 0.67, 0.73, 0.80, 0.91 at 254 nm Figure 1.

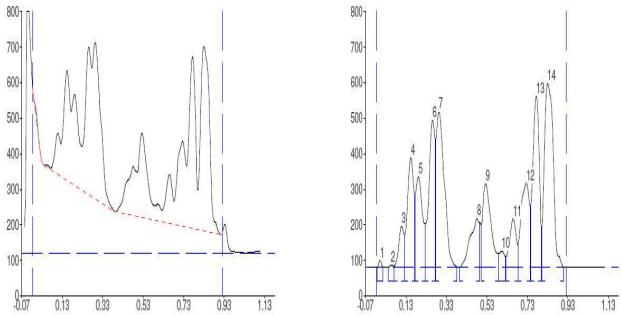
HPTLC finger printing of the ethyl acetate extract of *Azima tetracantha* with 14 peaks at Rf value 0.02, 0.07, 0.12, 0.17, 0.23, 0.28, 0.38, 0.49, 0.59, 0.63, 0.68, 0.75, 0.80, 0.91 at 254 nm was shown in the Figure 2. The methanol leaf extract of *Azima tetracantha* showed 14 peaks (Figure 3) with Rf value 0.14, 0.20, 0.23, 0.26, 0.29, 0.36, 0.45, 0.49, 0.53, 0.60, 0.70, 0.77, 0.83, 0.90 at 254 nm.

The image documentation of all the successive extracts (petroleum ether, ethyl acetate and methanol) of *Azima tetracantha* at four different volumes of the sample (2.5µl, 5 µl, 10 µl and 20 µl) at 366 nm was shown in Figure 4. The HPTLC chromatogram of *Azima tetracantha* leaf extracts confirms the presence of nearly 14 phytochemicals. In the present scenario, HPTLC finger printing is globally accepted and considered as very important, essential and viable tool for qualitative and quantitative analysis of herbal products.



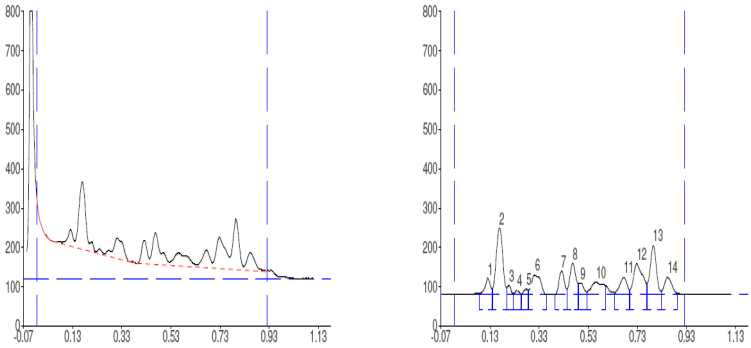
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.03	0.3	0.04	10.4	0.44	0.05	0.1	111.4	0.16
2	0.06	0.3	0.08	9.0	0.38	0.09	6.5	120.3	0.17
3	0.09	6.5	0.11	25.0	1.05	0.13	7.9	388.6	0.56
4	0.13	8.6	0.16	169.0	7.10	0.18	37.3	3873.8	5.62
5	0.19	37.4	0.20	42.7	1.80	0.22	20.0	793.1	1.15
6	0.22	20.3	0.27	176.6	7.42	0.28	130.8	4355.6	6.32
7	0.28	131.4	0.30	189.5	7.96	0.36	0.6	5825.3	8.45
8	0.38	0.3	0.49	160.5	6.74	0.53	47.2	7072.2	10.26
9	0.53	47.2	0.58	152.2	6.39	0.62	55.8	6098.5	8.84
10	0.63	56.3	0.66	139.6	5.87	0.67	106.4	3796.6	5.51
11	0.68	107.8	0.70	440.2	18.49	0.73	100.5	9706.0	14.08
12	0.73	100.8	0.77	474.9	19.95	0.80	66.7	12836.4	18.62
13	0.80	70.1	0.85	390.5	16.41	0.91	0.2	13975.8	20.27

Figure 1 HPTLC finger printing of petroleum ether leaf extract of *Azima tetracantha* at 254 nm



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.01	0.5	0.00	21.0	0.62	0.02	0.5	225.2	0.22
2	0.04	0.1	0.06	8.2	0.24	0.07	5.0	109.1	0.11
3	0.07	5.3	0.11	116.4	3.46	0.12	91.9	2529.7	2.49
4	0.13	92.0	0.16	309.5	9.19	0.17	203.2	8128.0	7.99
5	0.18	204.2	0.19	256.1	7.60	0.23	123.4	7267.1	7.14
6	0.23	123.4	0.26	415.3	12.33	0.28	361.9	10325.2	10.15
7	0.28	361.9	0.29	439.3	13.05	0.38	2.0	14933.5	14.67
8	0.40	0.2	0.48	138.4	4.11	0.49	127.0	5086.7	5.00
9	0.50	122.8	0.53	236.4	7.02	0.59	39.7	8404.4	8.26
10	0.59	39.8	0.61	48.0	1.42	0.63	32.3	1083.5	1.06
11	0.63	32.9	0.66	138.0	4.10	0.68	65.7	3797.6	3.73
12	0.69	67.1	0.73	238.8	7.09	0.75	172.3	8220.9	8.08
13	0.75	174.0	0.78	482.7	14.33	0.80	111.2	12859.2	12.64
14	0.80	112.1	0.83	519.3	15.42	0.91	0.4	18794.9	18.47

Figure 2 HPTLC finger printing of ethyl acetate leaf extract of *Azima tetracantha* at 254 nm



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.09	3.1	0.12	42.7	5.18	0.14	15.6	820.6	4.29
2	0.14	16.4	0.17	171.2	20.78	0.20	19.1	3742.5	19.54
3	0.20	19.5	0.21	24.1	2.93	0.23	3.4	309.6	1.62
4	0.23	3.5	0.24	12.8	1.55	0.26	2.3	170.6	0.89
5	0.26	2.6	0.28	15.5	1.88	0.29	13.2	214.8	1.12
6	0.29	13.4	0.31	52.2	6.33	0.36	0.2	1634.1	8.53
7	0.40	0.3	0.42	60.7	7.36	0.45	15.2	1145.4	5.98
8	0.45	15.9	0.47	81.4	9.88	0.49	28.3	1630.0	8.51
9	0.49	28.4	0.50	31.1	3.77	0.53	8.4	569.8	2.98
10	0.53	8.5	0.56	33.9	4.12	0.60	22.8	1352.8	7.06
11	0.64	5.8	0.68	45.2	5.49	0.70	12.6	1141.3	5.96
12	0.70	13.1	0.73	80.0	9.71	0.77	29.8	2484.0	12.97
13	0.77	30.1	0.80	127.8	15.51	0.83	9.3	2770.9	14.47
14	0.83	9.7	0.86	45.5	5.52	0.90	3.8	1163.1	6.07

Figure 3 HPTLC finger printing of methanol leaf extract of *Azima tetracantha* at 254 nm

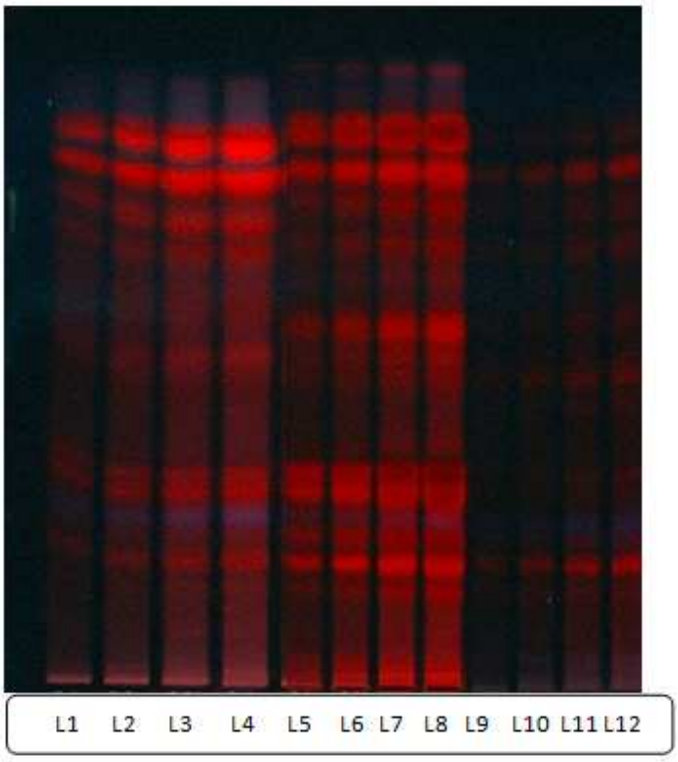


Figure 4. Image documentation of HPTLC chromatogram of *Azima tetracantha* leaf extracts at 366 nm. (L1-L4: 2.5µl, 5 µl, 10 µl and 20 µl of pet.ether extract; L5-L8: 2.5µl, 5 µl, 10 µl and 20 µl of ethyl acetate extract; L9-L12: 2.5µl, 5 µl, 10 µl and 20 µl of methanol extract)

***In vitro* mast cell stabilization assay**

The petroleum ether, ethyl acetate and methanol leaf extracts of *Azima tetracantha* stabilizes the mast cell degranulation induced by compound 48/80 in the rat peritoneal mast cells in a dose dependent manner as shown in the Table 1. Compound 48/80, a mast cell secretagogue brings out maximum degranulation of mast cells when compared to saline. When compared with all the extracts, treatment with methanolic extract of *Azima tetracantha* showed a significant maximum dose dependent beneficial effect on degranulation of peritoneal mast cells in rats when challenged with compound 48/80 by showing 71 intact cells at the dose of 1000 µg/ml. Both petroleum ether (49%) and ethyl acetate (66%) extracts also exhibited a significant dose dependent stabilizing effect when compared with compound 48/80 group (Figure 5(a-e)).

The present study shows the *in vitro* mast cell stabilizing effect of *Azima tetracantha* extracts (petroleum ether, ethyl acetate and methanol) under the influence of compound 48/80. Compound 48/80 increased the membrane permeability of the mast cell by acting as calcium ionophore causing a perturbation of the membrane [15] and induces signal transduction pathways, leading to exocytosis and degranulation of mast cells [16]. Results of the present investigation showed that all the extracts of *Azima tetracantha* stabilized the mast cell while the methanolic extract possessed significant activity in preventing compound 48/80-induced mast cell degranulation.

Table 1 Stabilizing effect of various extracts of the leaves of *Azima tetracantha* in compound 48/80 induced mast cell degranulation in rat peritoneal mast cells

Group	Number of Intact Cells (%)		
	250 (µg/ml)	500 (µg/ml)	1000 (µg/ml)
Saline (Control)	72 ± 3.52		
Compound 48/80	23 ± 1.75*a		
Petroleum ether extract	23 ± 1.62*a	43 ± 3.09*(a,b)	49 ± 2.54*(a,b)
Ethyl acetate extract	32 ± 2.04*a	49 ± 2.89*(a,b)	66 ± 3.47*b
Methanol extract	40 ± 2.76*(a,b)	52 ± 2.65*(a,b)	71 ± 3.76*b

Values are expressed as mean ±S.E.M (n=6).

*Statistical significant test for comparison was done by one way Analysis of Variance (ANOVA) followed by Tukey multiple range test. Statistically significant variations are expressed as * p<0.001.[a] – Group I compared with Group II, Group III, Group IV and Group V ;[b] – Group II compared with Group III, Group IV and Group V*

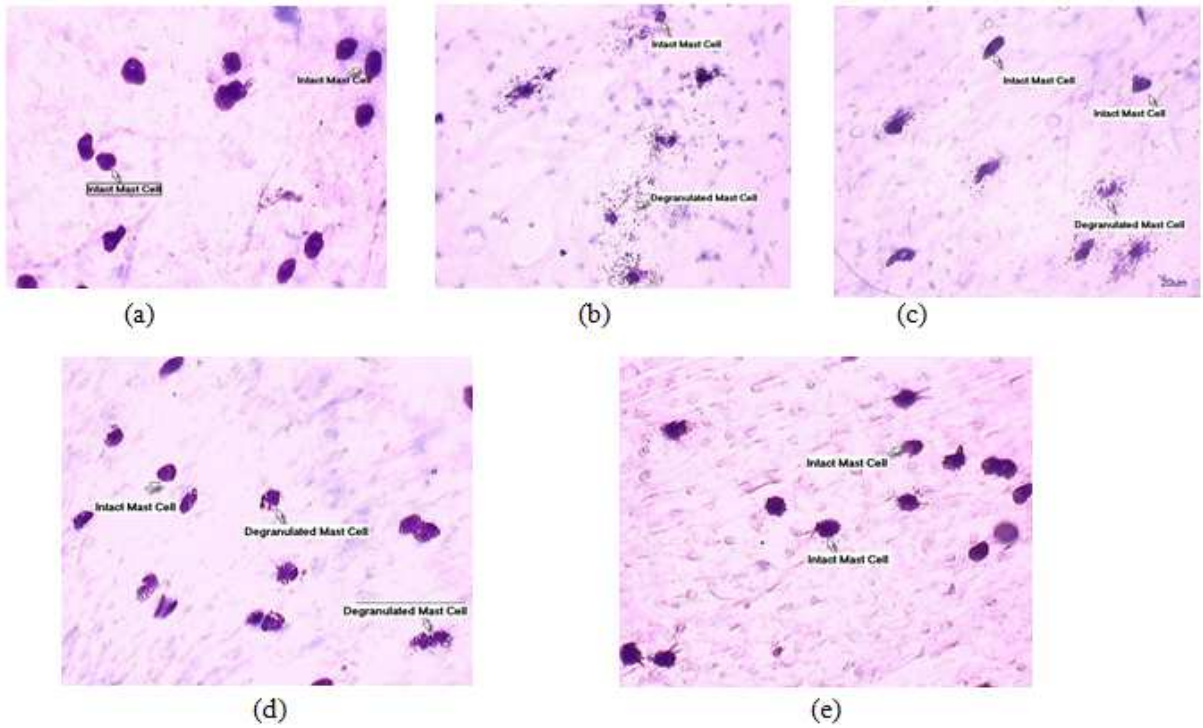


Figure 5. (a-e) Rat peritoneal mast cells treated with (a) saline (b) compound 48/80 (c) petroleum ether (d) ethyl acetate (e) methanol leaf extract of *Azima tetracantha* (1000µg/ml) stained with O-Toluidine blue (40X)

The phytochemical analysis showed that the methanolic extract of *Azima tetracantha* possessed considerable quantities of phenolic compounds and flavonoids [8] which might act on the lipid bilayer membrane preventing the perturbation induced by compound 48/80, stabilizing the mast cell and thus influencing the course of the inflammatory disease and preventing the harmful effects of the released mediators. Similar results on mast cells were observed by ethanolic leaf extract of *Lecythis pisonis* Camb [17] and methanol extracts of *Matricaria recutita* L. showed significant dose dependent activity [18]. The phytoconstituents like saponins, glycosides and flavonoids confers mast cell stabilizing and antiallergic properties [19]. Flavonoids are reported to possess antihistaminic, antiallergic and mast cell stabilizing properties [20; 21]. Phenolic compounds also contribute to the prevention of mast cell degranulation as evident with *Onosma* extract [22]. The methanol extract of *Olax subscorpioidea* leaves possessed rich amount of phytoconstituents [23].

The mast cell membrane stabilizing effect of *Azima tetracantha* leaf extracts might be attributed to the reduction in the Ca2+ influx in to the mast cells [24], inhibition of mast cell tryptase activity [25] that induces degranulation. Thus by inhibiting the histamine release due to degranulation the extracts halts mast cell-dependent immediate allergic reactions. These results suggest a potential therapeutic role of *Azima tetracantha* leaf extracts for the treatment of asthma in which activated mast cells play a crucial role.

CONCLUSION

Medicinal plants are effective against life style inflammatory disease like asthma. Our data corroborate the folkloric use of *Azima tetracantha* leaves for respiratory diseases. The results suggest that leaves of *Azima tetracantha* stabilize the mast cell membrane and hence could reduce Type I hypersensitivity mediated diseases like asthma and rhinitis. Further studies on animal models are warranted to explore the activity of active constituents.

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