Effect of Morinda umbellata Linn. (Rubiaceae) Leaf Extracts on Antigen Induced Mast Cell Degranulation and Active Paw Anaphylaxis in Wistar rats

N M Krishnakumar*, P G Latha, S R Suja
Division of Ethnomedicine and Ethnopharmacology, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram-695 562, Kerala, India

Received on: 14-01-2017; Revised on: 26-02-2017; Accepted on: 25-03-2017

ABSTRACT

Aim: Morinda umbellata Linn. is a climbing shrub distributed in Khasi Hills, Deccan Peninsula, Eastern Ghats and Western Ghats regions of India up to an altitude of 1500 m. It is an important traditional medicinal plant having curative properties against stomach disorders. The aim of the present study is to scientifically evaluate the mast cell stabilizing and anti-anaphylactic effects of M. umbellata leaves.

Methods: The different solvent extracts of M. umbellata leaves were used to evaluate antigen induced mast cell degranulation and active paw anaphylaxis in Wistar albino rats. Results and Discussion: The leaf ethanolic extract (200 mg/kg and 400 mg/kg), n-hexane extract (200 mg/kg and 400 mg/kg) and chloroform extract at 400 mg/kg doses exhibited significant protection against egg albumin induced peritoneal and mesenteric mast cell degranulation. In mesenteric mast cell study in vitro, the ethanolic and n-hexane leaf extracts (2 mg/mL) showed significant activity against egg albumin induced mast cell degranulation. In active paw anaphylaxis, the animals treated with the ethanolic and n-hexane extracts (100, 200 and 400 mg/kg) exhibited significant reduction in ovalbumin induced paw edema. The preliminary phytochemical analysis revealed the presence of flavonoids, phenolic compounds, alkaloids, steroids, tannins and carbohydrates in the leaf extracts. Conclusion: From the present study, it can be concluded that the phenolic compounds and flavonoids present in M. umbellata leaves may contribute to the inhibitory effect on antigen induced mast cell degranulation and active paw anaphylaxis in Wistar rats.

KEY WORDS: Mast cell degranulation, Active paw anaphylaxis, antigen, Morinda umbellata

1. INTRODUCTION:

Medicinal plants are the invaluable, incredible and traditional source of drugs that are being used from ancient times as herbal remedies for the healthcare, prevention and cure of various diseases. About 15000 medicinal plants are known for medicinal purpose in India and this is the highest proportion of plants known for medical purpose in any country of the world[1]. There has been a mounting interest all over the world as to the use of herbal remedies for the cure of different diseases. This stems from the fact that herbal medicines are cheaper, easily accessible and are devoid of side effects. According to World Health Organization (WHO), about three-quarters of the population in the developing countries depend on plant based products for meeting their primary health care needs[2].

Morinda umbellata Linn. (Family: Rubiaceae) is a climbing shrub and it is native plant of south-east Asia and north Australia. It is distributed in Khasi Hills, Deccan Peninsula, Eastern Ghats and Western Ghats regions of India up to an altitude of 1500 m. The leaves are rather small, elliptic, usually oblong-lanceolate but sometimes broadly oval, tapering or acute at base, shortly acuminate, acute, glabrous, thin, dark green above and green below. The flowers are white coloured, few together and flower heads in terminal sessile umbels. The fruits are small, lobed, smooth, scarlet and fused into a syncarpium[3]. The plant is locally known as “Neyvalli” or “Kudalchurukki” in Kerala, and the tribal communities of Western Ghats region of Kerala use the plant for various ailments such as diabetes, high blood pressure, muscle aches and pain. According to Indian traditional systems of medicine, a decoction of roots and leaves is considered useful in treating pains of intestine, diarrhoea, dysentery, indigestion, syphilis and gonorrhea[4]. The roots of the plant are used in Vietnamese folk medicine against furuncle, dysentery and skin diseases. The aerial parts are used against fever, cough, stomach ache, rheumatism and acute hepatitis[5].

*Corresponding author.
N M Krishnakumar
Senior Research Fellow
Division of Ethnomedicine and Ethnopharmacology
Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram-695 562, Kerala, India
According to the earlier pharmacological studies, the alcoholic extract of *M. umbellata* excluding the roots showed diuretic and anti-inflammatory effects\(^9\). The leaf powder was found to possess excellent antioxidant and antileukaemic effects and it exhibited potent cytotoxic activity against human oral epidermoid carcinoma KB cell line\(^7\). The cytotoxic effects of two 11-noriridoids isolated from the aerial parts of the plant was evaluated against human KB epidermoid carcinoma, LU-1 lung adenocarcinoma and MCF-7 breast adenocarcinoma cell lines and protective effect on oxidative stress injury induced by \(\text{H}_2\text{O}_2\) in murine hepatocytes\(^8\). The anthraquinones isolated from the ethyl acetate extract displayed significant cytotoxic effect against A549, HepG2, HT29 and PC3 tumour cell lines\(^9\). The antibacterial effects of the leaves were evaluated by resazurin redox method\(^10\). A comparative study on *in vitro* free radical scavenging effect of different solvent extracts of *M. umbellata* leaves, and the estimation of its phytochemical constituents such as total phenolic, flavonoid and condensed tannin content were also carried out\(^11\). Mast cells are considered as one of the integral parts of the immune system and they are the central mediators of allergic and anaphylactic responses\(^12\). Mast cells have been used for testing agents against allergic disorders because it can contribute to a broad spectrum of immunologic and pathologic reactions\(^13\). The prevalence of allergic disorders and asthma is rising throughout the world with more than 300 million people suffering from these types of diseases, especially in the industrialized countries\(^14\). Anti-allergic drugs such as antihistamines and anticholinergics besides topical corticosteroids for specific allergens are available for the management of allergic disorders. But they exhibited adverse side effects like drowsiness, sedation, lethargy, angioedema, immunosuppression etc. in patients and provide only symptomatic relief\(^15\).

Several investigators have selected antigen induced mast cell degranulation study to assess the anti-allergic effects of medicinal plants\(^16\). A perusal of literature revealed that *M. umbellata* has not been scientifically evaluated for its anti-allergic and anti-anaphylactic effects and the present study was undertaken to scientifically validate these properties.

### 2. MATERIALS AND METHODS

#### 2.1. Chemicals

RPMI-1640 and dimethyl sulphoxide (DMSO) were purchased from Hi-Media, Mumbai, India. Disodium chromoglycate (DSCG) and egg albumin were purchased from Sigma Aldrich, USA. All the other chemicals used for the experiments were of analytical reagent grade.

#### 2.2. Plant material

The whole plants excluding the roots of *M. umbellata* were collected in September 2013 from Karunagappally, Kollam District, Kerala. They were authenticated by the plant taxonomist of the Institute and a voucher specimen (TBGT 57055 dated 17/10/2013) was deposited at the Institute’s Herbarium. The fresh leaves were washed thoroughly, shade-dried and powdered. The powdered plant material was stored at room temperature in airtight containers under dark conditions.

#### 2.3. Preparation of plant extracts

The dried leaf powder (50 g) was extracted with 500 mL of 95% distilled ethanol for 24 h at room temperature with constant stirring. The extract was filtered and the filtrate concentrated under reduced pressure using rotary evaporator (Buchi, Switzerland) to yield 2.62 g (5.24%) of the ethanolic leaf extract (MU). The dried leaf powder was successively extracted with n-hexane and chloroform using a Soxhlet apparatus, filtered and the dried powder was refluxed with 10% aqueous ethanol and again filtered. All the filtrates were finally evaporated to dryness under reduced pressure using rotary evaporator to get n-hexane extract (MUH), chloroform extract (MUCH) and 10% aqueous ethanol extract (MUAE) respectively. The water extract (MUW) was prepared by the lyophilization method.

#### 2.4. Animals

Wistar albino male rats weighing 200-250 g were obtained from the Institute’s Animal House. They were housed in polycrystalline cages under standard conditions (temperature 24°-28°C, relative humidity 60-70% and 12 h dark-light cycles), fed commercial rat feed (Lipton India Ltd., Mumbai, India) and boiled water *ad libitum*. Animals were acclimatized for one week before starting the experiments. All experiments involving animals were carried out according to guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, after getting the approval of the Institute’s Animal Ethics Committee.

#### 2.5. Preliminary phytochemical screening

The preliminary phytochemical analysis of the extracts was carried out according to the standard methods\(^17\).

#### 2.6. Peritoneal mast cell degranulation study (in vivo)

Wistar male rats of body weight 200-250 g were treated with different solvent extracts of *M. umbellata* leaves (MU, MUH, MUCH, MUAE and MUW) at 100, 200 and 400 mg/kg doses and disodium chromoglycate (DSCG) at 10 mg/kg (i.p) as the standard drug for 4 days prior to the collection of mast cells. One group received 0.5% Tween-80 in an identical manner and served as control group. After 4 days of treatment, 10 mL of normal saline was injected into the
peritoneal cavity and after a gentle massage, the peritoneal fluid was collected and transferred to siliconized test tubes containing 7-10 mL of RPMI-1640 (pH 7.2-7.4). The collected mast cells were washed three times by centrifugation at low speed (400-500 rpm) discarding the supernatant and taking the pellets of mast cells. The mast cells from normal or sensitized groups of control and treated rats were incubated with egg albumin (1.0 mg/mL) respectively at 37°C in a water bath for 10 minutes. After incubation, the mast cells were stained with 0.1% toluidine blue and percentage protection against degranulation was counted under compound microscope\(^\text{19}\).

2. 7. Mesenteric mast cell degranulation study (in vivo)

Wistar rats, males weighing 200-250 g were sensitized with egg albumin (1 mg/rat) intramuscularly. After 10 days, another dose of egg albumin was given as above. The rats were then divided into different groups with 6 rats in each group. The extracts in 0.5% Tween-80 were administered orally for 3 consecutive days, following the second dose of antigen sensitization. The extracts MU, MUH, MUCH, MUAE and MUW were administered at 100, 200 and 400 mg/kg doses to the treatment groups. One group received 0.5% Tween-80 in an identical manner and served as control group. The standard drug treated group received DSCG (10 mg/kg, i. p.). 24 h after the last dose of the treatment, rats were sacrificed by carbon dioxide inhalation and the mesentery was collected. The intestinal mesentery of overnight fasted rats was dissected out and separated along with gut and washed in normal saline and suspended in RPMI-1640 medium. The mesentery was then cut into pieces of 1 cm\(^2\) each and used for the study. Two pieces from each animal were incubated in 5 mL RPMI-1640 medium with or without egg albumin (0.1 mg/mL) for 15 minutes at 37°C. The mesentery was removed and spread on a clean glass slide, dried in air and separated carefully from the gut using a sharp blade. The dried slide was stained with toluidine blue and counterstained with light green stain and observed under the microscope\(^\text{19}\).

2. 8. Mesenteric mast cell degranulation study (in vitro)

The in vitro effects of the leaf extracts on antigen induced mast cell degranulation were carried out in egg albumin induced mast cell degranulation study. Five groups of animals were sensitized with egg albumin. The mesenteric pieces (six pieces from each rat) were pooled in 50 mL RPMI-1640 medium. They were randomly divided in to six test tubes each containing six pieces in 10 mL medium. The extracts MU, MUH, MUCH, MUAE and MUW were dissolved in 50% DMSO and added in tubes at three different concentrations (0.5, 1 and 2 mg/mL). In another tube, only DMSO was added and served as DMSO control. The tubes were pre-incubated for 10 minutes at 37°C and egg albumin (0.1 mg/mL) was added in all test tubes except the control group. Then the mesenteric pieces were fixed on clean glass slides, stained and observed under the microscope for mast cells. The granulated and degranulated mast cells were counted and the percentage degranulation was calculated\(^\text{20}\).

2. 9. Active paw anaphylaxis

Wistar rats, males weighing 200-250 g were sensitized by injecting 0.25 µg/animal ovalbumin adsorbed on 6 mg aluminium hydroxide gel on the back of the animal subcutaneously on day 0. The leaf extracts MU, MUH, MUCH, MUAE and MUW were administered at 100, 200 and 400 mg/kg doses to the treatment groups administered from day 1 to day 11. On 11th day, the animals were challenged with 10 µg (0.05 mL of 200 µg/mL) ovalbumin in normal saline s. c. in the plantar region of hind paw. The contra-lateral paw received an equal volume of saline. The paw thickness was measured using a plethysmometer at 24 h after the challenge. The difference in paw volume reflects the edema due to antigen-antibody interaction. Disodium chromoglycate (DSCG) (5 mg/kg) was used as the reference standard for comparison\(^\text{21}\).

2.10. Statistical analysis

All the analyses were carried out in triplicate. The statistical significance between the samples were determined by the analysis of variance (ANOVA) and the data were recorded as mean ± standard deviation (SD), \(P\leq0.05\) was considered to be statistically significant. Significant differences between means were determined by Dunnett’s multiple comparison test\(^\text{22}\). The computer software employed for the statistical analysis was IBM SPSS Statistics, version 20 (USA).

3. RESULTS

The preliminary phytochemical screening of the extracts revealed the presence of carbohydrates, flavonoids, phenolic compounds, alkaloids, tannins and steroids. The antigen egg albumin (1 mg/mL) produced significant (\(P\leq0.05\)) degranulation of peritoneal mast cells as evidenced by the percentage of degranulated mast cells (77%) in egg albumin control group. *M. umbellata* leaf ethanolic extract (MU) at 200 mg/kg and 400 mg/kg doses produced significant (\(P\leq0.05\)) protection of mast cells (64.67±0.82 and 80.00±0.63 respectively) against egg albumin induced degranulation (Fig. 1). The n-hexane extract (MUH) at 200 mg/kg and 400 mg/kg doses also produced significant (\(P\leq0.05\)) protection of mast cells (72.33±1.03 and 85.00±0.89 respectively). The leaf chloroform extract (MUCH) at 400 mg/kg exhibited 52.17±0.75 protection. Disodium chromoglycate (DSCG) (10 mg/kg), the standard mast cell stabilizing drug produced significant (\(P\leq0.05\)) protection (92%). It was observed that the extracts (MU, MUH and MUCH) exhibited protection against mast cell degranulation in a dose dependent manner.
Egg albumin (0.1 mg/mL) produced disruption of mesenteric mast cells in vivo which was significantly ($P<0.05$) inhibited by the administration of MU (200 mg/kg and 400 mg/kg) and MUCH (400 mg/kg) in a dose dependent manner. The protection was comparable to the standard drug DSCG (10 mg/kg) (Fig. 2).

In active paw anaphylaxis, the results are expressed as the percentage of inhibition of paw edema. The animals treated with MU and MUH at doses 100, 200 and 400 mg/kg showed significant ($P<0.05$) reduction in ovalbumin induced paw edema compared to the control. MU at doses 100, 200 and 400 mg/kg inhibited the paw edema by 65.35%, 70.68% and 73.32% respectively and MUH at doses 100, 200 and 400 mg/kg inhibited the paw edema by 61.48%, 69.92% and 74.00% respectively compared to DSCG (75.48%) (Table 1). Animals treated with MUCH, MUAE and MUW showed less reduction of paw edema compared to the standard drug DSCG.

In mesenteric mast cell study in vitro, MU and MUH at 2 mg/mL dose showed significant ($P<0.05$) protection against egg albumin (0.1 mg/mL) induced mast cell degranulation (52.50±1.05 and 54.17±1.47 respectively) in vitro compared to DMSO control (Fig. 3).
4. DISCUSSION

Allergies and anaphylactic responses are associated with T₄,₂ type immune responses in which the production of IgE and some cytokines like IL-4 occurs. Mast cells are the primary targets in anaphylactic and allergic reactions and the degranulation is triggered by the cross linking of high affinity IgE antibodies to the receptors on mast cells\(^{[23]}\). The pathological mechanism involved in Type 1 allergic reactions has been attributed to the degranulation of mast cells followed by the release of mediators such as histamine, heparin, proteases, leukotrienes, prostaglandins and cytokines causing bronchoconstriction, increased vascular permeability, changed blood vessel tone and other proinflammatory effects\(^{[24]}\). The process leading to histamine secretion is mediated by calcium release from the intracellular storage of mast cells and mast cell stabilizing drugs block calcium channels, which is essential for the degranulation to occur\(^{[26]}\). Egg albumin is an inciting antigen which induces the release of anaphylactic and inflammatory mediators such as histamine, heparin, proteases and proinflammatory cytokines from mast cells on activation\(^{[26]}\) and hence it is used as the antigen.

In the present study, MU and MUH exhibited significant protection against egg albumin induced degranulation of both peritoneal and mesenteric mast cells in vivo (Fig. 4). Both the extracts inhibited significantly \((P \leq 0.05)\) the degranulation of mast cells and release of mediators and thus they are active against Type 1 allergic conditions and this may be due to the influence of the extracts on the binding of IgE on mast cells. The allergic or anaphylactic agents can alter mast cell membrane integrity leading to the increased permeability and consequent release of anaphylactic mediators\(^{[27]}\). The extracts inhibited degranulation by stabilizing the membrane of mast cells. Antigen-antibody IgE interaction on the mast cell membrane receptors resulted in increased membrane permeability to calcium ions leading to the release of inflammatory mediators\(^{[28]}\). Disodium chromoglycate (DSCG) is the drug used for the treatment of bronchial asthma and it inhibits mast cell degranulation by raising the intracellular cyclic adenosine monophosphate (cAMP) levels and blocking the calcium channels\(^{[29]}\). DSCG inhibits late response and the subsequent bronchial hyper responsiveness, by acting on inflammatory phagocytic cells like eosinophilic and basophilic cells, macrophages etc. It exhibited infrequent and minor adverse reactions such as bronchospasm, cough or wheezing, joint swelling and pain, laryngeal edema, angioedema, rashes, head ache and nausea\(^{[30]}\).

The percentage protection of MU and MUH against degranulation was significant compared to the standard mast cell stabilizing drug DSCG. MU and MUH may alter the calcium fluxes across the membrane, thereby inhibiting degranulation. The membrane stabilizing effect of the extracts may also be due to the inhibition of egg albumin induced histamine release from mast cells or unavailability of antibodies to bind on mast cell membrane\(^{[31]}\). MU and MUH showed significant protection against degranulation of mast cells in vivo compared to DMSO control. This is because the extracts can act directly on the mast cells and inhibit antigen induced mast cell degranulation and release of anaphylactic mediators from the sensitized cells.

Active paw anaphylaxis is another in vivo model to evaluate the modulatory effect on IgE antibody mediated immune hyperactivity using ovalbumin as antigen\(^{[30]}\). There is a significant increase in paw edema of ovalbumin control group. The extracts MU, MUH and MUCH exhibited significant inhibition of ovalbumin induced paw edema in a dose dependent manner compared to standard drug DSCG. The edema inhibitory effect of the extracts may be due to the regulation of IgE mediated Type 1 hypersensitivity.

From the present study, it is clear that the ethanolic (MU) and n-hexane (MUH) leaf extracts of *Morinda umbellata* showed significant inhibition of antigen induced mast cell degranulation and active paw anaphylaxis in Wistar rats. This may be achieved by the mechanisms such as (1) mast cell membrane stabilization (2) inhibition of antigen induced degranulation and release of anaphylactic and inflammatory
mediators such as histamine from sensitized mast cells (3) altering the calcium fluxes across mast cell membrane by increasing the cyclic AMP (cAMP) levels and decreasing cAMP phosphodiesterase and thereby inhibiting degranulation. It has been reported that flavonoids such as quercetin, apigenin and luteolin can inhibit the release of histamine and neutrophil β-glucuronidase and thereby exhibiting in vivo anti-allergic and anti-histaminic effect[33] or by inhibiting G-protein activation[34].

5. CONCLUSION
In conclusion, the mast cell stabilizing and anti-anaphylactic effects of Morinda umbellata leaf extracts can be attributed to the presence of flavonoids and phenolic compounds present in it and the findings substantiates various traditional medicinal uses of the plant. Thus M. umbellata can be considered as a good candidate for the management of allergic diseases. Further studies are in progress in our laboratory to explore its active compounds and detailed mechanism of action.

ACKNOWLEDGEMENT
The authors acknowledge the Director, JNTBGRI for providing facilities. The funding from University of Kerala, Thiruvananthapuram in the form of Senior Research Fellowship (SRF) to the first author is gratefully acknowledged.

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Source of support: University of Kerala; Conflict of interest: None Declared