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Anti-allergic and anti-inflammatory properties of *Zizyphus mauritiana* root bark

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Abstract

Allergy sometimes might be very dangerous and one of the main factors responsible for allergy is complement system which can lead to life threatening reaction called anaphylaxis. Cycloxygenase-1 (COX-1), Cycloxygenase-2 (COX-2) and 5-Lipoxygenase (5-LOX) trigger allergic and inflammatory reactions. A number of anti-allergic synthetic drugs are available but are costly and show many side effects. Here is the place where ancient traditional system of medication mentioned in *Ayurveda* finds an edge on various synthetic drugs. *Zizyphus mauritiana* is referred as the store house of phytochemicals in *Ayurveda*. Stem and root barks of *Zizyphus mauritiana* were dried and powdered under controlled conditions. Extractions of dried powders were performed separately in different solvents in the increasing order of their polarity and were tested for their ability to inhibit complement system. Aqueous extract of root bark was found to be more effective in inhibiting complement system. Fractionation of aqueous extract resulted in the isolation of Most Active Fraction (MAF) which inhibited complement system, COX-1, COX-2 and 5-LOX with IC_{50} values of 0.006 µg/ml, 0.065 µg/ml, 0.008 µg/ml and 0.083 µg/ml, respectively. MAF was proved to be successful in down regulating the pro-inflammatory mediators like TNF-α, COX-2 and iNOS when tested on RAW 264.7 cell line. *In vivo*, MAF was found to be preventive against anaphylactic shock and Arthus reaction, when daily orally administrated to Wistar rats. Phytochemical analysis of MAF fraction had indicated that it is rich in tannins. Results indicate that MAF, a fraction isolated from aqueous extract of root bark of *Zizyphus mauritiana*, has potent anti-allergic and anti-inflammatory properties.

Introduction

In *Ayurveda* different plants are said to possess various medicinal properties. Many of them have ability to modulate the immune system [1, 2]. Many natural immunomodulators obtained from plants are investigated by researchers [3]. In many preparations of *Ayurveda*, stem & root bark is included because of their active phytochemical contents.

According to World Health Organization (WHO), nearly 80% of the population in developing countries is dependent upon natural compounds isolated from medicinal plants [4]. In many countries with large village and tribal population, due to poverty, it is not possible to provide costly synthetic medication to each and every individual. Medicinal plants mentioned in *Ayurveda* can thus be a better replacement of synthetic and costly medicine. For this purpose, medicinal plants mentioned in *Ayurveda* should be studied systematically. Also, natural compounds obtained from medicinal plants have fewer side effects than synthetic drugs. Therefore, researchers are turning their attention towards traditional and natural medicinal plants to develop potent, side effect free, cheaper, specific and effective drugs against diseases [5]. Fresh fruits of *Zizyphus mauritiana* and their dried powder called *Borkut* are considered to be nutritious in Asia. Use of stem bark and root bark powder of *Zizyphus mauritiana* as spices in the preparation of food by the tribal people in Gadchiroli district of India is a well known fact. Researchers have explored the presence of flavonoids, saponins, tannins, alkaloids, essential oils, phenols and their derivatives in *Zizyphus* species. Also, *Zizyphus* is one of the richest genera with cyclopeptide alkaloids [6]. *Zizyphus mauritiana* leaves are well known for their antioxidant properties [7] while leaves and stem bark of *Zizyphus mauritiana* have been reported for their antimicrobial effects [8, 9]. It is generally seen that plants rich in alkaloids, quinones, terpenes, tannins, saponins with antimicrobial and antioxidant activity possess good immunomodulating

potential [10]. Hence, in the present work, *Zizyphus mauritiana* is selected to study its immunomodulating potential.

Allergy is a hypersensitivity disorder of immune system. It occurs when immune system reacts to normally harmless substances (allergen) in environment. Sometimes, severe allergies due to environmental or dietary allergens may result in life-threatening reactions called anaphylaxis. It is a shock which occurs within seconds as susceptible subject has specific IgE antibodies for the particular allergen [11]. Arthus reaction is a dermal inflammatory response (Type III hypersensitivity) caused by reaction of precipitating antibody with antigen present in skin [12]. Complement system mediators such as C3a, C4a etc. are called anaphylatoxins because of their ability to elevate anaphylactic shock and other allergic responses. Allergy can take serious form when complement system participates in it [13, 14]. Cyclooxygenases (COX-1 and COX-2) and 5-lipoxygenase (5-LOX) synthesize prostanoids and eicosanoids from poly unsaturated fatty acids (PUFAs) which are involved in various inflammatory and allergic disorders [15, 16]. COX has two isoforms; COX-1 is constitutive while COX-2 is an inducible enzyme. COX-1 plays some specific roles in cell, hence is always present in cell. But, even a very low level of COX-2 has always been a warning for its involvement in inflammatory and allergic reactions. Same is true with 5-LOX, whose elevated level is a cause of concern. Inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α) are proinflammatory mediators [17]. TNF-α is chiefly formed by activated macrophages, although it can also be produced by many other cell types. It mainly works by activating NF-κB which in turn mediates transcription of vast array of proteins involved in allergic and inflammatory responses [18].

Here, effective anti-allergic and anti-inflammatory fraction, named Most Active Fraction (MAF) was isolated from aqueous extract of root bark of *Zizyphus mauritiana* by adsorption column

chromatography. MAF was tested for its efficiency to inhibit complement system, COX-1, COX-2 and 5-LOX and to down regulate gene expression of iNOS, COX-2 and TNF-α. *In vivo*, immunomodulation by MAF was explored by investigating its effect on systemic anaphylaxis and Arthus reaction in Wistar rats.

Materials and methods

Chemicals

Dulbecco's phosphate buffered saline (DPBS), RPMI-1640, fetal calf serum (FCS), were purchased from Gibco laboratories; antibiotic-antimycotic solution was purchased from Himedia laboratories, Mumbai. Trypsin, β-actin antibody and lipopolysaccharide (LPS), nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Sigma Aldrich chemical company, St. Louis, USA. Antibodies for COX-2, TNF-α and iNOS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), USA. DMSO, silica gel, solvents for soxhlet extraction were of analytical grade. HPLC grade solvents were purchased from authorized standard companies especially for column chromatography.

Plant material

Stem & root barks of plants were collected from forest region of Gadchiroli district of Maharashtra (India) in May (summer). While taking out barks complete care was taken to prevent any injury to plants. Authentication was done at University Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur by expert taxonomists and voucher specimens were deposited in herbarium. Botanical name of the plant was identified to be *Zizyphus mauritiana* (Family Rhamnaceae) and relevant voucher specimen number was 9483.

Animals

Wistar rats (200-225 g) of either sex were procured from NCLAS (National Centre for Laboratory Animal Sciences, Hyderabad, India). Animals were kept in standard conditions (temperature $25 \pm 2°C$) and 12 h light/ 12 h dark cycle was maintained. They were fed *ad libitum* with standard pellet diet and purified water with free access to food and water. All the animals received human care. All experiments were performed in compliance with the relevant laws and guidelines prescribed by Institutional Animal Ethics Committee (IAEC) (vide permission letter from Institutional Animal Ethics Committee ref. no. PGTD/BC/53). During oral administration of MAF, utmost care was taken to prevent any stress or injury to animals. Animals were weighed on electronic balance (Essae Teraoka Ltd. FB200) before experiments.

Preparation of extracts

Stem and root barks of *Zizyphus mauritiana* were carefully cleaned and shed dried separately for approximately two months. Dried barks of both stem and root were crushed separately in grinder. Using soxhlet apparatus, powdered barks were separately extracted successively in increasing order of polarity by petroleum ether (polarity = 0.0), toluene (polarity = 2.7), chloroform (polarity = 4.1), ethanol (polarity = 5.2) and water (polarity = 9.0) to separate compounds in the increasing order of polarity [19]. The main purpose of starting extraction process from petroleum ether was defattying of the extract in which most of the steroidal and highly non-polar compounds were removed. Solvents were evaporated by using rotary vacuum evaporator (Superfit DB3135S). Dried extracts (1%) were dissolved in 0.1% Dimethyl sulphoxide (DMSO)

in PBS (phosphate buffered saline), mixed and vortexed for 1 min. Supernatants obtained after centrifugation at 100 g for 2 min were tested for their efficiency to inhibit complement system.

Adsorption Column Chromatography

Aqueous extract of root bark was found to be better in complement system inhibition. Hence, aqueous extract of root bark was further purified by adsorption column chromatography. Silica gel (100-200 mesh) slurry was prepared in petroleum ether and glass column (5 x 30 cm) was packed using silica gel slurry. Packed column was kept undisturbed for 1 h [20, 21]. For column chromatography, 20 g dried powder of aqueous extract of root bark was applied on the silica gel column. Petroleum ether, toluene, chloroform, ethyl acetate, acetone, ethanol, water and their mixtures in various proportions in the increasing order of polarity (50 ml petroleum ether, 40 ml petroleum ether + 10 ml toluene, 30 ml petroleum ether + 20 ml toluene, 20 ml petroleum ether + 30 ml toluene, 10 ml petroleum ether + 40 ml toluene, 50 ml toluene, 40 ml toluene + 10 ml chloroform and so on upto 50 ml water) were introduced successively in continuation. Fractions of 5 ml were collected (approx. 2 ml/min.) from the bottom of the column. Thin layer chromatography of each fraction was performed and similar fractions were mixed. Such 17 different fractions were obtained. All the 17 fractions were dried by evaporating the solvents. Dried fractions were dissolved in 0.1% DMSO in PBS and further tested. Fraction number 11 was found to be most effective in inhibiting complement system. Hence it was named as most active fraction (MAF).

Phytochemical Screening

Phytochemical screening of MAF fraction (1 mg/ml) was performed as per the methods proposed in Trease and Evans [22] for almost all known phytochemicals. For tannin, following tests were performed.

Ferric chloride test: To 1 ml solution of MAF, 2-3 drops of dilute ferric chloride solution were added, development of green black color; indicated the presence of tannin.

Gelatin test: To 1 ml solution of MAF, 2-3 drops of gelatin solution was added, formation of white precipitate gave an indication of tannin.

Lead acetate test: To 2 ml solution of MAF, 2-3 drops of 10% lead acetate solution was added, formation of red precipitate; confirmed the presence of tannin.

Complement system inhibition assay

Complement inhibiting ability of all crude extracts prepared in different solvents was tested to take a reference of active fractions [23]. Human serum from healthy volunteers, with no history of any disease, was used as a source of complement system (vide permission letter number: NU/BC/449; from Clinical Biochemistry Department of RTM Nagpur University, Nagpur) while sheep RBCs (SRBCs) were collected from Animal Husbandry Department of Veterinary College, Nagpur. Sheep blood was withdrawn from external jugular vein of sheep with the help of intravenous set and was directly mixed in freshly prepared Alsever's solution in 1:1 proportion. Mixture was kept at 4°C till separation of SRBCs [24]. After separation, SRBCs were counted in haemocytometer and the cell number was adjusted to 1×10^9 SRBCs/ml. Human serum was incubated separately with all the extracts (1% in 0.1% DMSO in PBS) at 37°C for 10 min. Human serum with 0.1% DMSO in PBS (without plant extract) was used as control. After incubation, 1×10^9 SRBCS were added in each tube and incubated at 37°C for 30 min. All the sets

were centrifuged at 1000 g for 15 min at 4°C following incubation time of 30 min. Absorbance of supernatants were measured at 405 nm in microplate reader (Thermo electron Corp. 358). The activity of complement system in control was considered as 100%.

Similarly, effect of MAF on complement inhibition was tested at various concentrations (1, 0.5, 0.1, 0.01 µg/ml). Below 0.01 µg/ml concentration, MAF showed very little complement inhibition.

Extraction and isolation of cyclooxygenase-1 enzyme

Microsomal fraction from Ram seminal vesicles was prepared as a source of COX-1 [25]. Ram seminal vesicles were ground in a grinder and homogenized in buffer containing 0.05 M Tris-HCl (pH 8.0), 5 mM EDTA disodium salt, 5 mM diethyl dithiocarbamate and 0.01% sodium azide. The homogenate was centrifuged at 13000 g for 15 min, at 4°C. The supernatant was again centrifuged at 100,000 g for 1 h 10 min, at 4°C by ultracentrifuge (Himac, CP-100, Hitachi) to obtain microsomal pellet. This microsomal fraction was stored at -80°C.

Extraction and isolation of cyclooxygenase-2 enzyme

Microsomal fraction from Sf9 insect cell line was prepared as a source of COX-2 [26]. *Spodoptera frugiperda* (Sf9) cell line with recombinant Human COX-2 gene was maintained at 28°C in Grace's insect culture medium. The cell line at 60% confluency was infected with recombinant baculovirus containing human COX-2. After 72 h of infection, the cells were centrifuged at 2000 g for 5 min at 4°C. The pellet was suspended in Tris-HCl buffer (50 mM, pH 7.2) containing 5 mM EDTA, 300 mM sucrose, 5 mM diethyl thiocarbamate, 1 µg/ml pepstatin, 1 mM phenol and

sonicated for 3 min. The cell lysate was centrifuged at 100,000 g for 1 h 10 min at 4°C by ultracentrifuge to obtain microsomal pellet. This microsomal fraction was stored at -80°C.

COX-1 and COX-2 assay

Ability of MAF to inhibit COX-1 and COX-2 were measured by chromogenic assay [27]. This assay is based on oxidation of N, N, N, N-tetra methyl-*p*-phenylene diamine (TMPD) during conversion of PGG2 to PGH2 [28, 29]. Assay mixture contained Tris-HCl buffer (0.5 M), hematin (5 mM), EDTA (0.5 M), enzyme (COX-1 or COX-2) and MAF (100, 10, 1, 0.1, 0.01, 0.001 μg/ml). Mixture was pre-incubated at 25°C for 5 min. Reaction was initiated by addition of substrate Arachidonic acid (AA) and TMPD, in total volume of 1 ml reaction mixture. Enzyme activity was determined by estimating the rate of TMPD oxidation for first 60 sec of reaction by following increase in absorbance at 603 nm. A low rate of non-enzymatic oxidation, observed in absence of COX-1 and COX-2, was subtracted from experimental value while calculating percent inhibition.

Purification and assay of 5-lipoxygenase (5-LOX)

5-LOX was obtained in purified form from potato tubers [30]. Capacity of MAF to inhibit activity of 5-LOX was measured by polarigraphic method with a Clark's oxygen electrode on Strathkelvin instrument (model 782, RC-300). Reaction mixture contained 50 μl of enzyme, 10 μl of substrate (Arachidonic acid, 40 mM), MAF (100, 10, 1, 0.1, 0.01, 0.001 μg/ml) and 100 mM potassium phosphate buffer (pH 6.3). Reaction was allowed to proceed at 25°C. Since LOX is an oxygen consuming enzyme, rate of decrease in oxygen was taken as a measure of enzyme

activity. Very low rate of non-enzymatic oxygen consumption, in absence of 5-LOX, was subtracted from experimental value while calculating percent inhibition.

Cell culture and treatment

RAW 264.7 cell line (macrophage cell line) was maintained in monolayer in 12 well tissue culture plates in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 1% antibiotic-antimycotic solution with passaging after every 3 days. Cell cultures were maintained in a humidified CO_2 incubator (Indian Equipment Corporation 3821) with 5% CO_2 at 37°C. Before treatment with MAF, cells were washed with PBS and fresh complete medium was added. Cell density of 5 x 10^5 cells per well was maintained [31]. Cells were incubated in the presence of different concentrations of MAF (10 µg/ml, 1 µg/ml, 0.1 µg/ml) for 3 h. Cells without MAF in the medium were used as control. Later all the sets except control were stimulated with lipopolysaccharide (1 µg/ml) for additional 24 h. At the end of the treatment, cells were washed with PBS and harvested to prepare cytosolic extract for western blot analysis.

Western blot analysis

Cell pellets were lysed in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1.0% Tween 20, 1 mM EDTA, 1 mM PMSF), sonicated for 5 sec and centrifuged at 10,000 g for 5 min. Bradford method was used to determine protein concentrations, using BSA as a standard protein. Cell extracts were boiled in Laemmli sample buffer, resolved by SDS-PAGE on polyacrylamide gel (12%) and electroblotted onto nitrocellulose membrane. Transfer of protein on membrane was confirmed by staining with 0.5% ponceau in 1% acetic acid. Non-specific binding sites were blocked by incubating the membrane in 5% casein in TBST solution overnight at 4°C. Blots

were incubated with primary antibodies of COX-2 (1:1000 dilution), iNOS (1:500 dilution), TNF-α (1:500 dilution) and β-actin (1:1000 dilution), dissolved in 5% BSA solution prepared in TBST, for overnight at 4°C. Finally, blots were probed with secondary antibodies (anti-goat) conjugated with alkaline phosphatase and incubated for 1 h at room temperature. After washing, western blot detection reagent, nitro blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) were introduced. NBT served as an oxidant and BCIP as substrate for alkaline phosphatase giving dark blue color. β-actin was used as loading control [31].

Acute toxicity study

Acute toxicity study was carried out according to Organization for Economic Corporation Development (OECD) guidelines [32]. A group of five Wistar rats (3 males and 2 females) was daily orally administered with MAF. In first week, 1 mg/kg body weight (bw) dose was provided and animals were regularly observed for acute toxicity signs like mortality and behavioral changes 1h post dosing and at least twice daily. As no change in behavior was observed, dose was shifted to 2 mg/kg bw. Similarly, graded doses of 5, 10, 25, 50 mg/kg bw were provided per week. Half of lethal dose LD_{50}, as obtained from this experiment was 50 mg/kg bw at which rats had shown a little different behavior than regular like itching and more aggression. At 100 mg/kg bw dose of MAF, one of the male rats became critical. High dose of any compound may lead to toxicity. That is why toxicity assessment of any medicinal preparation is essential for the development of safe but effective medicine. Hence, 100 mg/kg bw was determined as LD (Lethal Dose). The $1/10^{th}$ of LD_{50} value was taken as therapeutic dose. Thus, for *in vivo* studies, the therapeutic dose of MAF selected was 5 mg/kg b. w.

Systemic anaphylactic reaction

Total 10 Wistar rats (5 males and 5 females) were taken in each group (2 months old with average weight 200-225 g). Animals were divided into four groups; positive control (PC), negative control (NC), experimental 1 (E1) and experimental 2 (E2). Intraperitoneal injection (sensitization) of bovine serum albumin (BSA) (1 mg in 0.2 ml PBS) was administered to all four groups on *day 0* and thus all the four groups were expected to generate antibodies against BSA. Oral administration of MAF (5 mg/kg bw) was given to E1 by dissolving in PBS daily from *day -10* (10 days prior to sensitization injection) till *day +15* (15 days past the sensitization injection) [33]. Thus, total 25 doses of MAF were given to E1. Intravenous injection (shocking injection) of BSA (1 mg in 0.2 ml PBS) was given to PC and E1 while NC was given intravenous injection of ovalbumin (1 mg ovalbumin in 0.2 ml PBS) on *day +15*. To E2, along with intravenous shocking injection of BSA, 1 mg MAF was injected in combination on *day +15* to check whether MAF can prevent anaphylactic shock without any pretreatment [34, 35]. Systemic anaphylactic reaction was observed within 10 min after shocking injection and rated as: *Positive reaction*; animals died or rendered stationary at least for 1 min, *Negative reaction*; no changes were observed in activity and movement of animals were normal [36].

Arthus reaction

Total 10 rats (5 males and 5 females) were included in each group (2 months old with average weight 200-225 g). Animals were divided into four groups; positive control (PC), negative control (NC), experimental 1 (E1) and experimental 2 (E2). Subcutaneous injection of 1 mg bovine serum albumin (BSA) in 0.2 ml PBS was given to all four groups on *day 0* (sensitization). Oral administration of MAF (5 mg/kg bw) was given to E1 by dissolving in PBS daily from *day*

-10 (10 days prior to sensitization injection) till *day +15* (15 days past the sensitization injection). Thus, total 25 doses of MAF were given to E1. Intradermal injection (shocking injection) of 0.5 mg BSA in 0.2 ml PBS was given to PC and E1 in right foot pad. An intradermal injection (shocking injection) of ovalbumin (0.5 mg ovalbumin in 0.2 ml PBS) was injected in right foot pad of NC on *day +15*. Immediate effectiveness of MAF on Arthus reaction was tested in E2, where along with shocking injection of BSA, 1 mg MAF was injected in combination on *day +15* to check whether MAF can prevent anaphylactic shock without any pretreatment. Thickness of right footpad of each rat was recorded from four different angles by Vernier caliper at 2 h, 6 h, 1 day, 2 days and 3 days after shocking injection [36].

Statistical analysis

Sigma Plot 10 software was used for statistical analysis of experimental data. The experimental data were expressed as mean ± S.D. *P*-values were determined using the unpaired student's *t*-test. *P*-values less than 0.01 and 0.05 were considered as significant.

Results

Effect of different extracts on complement inhibition

All the crude extracts of both stem and root barks (1%) were found to inhibit complement system to some extent. But, as we move from non-polar compounds to the polar ones, the complement inhibition activity goes on increasing (Fig. 1 and Fig. 2). Aqueous crude extract of root bark was found to be better in complement inhibition showing nearly 81% inhibition. Hence, aqueous extract of root bark was selected for adsorption column chromatography for isolation of active fraction.

Purification and isolation of MAF

On application of 20 g of aqueous root bark extract for adsorption column chromatography, 6.14 g of MAF was obtained.

Phytochemical analysis of MAF

The results of the phytochemical tests of MAF are shown in table 1. MAF had shown positive tests for alkaloids, cardiac glycosides, reducing sugars but presence of tannins was the most prominent.

Effect of MAF on complement inhibition

At 1 μg/ml level, MAF has shown 93.05% complement inhibition (Fig. 3). IC_{50} value of MAF in complement system inhibition was found to be 0.006 μg/ml (Fig. 4).

Effect of MAF on COX-1, COX-2 and 5-LOX

MAF had shown a dose dependent inhibition of COX-1, COX-2 and 5-LOX activities (Fig. 5) with IC_{50} value of 0.065 μg/ml, 0.008 μg/ml and 0.083 μg/ml respectively. Especially in case of COX-2, MAF (10 μg/ml) was able to show approximately 98% inhibition.

Effect of MAF on expression of TNF-α, COX-2 and iNOS

On LPS treatment, levels of TNF-α, COX-2 and iNOS were found to be highly up regulated but in MAF treated cells, down regulation of these proteins in a dose dependent manner were prominently observed (Fig. 6). TNF-α is always expressed in RAW 264.7 cell line to some

extent because of its role in wound healing and angiogenesis. Hence, a little expression of TNF-α

was seen in control [37].

Effect of MAF in preventing systemic anaphylactic shock in Wistar rats

Results presented in Table 2 depict effect of MAF in preventing anaphylactic shock in Wistar

rats. All animals in PC (Positive Control) displayed symptoms of systemic anaphylactic shock

(animals remained stationary at least for 1min) while rats in NC (Negative Control) group did

not show any anaphylactic reaction as the antibodies (IgE) for BSA were unable to react and

form immune complex with ovalbumin. In E1, after 25 oral supplementations of MAF, animals

did not show systemic anaphylaxis. No pretreatment of MAF was given to rats in group E2. An

immediate effect of MAF in preventing anaphylactic shock was, however, not apparent when

injected in combination with BSA in shocking injection to E2.

Effect of MAF in preventing Arthus reaction in Wistar rats

All animals in PC (Positive Control) displayed positive footpad reaction within 2 h of shocking

injection (Fig. 7). However, in NC (Negative Control), rats did not show Arthus reaction as the

antibodies for BSA were unable to react and form immune complex with ovalbumin. After 25

oral supplementations of MAF to E1, no Arthus reaction was observed in the right foot pad. In

PC, the right footpad has shown an increase of approximately 0.11 cm within 2 h as compared to

E1 where an increase of just 0.005 cm was recorded. Thus, severity of Arthus reaction was found

to decrease to nearly 95.5% (Fig. 8). However, in E2, MAF was found to be comparatively less

effective in inhibiting Arthus reaction instantly but had decreased the severity of foot pad

reaction to approximately 30% and has brought foot pad to normal condition earlier as compared to PC.

Discussion

Complement system on activation; keep on integrating allergic and inflammatory symptoms through complement products like C3a, C4a etc. These are also called anaphylatoxins, which contributes to tissue damage in many allergic and inflammatory conditions. Similarly, COX-1, COX-2 and 5-LOX are the enzymes involved primarily in elevating allergic and inflammatory reactions. Complement system synergistically along with these mediators of inflammation (cycloxygenases and lipoxygenases) elevate the allergic and inflammatory reactions many folds [38]. MAF had shown quite good complement inhibition activity. MAF had also inhibited COX-1, COX-2 and 5-LOX, but the most satisfactory part of MAF is its ability to inhibit COX-2 to a much greater extent than COX-1. This is the sign of a good drug as drugs that inhibit COX-1 more than COX-2 are responsible for gastric injuries [39, 40]. From IC_{50} values of complement, COX-1, COX-2 and 5-LOX, it is clear that all these leaders of allergic and inflammatory responses are strongly inhibited by MAF.

Despite its beneficial roles in host defense, excessive NO production has been implicated in several allergic and inflammatory diseases. LPS induces IκB proteolysis and NF-κB nuclear translocation in RAW 264.7 cells [41, 42]. NF-κB plays a critical role in the regulation of cell survival genes and coordinates the expression of pro-inflammatory enzymes and cytokines such as iNOS, COX-2 and TNF-α. Therefore, RAW 264.7 cells provide an excellent model for drug screening and for subsequent evaluation of potential inhibitors of the pathway leading to iNOS and COX-2 induction and TNF-α production. From the results, it has appeared that MAF is an

excellent down regulator of TNF-α, iNOS and COX-2 indicating that the action of MAF occurs at the transcriptional level via blocking the NF-κB signaling pathway [43].

Many compounds give good results *in vitro* but fail to act *in vivo*. MAF, however, has prevented both anaphylactic shock and Arthus reaction in Wistar rats. In anaphylactic shock, immediate effect of the preformed IgE antibody is seen which can cause death of an individual in severe conditions. On the other hand, Arthus reaction is a local dermal inflammatory response. Underlying mechanisms might be several, but the capacity of MAF to inhibit complement system is the major one. It is reported that if complement system is inhibited, the allergic and inflammatory reactions like anaphylactic shock and Arthus reaction can be minimized to a great extent [44, 45]. It is also reported that inhibition or reduction in the activity of inflammatory enzymes such as COX-1, COX-2 and 5-LOX can decrease the severity of both anaphylactic shock and Arthus reaction to a great extent [46, 47]. Therefore, the ability of MAF to inhibit COX-1, COX-2 and 5-LOX must have played an important role in preventing anaphylactic shock and Arthus reaction. Parallel to it is the ability of MAF to down regulate proinflammatory factors especially iNOS and TNF-α. But, to be rightly effective, regular pretreatment of MAF is essential.

Conclusion

According to experimental results, MAF obtained from *Zizyphus mauritiana* is found to possess excellent anti-allergic and anti-inflammatory properties. Being natural, MAF fraction is free from side effects. Downregulation of TNF-α, iNOS and COX-2 provide the molecular basis of anti-allergic and anti-inflammatory action of MAF. Thus, MAF can act as effective immunosuppressant.

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Figure legends

Fig. 1. Effect of 1% crude stem bark extract (prepared in different solvents) on complement system inhibition. (PE: Petroleum Ether; To: Toluene, Ch: Chloroform, Et: Ethanol, W: Water)

[Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 (*$P<0.01$) and 0.05 ($^{\$}P<0.05$) were considered to be statistically significant].

Fig. 2. Effect of 1% crude root bark extract (prepared in different solvents) on complement system inhibition. (PE: Petroleum Ether; To: Toluene, Ch: Chloroform, Et: Ethanol, W: Water)

[Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 (*$P<0.01$) and 0.05 ($^{\$}P<0.05$) were considered to be statistically significant].

Fig. 3. Effect of the different concentrations of MAF (0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml) on complement inhibition. Dose dependent inhibition was observed.

[Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 (*P<0.01) were considered to be statistically significant].

Fig. 4. IC_{50} value of MAF in complement inhibition was found to be 0.006 µg/ml after using different concentrations of MAF (0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml,).

Fig. 5. Effect of MAF (100 µg/ml, 10 µg/ml, 1 µg/ml, 0.1 µg/ml, 0.01 µg/ml and 0.001 µg/ml) on COX-1, COX-2 and 5-LOX inhibition.

[Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 (*P<0.01) and 0.05 (\$*P*<0.05) were considered to be statistically significant].

Fig. 6. Effect of MAF on downregulation of TNF-α, COX-2 and iNOS against LPS stimulated RAW 264.7 cell line. Cells were preincubated with MAF (10 µg/ml, 1 µg/ml and 0.1 µg/ml) for 3 h and stimulated with LPS (1 µg/ml) for additional 24 h. TNF-α, COX-2 and iNOS expression levels were monitored by western blot analysis; β-actin was used as loading control.

Fig. 7. Effect of MAF in prevention of Arthus reaction in Wistar rats. In case of PC, the right footpad has shown an average increase of approximately 0.11 cm within 2 h as compared to E1 where just an average increase of 0.005 cm was recorded. Thus, severity of Arthus reaction was decreased to nearly 95.5%. However, in case of negative control where shocking injection was replaced with ovalbumin instead of BSA, no Arthus reaction was observed. While in case of E2,

the immediate effect of MAF was not so profound. Data expressed is mean of readings of all 10 rats.

Fig. 8. Photograph showing foot pad reaction in Wistar rats. In case of PC, positive reaction was observed within 2 h while in case of E1, prevention of the foot pad reaction was observed. In E1, no significant difference was observed between left and right foot pad after 2 h.
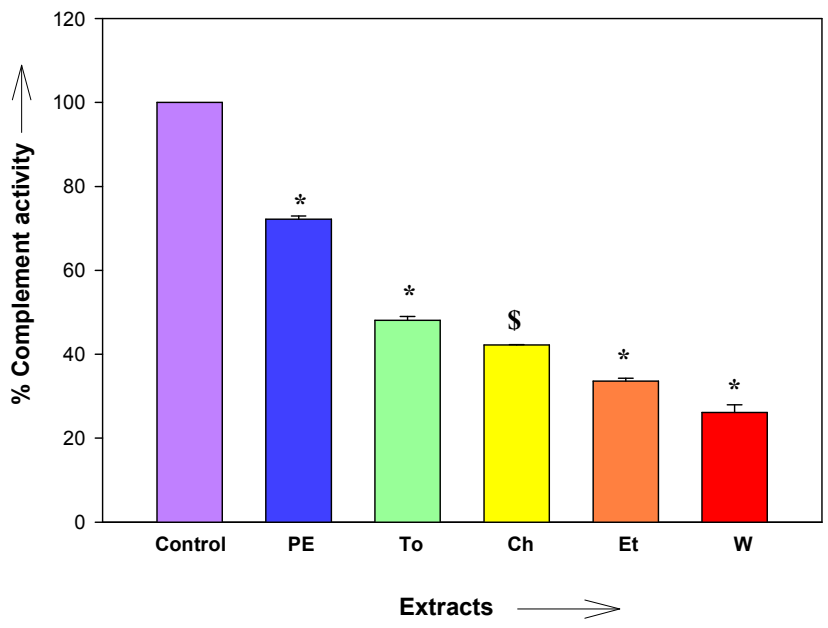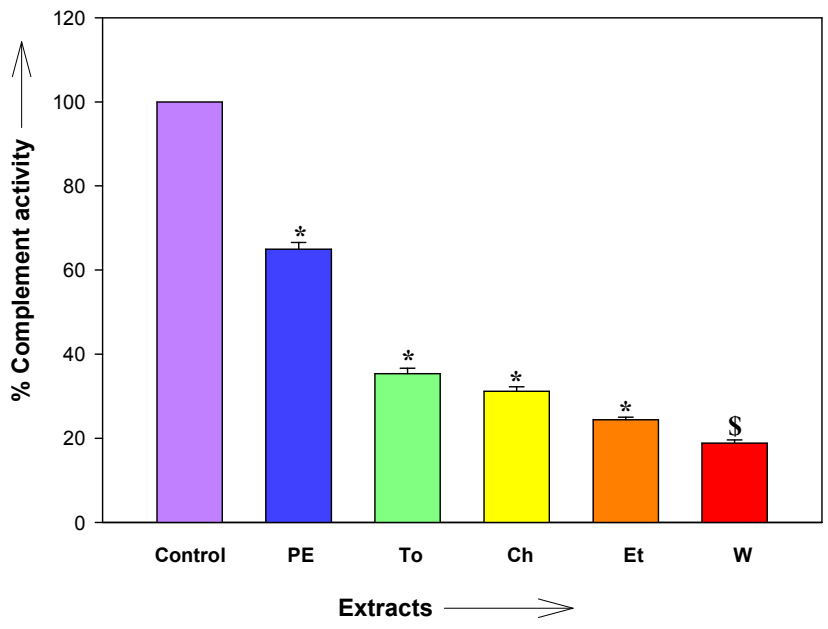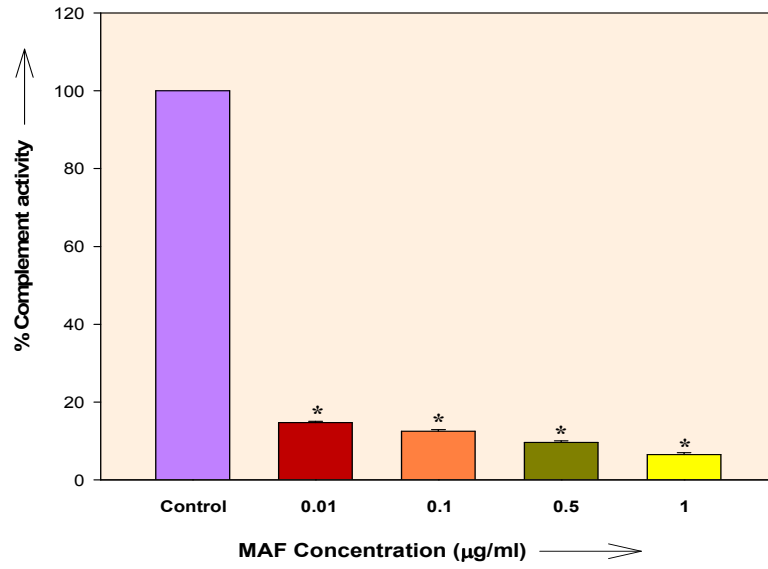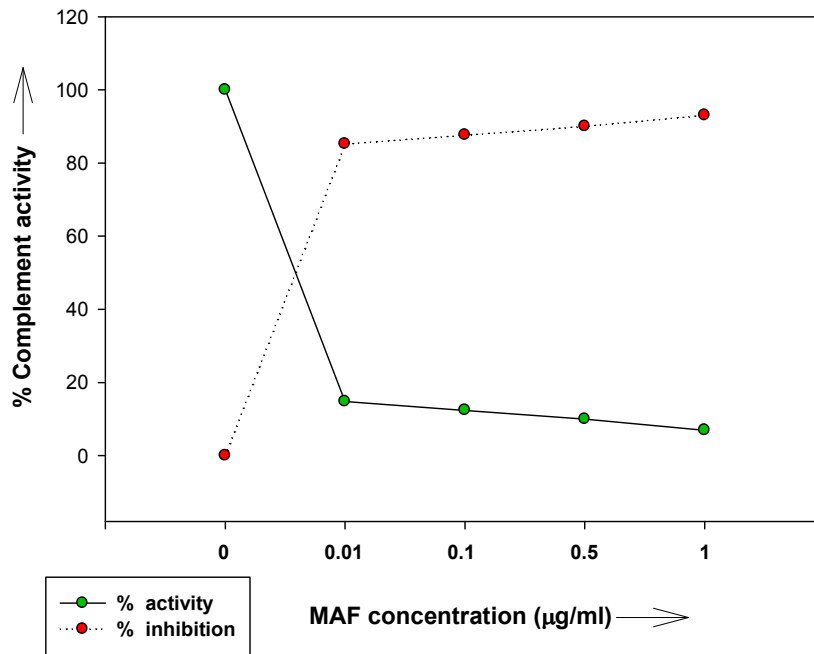
Fig.1.

Fig.2.

Fig. 3.

Fig. 4.

Fig. 5.
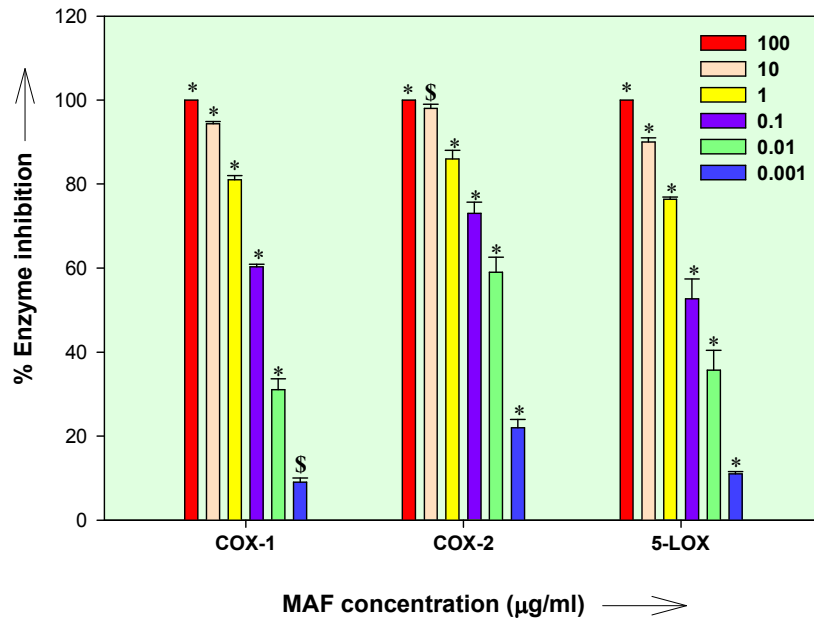

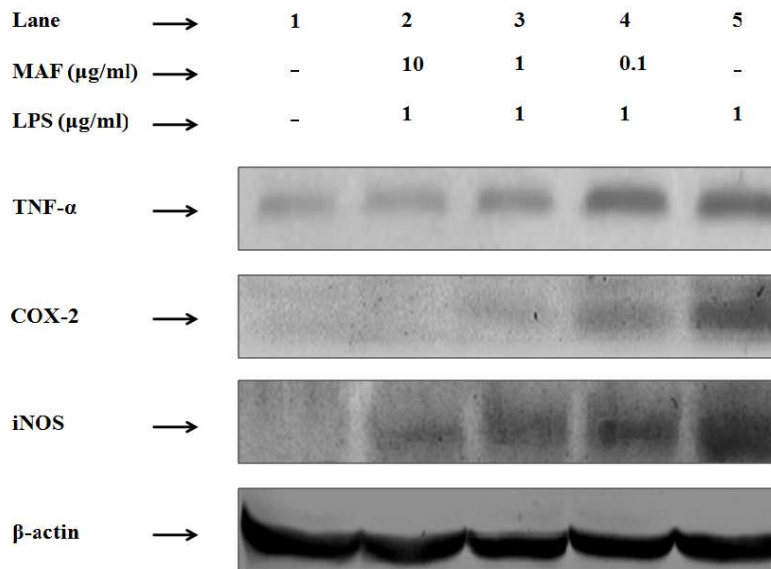
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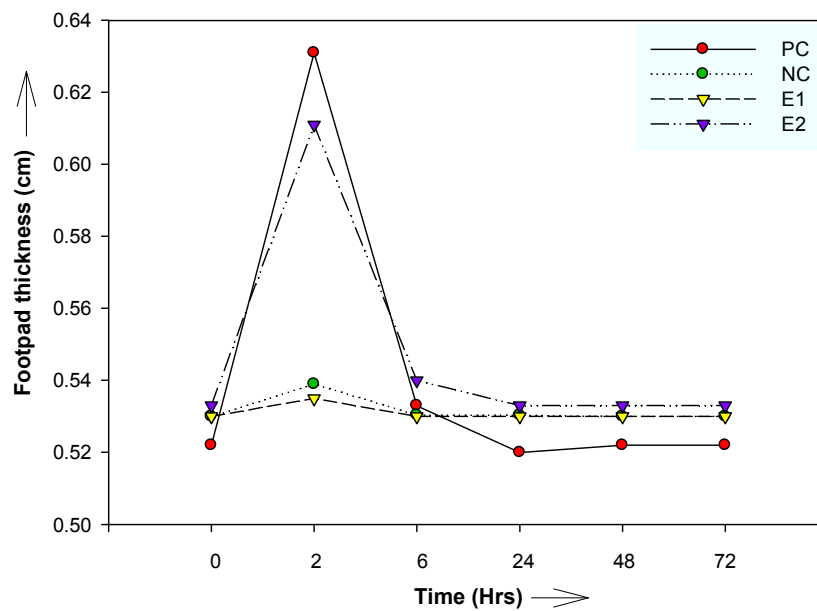
Fig. 7.

Positive Arthus reaction in PC'   Prevention of Arthus reaction
                                    Due to MAF treatment in E1

Fig. 8.

Table 1. Phytochemical analysis of fraction with Most Active Fraction (MAF) showing the presence of alkaloids, cardiac glycosides, reducing sugars and more prominantly tannins.

SN	Test	MAF
1)	Alkaloids	+
2)	Anthocyanins and Anthocyanidins	-
3)	Anthracene glycosides	-
4)	Anthraquinones	-
5)	Aucubins and Iridoids	-
6)	Carbohydrates	-
7)	Cardiac glycosides	++
8)	Carotenoids	-
9)	Coumarins	-
10)	Cyanogenic glycosides	-
11)	Emodins	-
12)	Flavonoids	-
13)	Polyoses	-
14)	Polyuronoids	-
15)	Reducing Sugars	+
16)	Saponins	-
17)	Starch	-
18)	Steroids	-
19)	Tannins	++++
20)	Triterpenoids	-

Table 2.   Effect of MAF on the inhibition of active systemic anaphylaxis in Wistar rats.

OVA: Ovalbumin;   i.p.: intraperitoneal;   i.v.: intravenous;   S/T: Number with anaphylactic symptoms/total no. of rats;   D/T: Number of anaphylactic deaths/total no. of rats.

In case of PC, all 10 rats had shown anaphylactic shock as compared to E1 where neither of the rats was recorded with anaphylactic shock. However, in case of negative control as shocking injection was replaced with ovalbumin instead of BSA, no anaphylactic shock was observed. While in case of E2, no immediate effect of MAF was seen.
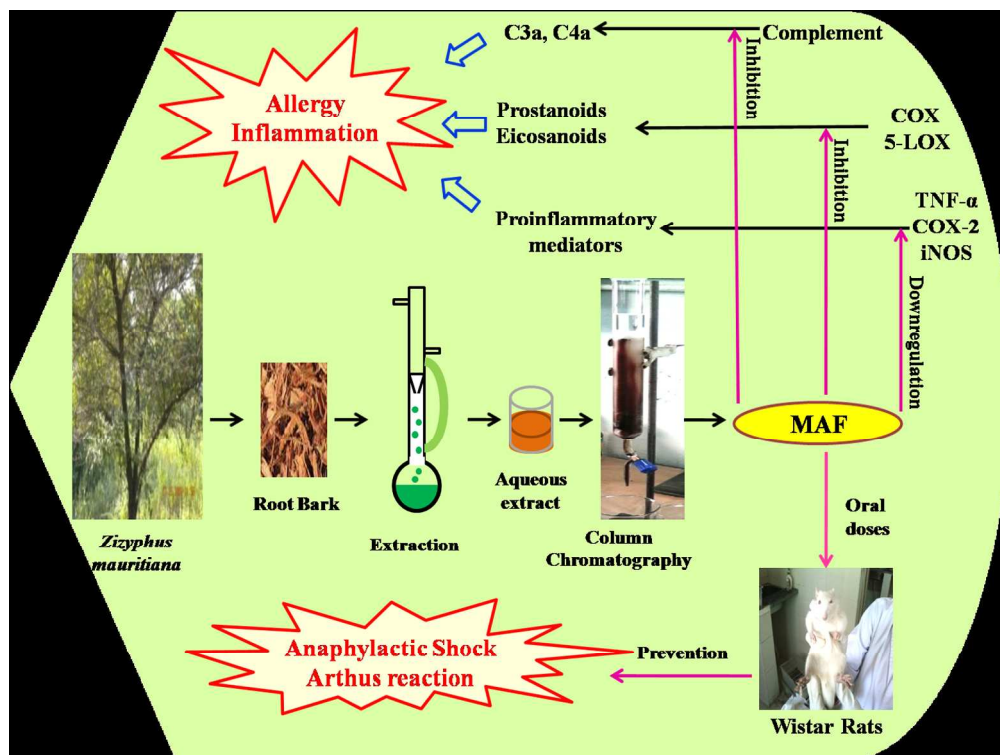
	Sensitizing injection	Shocking injection	Oral MAF treatment	Results S/T	Results D/T
PC	BSA (i.p.)	BSA (i.v.)	-	10/10	0/10
NC	BSA (i.p.)	OVA (i.v.)	-	0/10	0/10
E1	BSA (i.p.)	BSA (i.v.)	+	0/10	0/10
E2	BSA (i.p.)	BSA (i.v.) + MAF (i.v.)	-	9/10	0/10
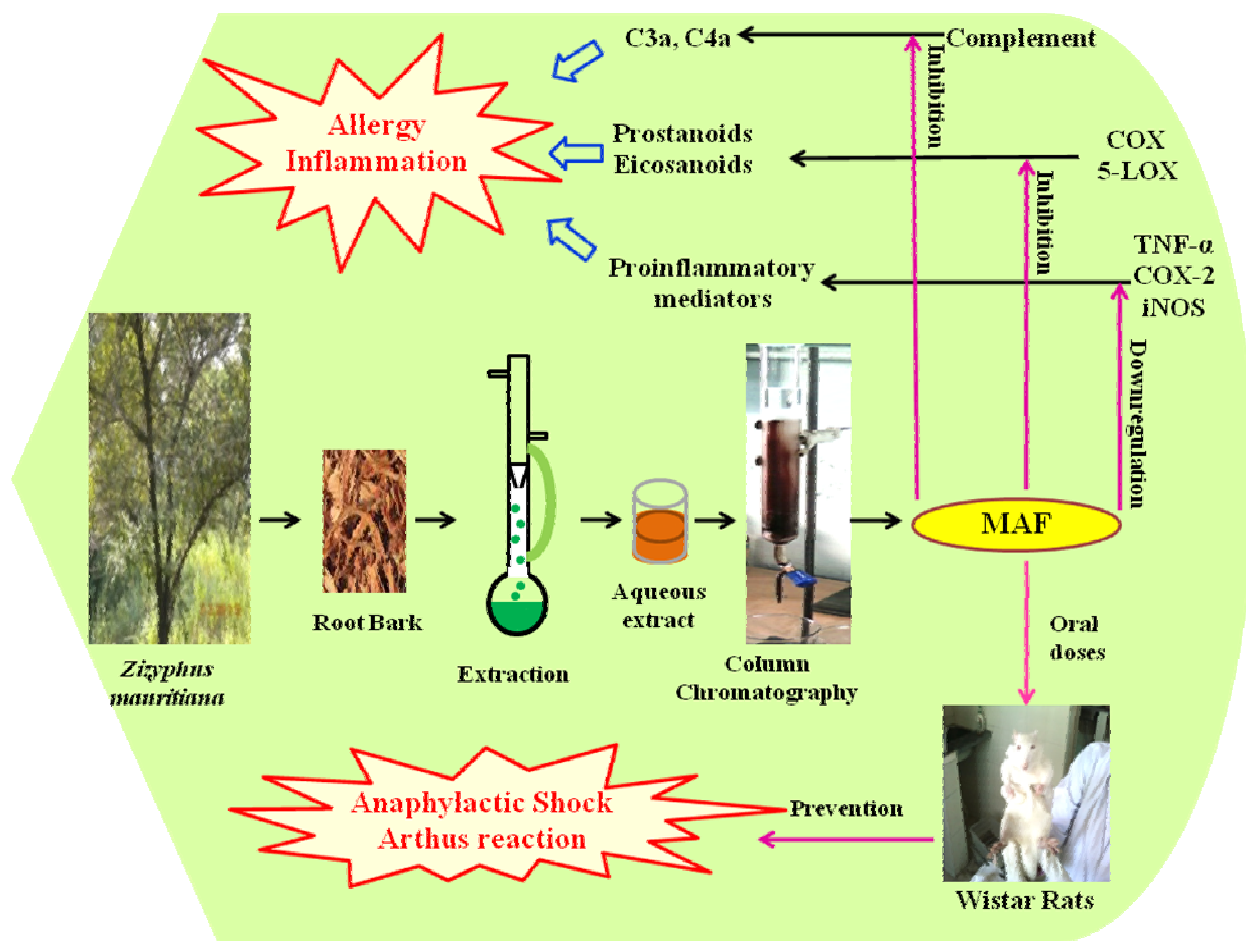
Graphical Abstract

MAF, a fraction with potent anti-allergic and anti-inflammatory compounds is isolated from *Zizyphus mauritiana* root bark. MAF has excellent ability to inhibit complement system, COX-1, COX-2 and 5-LOX. MAF is a very good downregulator of TNF-α, COX-2 and iNOS. MAF has the potential to prevent anaphylactic shock and Arthus reaction.

191x143mm (600 x 600 DPI)