



Cite this: *Food Funct.*, 2015, **6**, 2975

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

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An allergy may sometimes be very dangerous and one of the main factors responsible for allergy is the complement system which can lead to a life-threatening reaction called anaphylaxis. Cyclooxygenase-1 (COX-1), Cyclooxygenase-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. Hence, the ancient traditional system of medication mentioned in *Ayurveda* finds an edge over various synthetic drugs. *Zizyphus mauritiana* is referred to as the store house of phytochemicals in *Ayurveda*. The stem and root barks of *Zizyphus mauritiana* were dried and powdered under controlled conditions. Extractions of the dried powders were performed separately in different solvents in increasing order of their polarity and were tested for their ability to inhibit the complement system. The aqueous extract of the root bark was found to be more effective in inhibiting the complement system. Fractionation of the aqueous extract resulted in the isolation of the Most Active Fraction (MAF) which inhibited the complement system, COX-1, COX-2, and 5-LOX with IC₅₀ values of 0.006 $\mu\text{g ml}^{-1}$, 0.065 $\mu\text{g ml}^{-1}$, 0.008 $\mu\text{g ml}^{-1}$, and 0.083 $\mu\text{g ml}^{-1}$, respectively. The MAF was proven to be successful in down-regulating pro-inflammatory mediators like TNF- α , COX-2, and iNOS when tested on a RAW 264.7 cell line. *In vivo*, the MAF was found to be preventive against anaphylactic shock and the Arthus reaction, when orally administered daily to Wistar rats. Phytochemical analysis of the MAF has indicated that it is rich in tannins. Results indicate that the MAF, a fraction isolated from the aqueous extract of the root bark of *Zizyphus mauritiana*, has potent anti-allergic and anti-inflammatory properties.

Received 16th March 2015,

Accepted 26th June 2015

DOI: 10.1039/c5fo00270b

www.rsc.org/foodfunction

Introduction

In *Ayurveda*, different plants are said to possess various medicinal properties. Many of them have the 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 the 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 populations, it is not possible to provide costly synthetic medication to each and every individual due to poverty. Medicinal plants mentioned in *Ayurveda* can thus be better replacements of synthetic and costly medicines. 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 the stem bark and root bark powder of *Zizyphus mauritiana* as spices in the preparation of food by the tribal people in the Gadchiroli district of India is well known. Researchers have explored the presence of flavonoids, saponins, tannins, alkaloids, essential oils, phenols, and their derivatives in the *Zizyphus* species. Also, *Zizyphus* is one of the richest genera containing cyclopeptide alkaloids.⁶ *Zizyphus mauritiana* leaves are well known for their antioxidant properties,⁷ while the 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, and saponins with antimicrobial and antioxidant activities possess good immunomodulating potentials.¹⁰ Hence, in the present work, *Zizyphus mauritiana* is selected to study its immunomodulating potential.

Allergy is a hypersensitivity disorder of the immune system. It occurs when the immune system reacts to a normally harmless substance (allergen) in the environment. Sometimes, severe allergies due to environmental or dietary allergens may

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result in a life-threatening reaction called anaphylaxis. It is a shock which occurs within seconds as the susceptible subject has specific IgE antibodies for the particular allergen.¹¹ The Arthus reaction is a dermal inflammatory response (Type III hypersensitivity) caused by the reaction of a precipitating antibody with an antigen present in the skin.¹² The complement system mediators such as C3a, C4a, *etc.*, are called anaphylatoxins because of their ability to elevate anaphylactic shock and other allergic responses. An allergy can take a serious form when the complement system participates in it.^{13,14} Cyclooxygenases (COX-1 and COX-2) and 5-lipoxygenase (5-LOX) synthesize prostanoids and eicosanoids from polyunsaturated fatty acids (PUFAs) which are involved in various inflammatory and allergic disorders.^{15,16} COX has two isoforms; COX-1 is a constitutive enzyme while COX-2 is an inducible enzyme. COX-1 plays some specific roles in cells, hence it is always present in cells. But, even a very low level of COX-2 has always been a warning for its involvement in inflammatory and allergic reactions. The same is true for 5-LOX, whose elevated level is a cause for 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- κ B which in turn mediates the transcription of a vast array of proteins involved in allergic and inflammatory responses.¹⁸

Here, the effective anti-allergic and anti-inflammatory fraction, named the Most Active Fraction (MAF) was isolated from the aqueous extract of the root bark of *Zizyphus mauritiana* using adsorption column chromatography. The MAF was tested for its efficiency in inhibiting the complement system, COX-1, COX-2, and 5-LOX, and to down-regulate the gene expression of iNOS, COX-2 and TNF- α . *In vivo*, immunomodulation by the MAF was explored by investigating its effect on systemic anaphylaxis and the Arthus reaction in Wistar rats.

Materials and methods

Chemicals

Dulbecco's phosphate buffered saline (DPBS), RPMI-1640, and fetal calf serum (FCS), were purchased from Gibco laboratories; the 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 the 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 and the solvents for soxhlet extraction were of analytical grade. HPLC grade solvents were purchased from authorized standard companies, especially for column chromatography.

Plant materials

Stem & root barks of plants were collected from the forest region of the Gadchiroli district of Maharashtra (India) in May

(summer). While collecting barks, complete care was taken to prevent any injury to the plants. Authentication was done at the Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur by expert taxonomists and voucher specimens were deposited in the herbarium. The botanical name of the plant was identified to be *Zizyphus mauritiana* (Family Rhamnaceae) and the relevant voucher specimen number was 9483.

Animals

Wistar rats (200–225 g) of either sex were procured from the NCLAS (National Centre for Laboratory Animal Sciences, Hyderabad, India). The animals were kept in standard conditions (temperature 25 ± 2 °C) and a 12 h light/12 h dark cycle was maintained. They were fed *ad libitum* with a 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 the Institutional Animal Ethics Committee (IAEC) (*vide* permission letter from the Institutional Animal Ethics Committee ref. no. PGTD/BC/53). During oral administration of the MAF, utmost care was taken to prevent any stress or injury to the animals. The animals were weighed on an electronic balance (Essae Teraoka Ltd FB200) before the experiments.

The preparation of the extracts

The stem and root barks of *Zizyphus mauritiana* were carefully cleaned and shed-dried separately for approximately two months. The dried barks of both the stem and root were crushed separately in a grinder. Using soxhlet apparatus, the powdered barks were extracted separately, in increasing order of polarity using petroleum ether (polarity = 0.0), toluene (polarity = 2.7), chloroform (polarity = 4.1), ethanol (polarity = 5.2), and water (polarity = 9.0) to separate the compounds in increasing order of polarity.¹⁹ The main purpose of starting the extraction process with petroleum ether was defatting of the extract in which most of the steroidal and highly non-polar compounds were removed. The solvents were evaporated using a rotary vacuum evaporator (Superfit DB3135S). The 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 100g for 2 min were tested for their efficiency to inhibit the complement system.

Adsorption column chromatography

The aqueous extract of the root bark was found to be better in the complement system inhibition. Hence, the aqueous extract of the root bark was further purified using adsorption column chromatography. A silica gel (100–200 mesh) slurry was prepared in petroleum ether and a glass column (5 × 30 cm) was packed using the silica gel slurry. The packed column was kept undisturbed for 1 h.^{20,21} For column chromatography, 20 g of the dried powder of the aqueous extract of the 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 an increasing order of polarity (50 ml of petroleum ether, 40 ml of petroleum ether + 10 ml of toluene, 30 ml of petroleum ether + 20 ml of toluene, 20 ml of petroleum ether + 30 ml of toluene, 10 ml of petroleum ether + 40 ml of toluene, 50 ml of toluene, 40 ml of toluene + 10 ml of chloroform, and so on up to 50 ml of water) were introduced successively in continuation. Fractions of 5 ml were collected (approx. 2 ml min⁻¹) from the bottom of the column. Thin layer chromatography of each fraction was performed and similar fractions were mixed. As such, 17 different fractions were obtained. All of the 17 fractions were dried by evaporating the solvents. The dried fractions were dissolved in 0.1% DMSO in PBS and tested further. Fraction number 11 was found to be most effective in inhibiting the complement system. Hence it was named as the most active fraction (MAF).

Phytochemical screening

Phytochemical screening of the MAF fraction (1 mg ml⁻¹) was performed as per the methods proposed in Trease and Evans²² for almost all known phytochemicals. For tannins, the following tests were performed.

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

Gelatin test. To a 1 ml solution of the MAF, 2–3 drops of a gelatin solution were added, the formation of a white precipitate gave an indication of tannins.

Lead acetate test. To a 2 ml solution of the MAF, 2–3 drops of a 10% lead acetate solution were added, formation of a red precipitate confirmed the presence of tannins.

The complement system inhibition assay

The complement inhibiting ability of all crude extracts prepared in different solvents was tested to take a reference of the active fractions.²³ Human serum from healthy volunteers, with no history of any disease, was used as a source of the complement system (*vide* permission letter number: NU/BC/449; from the Clinical Biochemistry Department of RTM Nagpur University, Nagpur) while sheep RBCs (SRBCs) were collected from the Animal Husbandry Department of the Veterinary College, Nagpur. Sheep blood was withdrawn from the external jugular vein of sheep with the help of an intravenous set and was directly mixed in the freshly prepared Alsever's solution in a 1 : 1 proportion. The mixture was kept at 4 °C until the separation of the SRBCs.²⁴ After separation, the SRBCs were counted in a haemocytometer and the cell number was adjusted to 1 × 10⁹ SRBCs per 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 the control. After incubation, 1 × 10⁹ SRBCs were added to each tube and incubated at 37 °C for 30 min. All of the sets were centrifuged at 1000g for 15 min at 4 °C following an incubation time of 30 min. The

absorbance of the supernatants was measured at 405 nm in a microplate reader (Thermo electron Corp. 358). The activity of the complement system in the control was considered as 100%.

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

Extraction and isolation of the cyclooxygenase-1 enzyme

A 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 a 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 13 000g for 15 min, at 4 °C. The supernatant was again centrifuged at 100 000g for 1 h 10 min, at 4 °C using an ultracentrifuge (Himac, CP-100, Hitachi) to obtain a microsomal pellet. This microsomal fraction was stored at –80 °C.

Extraction and isolation of the cyclooxygenase-2 enzyme

A microsomal fraction from the Sf9 insect cell line was prepared as a source of COX-2.²⁶ The *Spodoptera frugiperda* (Sf9) cell line with the 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 a recombinant baculovirus containing human COX-2. After 72 h of infection, the cells were centrifuged at 2000g 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⁻¹ pepstatin, and 1 mM phenol and was sonicated for 3 min. The cell lysate was centrifuged at 100 000g for 1 h 10 min at 4 °C using an ultracentrifuge to obtain the microsomal pellet. This microsomal fraction was stored at –80 °C.

The COX-1 and COX-2 assay

The ability of the MAF to inhibit COX-1 and COX-2 was measured using a chromogenic assay.²⁷ This assay is based on the oxidation of *N,N,N,N*-tetra methyl-*p*-phenylene diamine (TMPD) during the conversion of PGG₂ to PGH₂.^{28,29} The 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⁻¹). The mixture was pre-incubated at 25 °C for 5 min. The reaction was initiated by the addition of substrates, arachidonic acid (AA) and TMPD, into a total volume of 1 ml of reaction mixture. The enzyme activity was determined by estimating the rate of TMPD oxidation for the first 60 s of the reaction by following the increase in absorbance at 603 nm. A low rate of non-enzymatic oxidation, observed in the absence of COX-1 and COX-2, was subtracted from the experimental value while calculating the percent inhibition.

The purification and assay of 5-lipoxygenase (5-LOX)

5-LOX was obtained in a purified form from potato tubers.³⁰ The capacity of the MAF to inhibit the activity of 5-LOX was

measured using a polarigraphic method with a Clark's oxygen electrode on a Strathkelvin instrument (model 782, RC-300). The reaction mixture contained 50 μl of the enzyme, 10 μl of the substrate (arachidonic acid, 40 mM), the MAF (100, 10, 1, 0.1, 0.01, 0.001 $\mu\text{g ml}^{-1}$) and 100 mM of potassium phosphate buffer (pH 6.3). The reaction was allowed to proceed at 25 °C. Since LOX is an oxygen consuming enzyme, the rate of decrease in oxygen was taken as a measure of enzyme activity. The very low rate of non-enzymatic oxygen consumption, in the absence of 5-LOX, was subtracted from the experimental value when calculating the percent inhibition.

Cell culture and treatment

The RAW 264.7 cell line (a macrophage cell line) was maintained as a 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. The cell cultures were maintained in a humidified CO₂ incubator (Indian Equipment Corporation 3821) with 5% CO₂ at 37 °C. Before treatment with the MAF, the cells were washed with PBS and fresh complete medium was added. A cell density of 5×10^5 cells per well was maintained.³¹ The cells were incubated in the presence of different concentrations of the MAF (10 $\mu\text{g ml}^{-1}$, 1 $\mu\text{g ml}^{-1}$, 0.1 $\mu\text{g ml}^{-1}$) for 3 h. Cells without the MAF in the medium were used as a control. Later, all the sets except the control were stimulated with lipopolysaccharide (1 $\mu\text{g ml}^{-1}$) for an additional 24 h. At the end of the treatment, the cells were washed with PBS and harvested to prepare the 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 s and centrifuged at 10 000g for 5 min. The Bradford method was used to determine protein concentrations, using BSA as the standard protein. The cell extracts were boiled in Laemmli sample buffer, resolved using SDS-PAGE on polyacrylamide gel (12%) and electroblotted onto a nitrocellulose membrane. Transfer of the protein on the 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 a TBST solution overnight at 4 °C. The blots were incubated overnight at 4 °C with the 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 a 5% BSA solution prepared in TBST. Finally, the blots were probed with secondary antibodies (anti-goat) conjugated with alkaline phosphatase and incubated for 1 h at room temperature. After washing, a 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 a substrate for alkaline phosphatase, giving a dark blue color. β -actin was used as the loading control.³¹

Acute toxicity study

Acute toxicity study was carried out according to the Organization for Economic Corporation Development (OECD) guidelines.³² A group of five Wistar rats (3 males and 2 females) were orally administered daily with the MAF. In the first week, a 1 mg per kg body weight (bw) dose was provided and the animals were regularly observed for acute toxicity signs like mortality and behavioral changes 1 h post dosing and at least twice daily. As no change in behavior was observed, the dose was shifted to 2 mg per kg bw. Similarly, graded doses of 5, 10, 25, and 50 mg per kg bw were provided per week. Half of the lethal dose LD₅₀ as obtained from this experiment was 50 mg per kg bw, at which the rats had shown slightly different behavior than regular, like itching and more aggression. At a 100 mg per kg bw dose of the MAF, one of the male rats became critical. A high dose of any compound may lead to toxicity. That is why a toxicity assessment of any medicinal preparation is essential for the development of safe but effective medicine. Hence, 100 mg per kg bw was determined as the LD (Lethal Dose). 1/10th of the LD₅₀ value was taken as the therapeutic dose. Thus, for *in vivo* studies, the therapeutic dose of the MAF selected was 5 mg per kg bw.

The systemic anaphylactic reaction

A total of 10 Wistar rats (5 males and 5 females) were included in each group (2 months old with average weights of 200–225 g). Animals were divided into four groups; the positive control (PC), negative control (NC), experimental 1 (E1), and experimental 2 (E2). An 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 four groups were expected to generate antibodies against BSA. Oral administration of the MAF (5 mg per kg bw) was given to the E1 group by dissolving in PBS daily from *day -10* (10 days prior to the sensitization injection) until *day +15* (15 days past the sensitization injection).³³ Thus, a total of 25 doses of the MAF were given to the E1 group. An intravenous injection (shocking injection) of BSA (1 mg in 0.2 ml PBS) was given to the PC and E1 groups while the NC group was given an intravenous injection of ovalbumin (1 mg of ovalbumin in 0.2 ml PBS) on *day +15*. To E2, along with the intravenous shocking injection of BSA, 1 mg of the MAF was injected in combination on *day +15* to check whether the MAF can prevent an anaphylactic shock without any pretreatment.^{34,35} A systemic anaphylactic reaction was observed within 10 min after the shocking injection and rated as: *Positive reaction*; animals died or rendered stationary at least for 1 min, or *Negative reaction*; no changes were observed in activity and movements of the animals were normal.³⁶

Arthus reaction

A total of 10 rats (5 males and 5 females) were included in each group (2 months old with average weights of 200–225 g). The animals were divided into four groups; the positive control (PC), negative control (NC), experimental 1 (E1), and

experimental 2 (E2). A 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 the MAF (5 mg per kg bw) was given to the E1 by dissolving in PBS daily from *day -10* (10 days prior to sensitization injection) until *day +15* (15 days past the sensitization injection). Thus, a total of 25 doses of the MAF were given to the E1. Intradermal injection (shocking injection) of 0.5 mg BSA in 0.2 ml PBS was given to the PC and E1 in the right foot pad. An intradermal injection (shocking injection) of ovalbumin (0.5 mg ovalbumin in 0.2 ml PBS) was injected in the right foot pad of the NC on *day +15*. Immediate effectiveness of the MAF on the Arthus reaction was tested in the E2, where along with the shocking injection of BSA, 1 mg of the MAF was injected in combination on *day +15* to check whether the MAF can prevent anaphylactic shock without any pretreatment. The thickness of the right foot pad of each rat was recorded from four different angles using a Vernier caliper at 2 h, 6 h, 1 day, 2 days and 3 days after the shocking injection.³⁶

Statistical analysis

Sigma Plot 10 software was used for the statistical analysis of experimental data. The experimental data were expressed as the means $\pm$ 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 the complement system to some extent. But, as we move from non-polar compounds to polar ones, the complement inhibition activity keeps on increasing (Fig. 1 and 2). The aqueous crude extract of the root bark was found to be better at complement inhibition showing nearly 81% inhibition. Hence, the aqueous extract of the root bark was selected for adsorption column chromatography for the isolation of the active fraction.

The purification and isolation of the MAF

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

The phytochemical analysis of the MAF

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

The effect of the MAF on complement inhibition

At a 1 $\mu\text{g ml}^{-1}$ level, the MAF showed 93.05% complement inhibition (Fig. 3). The IC_{50} value of the MAF in the inhibition of the complement system was found to be 0.006 $\mu\text{g ml}^{-1}$ (Fig. 4).

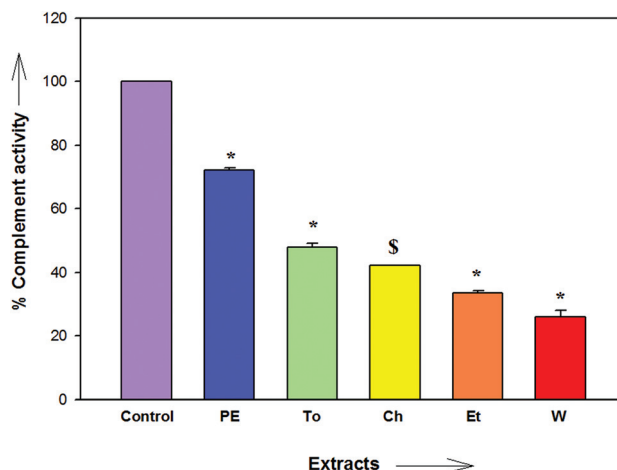


Fig. 1 The effect of 1% crude stem bark extract (prepared in different solvents) on complement system inhibition. (PE: petroleum ether; To: toluene, Ch: chloroform, Et: ethanol, W: water). [Each value has been expressed as the mean $\pm$ SD of five independent experiments. *P* values less than 0.01 (**P* < 0.01) and 0.05 ($^{\$}P$ < 0.05) were considered to be statistically significant].

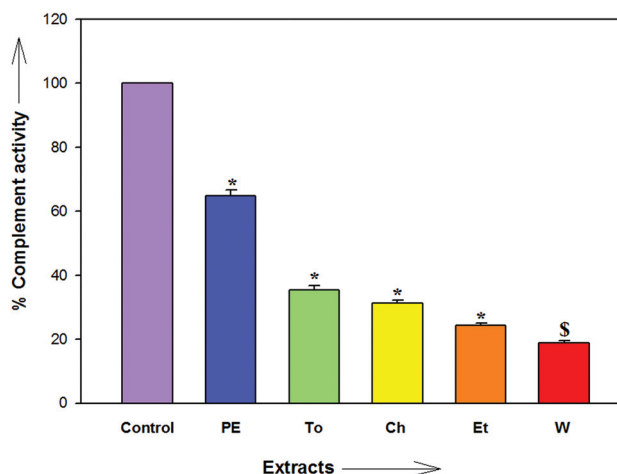


Fig. 2 The effect of 1% crude root bark extract (prepared in different solvents) on complement system inhibition. (PE: petroleum ether; To: toluene, Ch: chloroform, Et: ethanol, W: water). [Each value has been expressed as the mean $\pm$ SD of five independent experiments. *P* values less than 0.01 (**P* < 0.01) and 0.05 ($^{\$}P$ < 0.05) were considered to be statistically significant].

The effect of the MAF on COX-1, COX-2 and 5-LOX

The MAF showed a dose-dependent inhibition of COX-1, COX-2 and 5-LOX activities (Fig. 5) with IC_{50} values of 0.065 $\mu\text{g ml}^{-1}$, 0.008 $\mu\text{g ml}^{-1}$ and 0.083 $\mu\text{g ml}^{-1}$, respectively. Especially in the case of COX-2, the MAF (10 $\mu\text{g ml}^{-1}$) was able to show approximately 98% inhibition.

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

SN	Test	MAF
(1)	Alkaloids	+
(2)	Anthocyanins and anthocyanidins	—
(3)	Anthracene glycosides	—
(4)	Antraquinones	—
(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	—

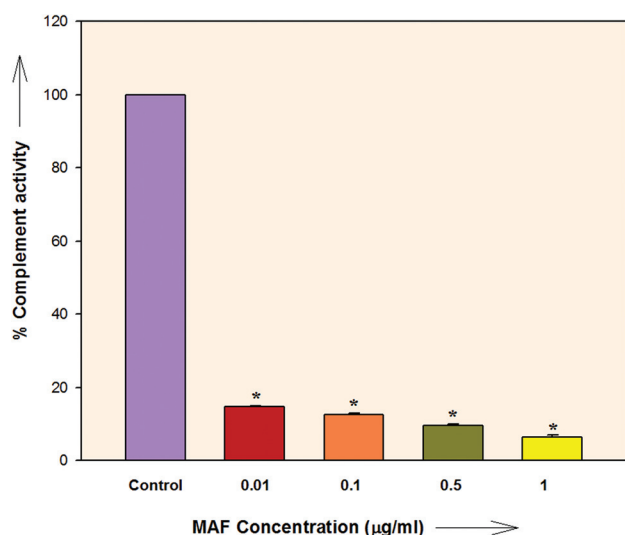


Fig. 3 The effect of the different concentrations of the MAF ($0.01 \mu\text{g ml}^{-1}$, $0.1 \mu\text{g ml}^{-1}$, $0.5 \mu\text{g ml}^{-1}$, $1 \mu\text{g ml}^{-1}$) on complement inhibition. A dose-dependent inhibition was observed. [Each value has been expressed as the mean $\pm$ SD of five independent experiments. The P values less than 0.01 ($*P < 0.01$) were considered to be statistically significant].

The effect of the MAF on the expression of TNF- α , COX-2 and iNOS

On LPS treatment, the levels of TNF- α , COX-2 and iNOS were found to be highly upregulated but in the MAF-treated cells, the downregulation of these proteins in a dose-dependent manner was prominently observed (Fig. 6). TNF- α is always expressed in the 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 the control.³⁷

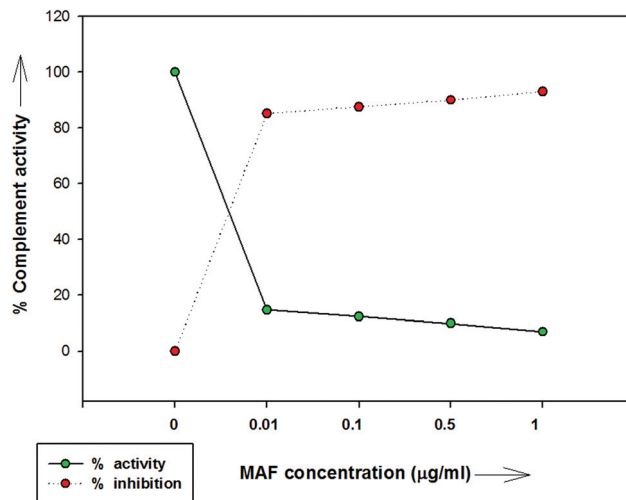


Fig. 4 The IC_{50} value of the MAF in complement inhibition was found to be $0.006 \mu\text{g ml}^{-1}$ after using different concentrations of the MAF ($0.01 \mu\text{g ml}^{-1}$, $0.1 \mu\text{g ml}^{-1}$, $0.5 \mu\text{g ml}^{-1}$, $1 \mu\text{g ml}^{-1}$).

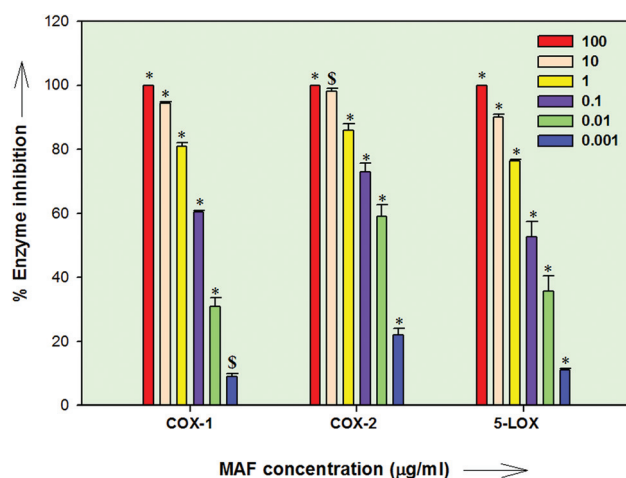


Fig. 5 The effect of the MAF ($100 \mu\text{g ml}^{-1}$, $10 \mu\text{g ml}^{-1}$, $1 \mu\text{g ml}^{-1}$, $0.1 \mu\text{g ml}^{-1}$, $0.01 \mu\text{g ml}^{-1}$ and $0.001 \mu\text{g ml}^{-1}$) on COX-1, COX-2 and 5-LOX inhibition. [Each value has been expressed as the mean $\pm$ SD of five independent experiments. The P values less than 0.01 ($*P < 0.01$) and 0.05 ($^{\circ}P < 0.05$) were considered to be statistically significant].

The effect of the MAF in preventing systemic anaphylactic shock in Wistar rats

The results presented in Table 2 depict the effect of the MAF in preventing anaphylactic shock in Wistar rats. All animals in the PC (positive control) group displayed symptoms of systemic anaphylactic shock (animals remained stationary at least for 1 min) while the rats in the NC (negative control) group did not show any anaphylactic reactions as the antibodies (IgE) for BSA were unable to react and form an immune complex with ovalbumin. In the E1, after 25 oral supplementations of the MAF, the animals did not show systemic anaphylaxis.

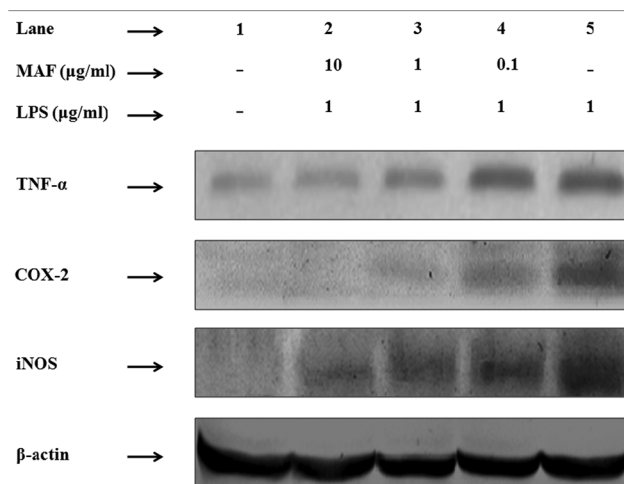


Fig. 6 The effect of the MAF on the downregulation of TNF- α , COX-2 and iNOS against the LPS stimulated RAW 264.7 cell line. Cells were pre-incubated with the MAF (10 $\mu\text{g ml}^{-1}$, 1 $\mu\text{g ml}^{-1}$ and 0.1 $\mu\text{g ml}^{-1}$) for 3 h and stimulated with LPS (1 $\mu\text{g ml}^{-1}$) for an additional 24 h. TNF- α , COX-2 and iNOS expression levels were monitored using western blot analysis; β -actin was used as the loading control.

Table 2 The effect of the MAF on the inhibition of active systemic anaphylaxis in Wistar rats^a

	Sensitizing injection	Shocking injection	Oral MAF treatment	Results	
				S/T	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

^a 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 the case of the PC group, all 10 rats showed anaphylactic shock as compared to the E1 group where none of the rats were recorded with anaphylactic shock. However, in the case of the negative control, as the shocking injection was replaced with ovalbumin instead of BSA, no anaphylactic shock was observed. While in the case of the E2 group, no immediate effect of the MAF was seen.

No pretreatment of the MAF was given to rats in the E2 group. An immediate effect of the MAF in preventing anaphylactic shock was, however, not apparent when injected in combination with BSA in the shocking injection to the E2 group.

The effect of the MAF in preventing the Arthus reaction in Wistar rats

All animals in the PC (positive control) group displayed a positive foot pad reaction within 2 h of the shocking injection (Fig. 7). However, in the NC (negative control) group, the rats did not show the Arthus reaction as the antibodies for BSA were unable to react and form the immune complex with ovalbumin. After 25 oral supplementations of the MAF to the

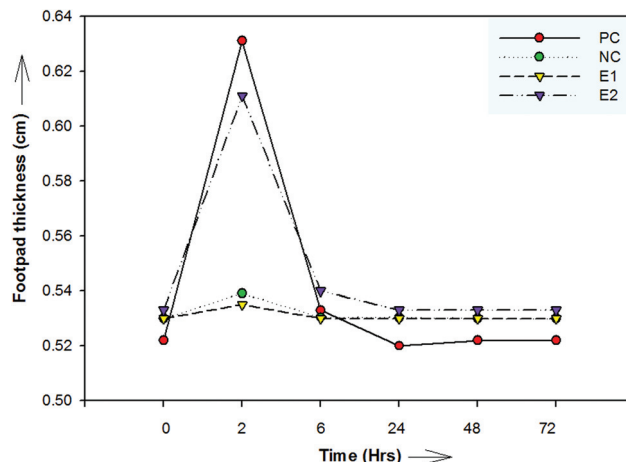


Fig. 7 The effect of the MAF in the prevention of the Arthus reaction in Wistar rats. In the case of the PC group, the right foot pad showed an average increase of approximately 0.11 cm within 2 h as compared to the E1 group where just an average increase of 0.005 cm was recorded. Thus, the severity of the Arthus reaction decreased to nearly 95.5%. However, in the case of the negative control group, where the shocking injection was replaced with ovalbumin instead of BSA, no Arthus reaction was observed. While in the case of the E2 group, the immediate effect of the MAF was not so profound. The data expressed are the means of the readings of all 10 rats.

E1 group, no Arthus reaction was observed in the right foot pad. In the PC group, the right foot pad showed an increase of approximately 0.11 cm within 2 h as compared to the E1 group where an increase of just 0.005 cm was recorded. Thus, the severity of the Arthus reaction was found to decrease to nearly 95.5% (Fig. 8). However, in the E2 group, the MAF was found to be comparatively less effective in inhibiting the Arthus reaction instantly, but had decreased the severity of the foot pad reaction to approximately 30% and brought the foot pad to its normal condition earlier as compared to the PC group.

Discussion

On activation, the complement system keeps on integrating allergic and inflammatory symptoms through complement products like C3a, C4a, etc. These are also called anaphylatoxins, which contribute 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. The complement system synergistically along with these mediators of inflammation (cyclooxygenases and lipoxygenases) highly elevate the allergic and inflammatory reactions.³⁸ The MAF showed quite good complement inhibition activity. The MAF also inhibited COX-1, COX-2 and 5-LOX, but the most satisfactory part of the MAF is its ability to inhibit COX-2 to a much greater extent than COX-1. This is a sign of a good drug as drugs that inhibit COX-1 more than COX-2 are responsible for gastric injuries.^{39,40} From the IC₅₀ values of the complement system, COX-1, COX-2 and 5-LOX, it is clear that all



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

these leaders of allergic and inflammatory responses are strongly inhibited by the MAF.

Despite its beneficial roles in host defense, excessive NO production has been implicated in several allergic and inflammatory diseases. LPS induces I κ B proteolysis and NF- κ B nuclear translocation in RAW 264.7 cells.^{41,42} NF- κ B 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 the MAF is an excellent downregulator of TNF- α , iNOS, and COX-2, indicating that the action of the MAF occurs at the transcriptional level *via* blocking of the NF- κ B signaling pathway.⁴³

Many compounds give good results *in vitro* but fail to act *in vivo*. The MAF, however, has prevented both anaphylactic shock and the Arthus reaction in Wistar rats. In anaphylactic shock, an immediate effect of the preformed IgE antibody is seen which can cause death of an individual in severe conditions. On the other hand, the Arthus reaction is a local dermal inflammatory response. There may be several underlying mechanisms, but the capacity of the MAF to inhibit the complement system is the major one. It is reported that if the complement system is inhibited, the allergic and inflammatory reactions like anaphylactic shock and the 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 the anaphylactic shock and Arthus reaction to a great extent.^{46,47} Therefore, the ability of the MAF to inhibit COX-1, COX-2 and 5-LOX must have played an important role in preventing anaphylactic shock and the Arthus reaction. Parallel to it is the ability of the MAF to downregulate proinflammatory factors, especially iNOS and TNF- α . But, to be rightly effective, a regular pretreatment of the MAF is essential.

Conclusion

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

Acknowledgements

Authors express sincere gratitude to Dr Itankar (Department of Pharmacy, RTMNU, Nagpur) for providing the necessary facilities for this work. Special thanks to Prof. G.B. Shinde, Head, University Department of Biochemistry, RTM Nagpur University, Nagpur, for encouragement.

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