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STUDIES ON THE ANTI-ALLERGIC AND ANTI-INFLAMMATORY ACTIVITIES OF THE COMPOUNDS ISOLATED FROM *ZIZYPHUS MAURITIANA*

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ABSTRACT

Allergy is a type-I hypersensitivity and occurs when body gives immune response against those antigens which are normally nonpathogenic. It might take a dangerous turn on involvement of complement system. Anaphylactic shock is one of such dreadful reactions. Serious roles are also played by inflammatory enzymes such as Cycloxygenase-1, Cycloxygenase-2 and 5-Lipoxygenase in elevating allergic and inflammatory reactions. Though synthetic anti-allergic and anti-inflammatory drugs are available, but show abnormal effect on other body systems. Natural compounds on the other hand are free from side effects. *Zizyphus mauritiana*, Lam (Rhamnaceae) is considered to be rich in phytochemicals in *Ayurveda*. Root barks of *Zizyphus mauritiana* were dried and powdered under controlled conditions followed by extraction in different solvents with increasing polarity. The ethanolic extract of root barks of *Zizyphus mauritiana* was found to be rich in many phytochemicals such as alkaloids, emodins, flavonoids, tannins, tritepenoids and saponins. Hence, it was selected to study its anti-allergic and anti-inflammatory potential. Adsorption column chromatography of the ethanolic extract was performed to isolate a fraction called Anti-allergic and Anti-inflammatory Fraction (AAF). *In vitro*, AAF was found to be effective in inhibiting complement system, COX-1, COX-2 and 5-LOX with IC_{50} values of 0.052 µg/ml, 0.584 µg/ml, 6.33 µg/ml and 0.99 µg/ml, respectively. From the results it was clear that AAF fraction has good anti-allergic and anti-inflammatory ability.

Key words: Complement system, Cycloxygenase, Lipoxygenase, *Zizyphus mauritiana*, Phytochemicals.

INTRODUCTION

Zizyphus genus has been investigated widely for its medicinal properties. The extract of *Zizyphus jujuba* seed has been recommended for treatment of sleep disorders [1]. In the Indian system of medicine, *Zizyphus rugosa* is in use to cure diarrhea, menorrhagia and infection of teeth [2]. *Zizyphus jujuba* leaves and *Zizyphus spina* are identified to have hypoglycemic effect [3, 4]. *Zizyphus* plants are common in various medicinal preparations in India, Egypt and China for treatment of several diseases. Protective effects of seeds of *Zizyphus spinosa* help to overcome the endotoxin fever in mice induced by superoxide dismutase deficiency [5]. Compounds isolated from *Zizyphus* extracts have been shown to possess many therapeutic properties like treatment of malaria, fever and many more. Methanolic extract of the stem bark of *Zizyphus spina* has been recorded for anti diarrheal effect while aqueous extract of the root bark was shown to possess analgesic effect [6]. All the medicinal properties present in a plant are due to its phytochemical contents. *Zizyphus mauritiana* is considered as a treasure of phytochemicals in *Ayurveda* [7]. 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 [8]. Many pharmaceutical preparations involve compounds isolated from root bark which is an indication that root barks of medicinal plants must be rich in phytochemical contents.

Complement system products such as C5a, C2b etc. act as inflammatory mediators and amplify the allergic and inflammatory reactions several folds. Thus, involvement of complement system in allergic and inflammatory reactions can take severe form [9, 10]. Prostanoids and eicosanoids are some other inflammatory mediators produced and secreted in huge quantity by activated phagocytes and mast cells. Their synthesis is catalyzed by Cycloxygenases (COX-1 and COX-2) and 5-lipoxygenase (5-LOX) [11, 12]. COX-2 has been

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considered to be much more dangerous as even a very little increase in its level can lead to many fold increase in allergic and inflammatory reactions in body.

The present work includes the isolation of an active fraction from the ethanolic extract of the root bark of *Zizyphus mauritiana* called as the Anti-allergic and Anti-inflammatory Fraction (AAF) through adsorption column chromatography and investigation into its role in inhibition of complement system, COX-1, COX-2 and 5-LOX.

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. 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

Root barks of plants were collected from forest region of Gadchiroli district of Maharashtra (India) in May 2009 (summer). Authentication of the plant was done by expert taxonomists at University Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur and voucher specimens were deposited in herbarium. Plant was identified to be *Zizyphus mauritiana* (Family Rhamnaceae) with a voucher specimen number 9483 (Date: 09/10/2009).

Preparation of extracts

Root barks of *Zizyphus mauritiana* were properly cleaned and shed dried for approximately two months. Dried barks were crushed in grinder. Using soxhlet apparatus, powdered barks were extracted successively in solvents with increasing order of polarity with petroleum ether (0), toluene (2.7), chloroform (4.1), ethanol (5.2) and water (9). Solvents were evaporated by using rotary vacuum evaporator (Superfit DB3135S). Dried extracts 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.

Phytochemical analysis

Phytochemical analysis of all the extracts of the root barks of *Zizyphus mauritiana* was performed as per the methods proposed by Trease and Evans [13] for almost all known phytochemicals.

Adsorption Column Chromatography

Ethanolic extract of root bark was found to be rich in phytochemicals such as alkaloids, emodins, flavonoids, tannins, tritepenoids and saponins. Hence, it was further purified by adsorption column chromatography. Column (5 x 30 cm) was packed using silica gel (100-200 mesh). Silica gel slurry was prepared in petroleum ether and column was kept undisturbed for 1 h [14, 15]. For column chromatography, 20 g dried powder of ethanolic extract of root bark was applied. Petroleum ether (PE), toluene (To), chloroform (Ch), ethyl acetate, acetone, ethanol, water and their mixtures in various proportions in the increasing order of polarity (50 ml PE, 40 ml PE + 10 ml To, 30 ml PE + 20 ml To, 20 ml PE + 30 ml To, 10 ml PE + 40 ml To, 50 ml To, 40 ml To + 10 ml Ch and so on upto 50 ml Water) were introduced successively in continuation. Fractions of 5 ml each were collected (approx. 3 ml/min.). Thin layer chromatography of each fraction was performed and similar fractions were mixed. Such 14 different fractions were obtained. Dried fractions were dissolved in 0.1% DMSO in PBS and further tested for their ability to inhibit complement system.

Complement system inhibition assay

Complement inhibiting ability of all the 14 fractions (100 µg/ml) was tested [16]. 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 (India). 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 RBCs [17].

After separation from the sheep blood, SRBCs were counted in haemocytometer and the cell number was adjusted to $1x10^9$ SRBCs/ml. Human serum was incubated separately with all the extracts (100 µg/ml 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, $1x10^9$ 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 for 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%.

Out of the 14 fractions, fraction number 8 was found to be more effective in complement inhibition. Hence, the 8^{th} fraction was termed as the Anti-allergic and Anti-inflammatory Fraction (AAF). Effect of AAF on complement inhibition was tested in a similar way at various concentrations (100, 10, 1, 0.5, 0.1, 0.01 µg/ml).

Extraction and isolation of cyclooxygenase-1 enzyme

Microsomal fraction from Ram seminal vesicles was prepared as a source of COX-1 [18]. 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 [19]. *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 AAF to inhibit COX-1 and COX-2 were measured by chromogenic assay [20]. This assay is based on oxidation of N, N, N, N-tetra methyl-*p*-phenylene diamine (TMPD) during conversion of PGG2 to PGH2. Assay mixture contained Tris-HCl buffer (0.5 M), hematin (5 mM), EDTA (0.5M), enzyme (COX-1 or COX-2) and AAF (500, 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 1ml 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 [21]. Capacity of AAF 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), AAF (500, 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.

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

Phytochemical analysis of all the crude extracts

Results of the phytochemical analysis of all the crude extracts were shown in table 1. Ethanolic extract of the root bark was found to contain significant amount of phytochemicals such as alkaloids, emodins, flavonoids, tannins, tritepenoids and saponins.

Fractionation of the ethanolic extract by adsorption column chromatography and isolation of AAF

On application of 20 g of ethanolic extract of root bark for adsorption column chromatography, 4.11 g of active fraction (8^{th}) was obtained which was termed as Anti-allergic and Anti-inflammatory Fraction (AAF).

Effect of various fractions of ethanolic extract of root bark on complement system

All the 14 fractions obtained from ethanolic extract of the root bark were tested for complement inhibition (100 µg/ml). The 8^{th} fraction named as Anti-allergic and Anti-inflammatory Fraction (AAF) was found to be a better in complement inhibition showing nearly 85% inhibition (Fig. 1).

Effect of AAF on complement inhibition

AAF was further studied for complement inhibition at various concentrations (100, 10, 1, 0.5, 0.1, 0.01 µg/ml). A concentration dependent decrease in the complement activity is seen. Even at 1 µg/ml level, AAF has shown approximately 70% complement inhibition (Fig. 2). IC_{50} value of AAF in complement system inhibition was found to be 0.052 µg/ml (Fig. 3).

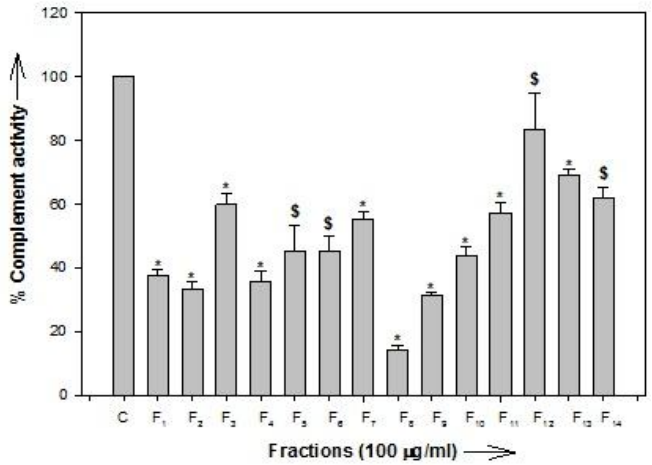
Effect of AAF on COX-1, COX-2 and 5-LOX inhibition

AAF has shown a dose dependent inhibition of COX-1, COX-2 and 5-LOX activities (Fig. 4) with IC_{50} values of 0.584 µg/ml, 6.33 µg/ml and 0.99 µg/ml, respectively (Fig. 5, Fig. 6 and Fig. 7).

Table 1. Phytochemical analysis of all the crude extracts of root bark of *Zizyphus mauritiana*

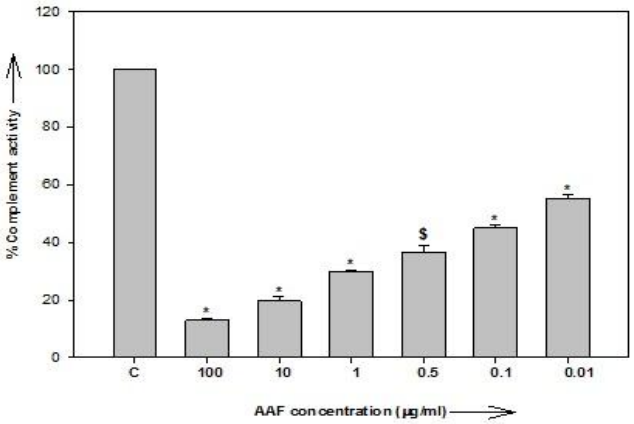
Test	PE	To	Ch	Et	W
Alkaloids	+	+	-	++	++
Emodins	-	-	+	++	-
Flavonoids	-	-	+++	++	+
Tannins	-	-	+	++	+++
Tritepenoids	-	+	+	++	+
Saponins	+	-	+	+	-

Fig 1. Effect of all the fractions (100 µg/ml) of ethanolic extract of root bark on complement system inhibition. Fraction number 8 was found to be more effective



[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 the Anti-allergic and Anti-inflammatory Fraction (AAF) (100, 10, 1, 0.5, 0.1, 0.01 µg/ml) on complement system 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) and 0.05 ($*P*<0.05) were considered to be statistically significant].

Fig 3. IC_{50} value of AAF in complement inhibition was found to be 0.052 µg/ml

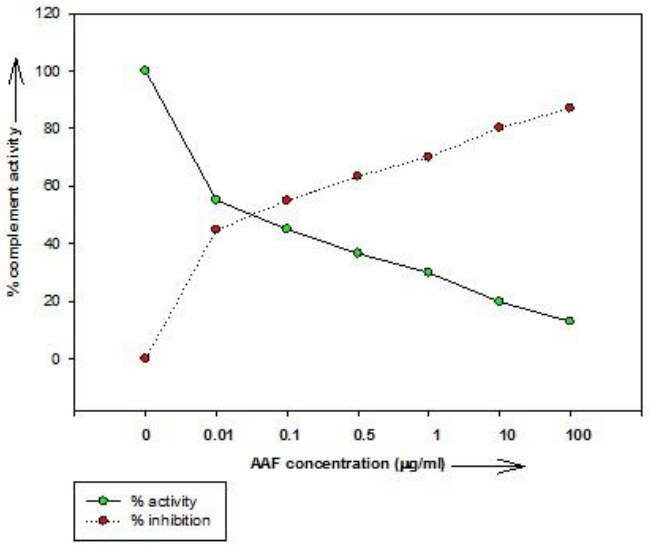
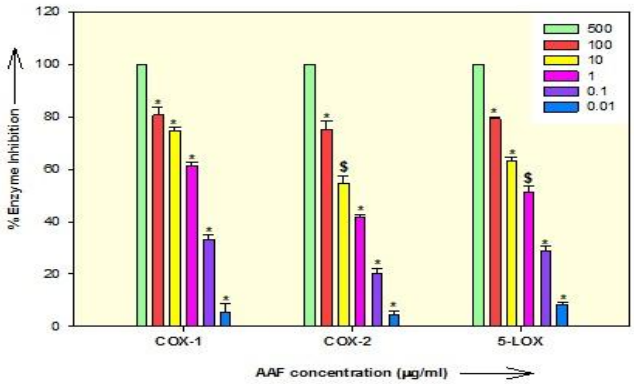


Fig 4. Effect of AAF (500µg/ml, 100µg/ml, 10µg/ml, 1µg/ml, 0.1µg/ml and 0.01µg/ml) on COX-1, COX-2 and 5-LOX inhibition.



[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 5. IC_{50} value of AAF in COX-1 inhibition was found to be 0.584 µg/ml

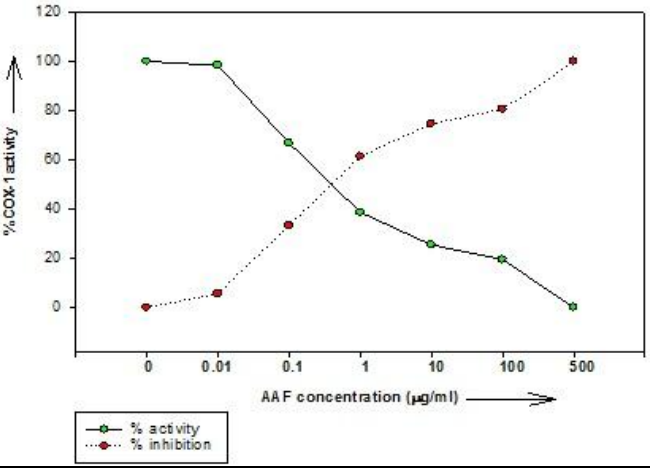


Fig 6. IC_{50} value of AAF in COX-2 inhibition was found to be 6.33 µg/ml

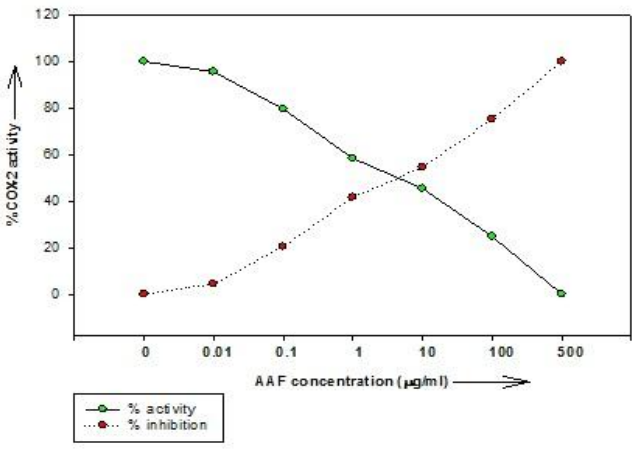
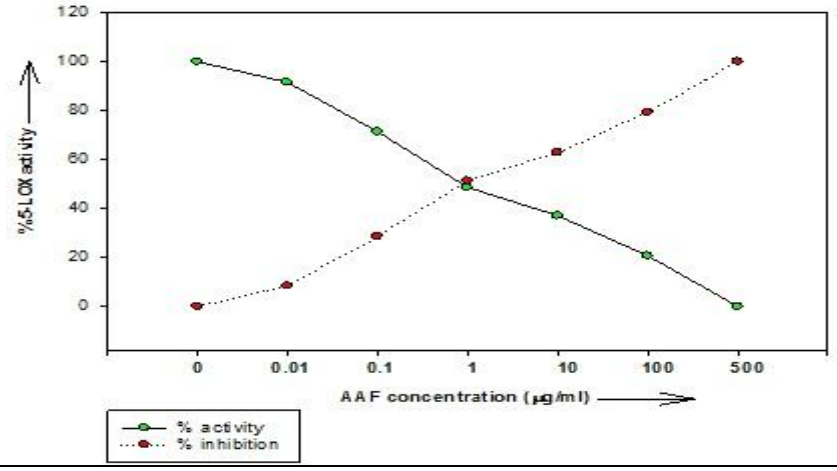


Fig 7. IC_{50} value of AAF in 5-LOX inhibition was found to be 0.99 µg/ml.



DISCUSSION AND CONCLUSION

Most of the anti-allergic and anti-inflammatory drugs available today are synthetic immunosuppressants. They inhibit the immunological response of the body for the allergen and thus body gets rid of the allergic and inflammatory reactions. But these synthetic drugs are always found to be rich in side effects. Hence, researchers are now a days turning their attention towards finding new natural anti-allergic and anti-inflammatory compounds which are free from side effects. Hence, the present work is an effort to find natural compounds with anti-allergic and anti-inflammatory properties. *Zizyphus mauritiana* is a wild medicinal plant found throughout the temperate zone. Since, this plant is rich with phytochemicals, it was selected for the present work.

Complement system on activation; keep on amplifying allergic and inflammatory reactions by forming anaphylatoxins, thus it is a major cause of tissue damage. Similarly, COX-1, COX-2 and 5-LOX are the enzymes involved elevating inflammation. Complement system along with these mediators of inflammation (COX-1, COX-2 and 5-LOX) increase the damaging affects of allergic and inflammatory reactions many folds [22]. AAF isolated from ethanolic extract of root bark of *Zizyphus mauritiana* has shown quite good complement inhibition activity which is the very first signal of antiallergic and anti-inflammatory compounds. From IC_{50} values of COX-1, COX-2 and 5-LOX, it is clear that all the three enzymes of allergic and inflammatory responses are strongly inhibited by AAF. This was the most satisfactory part as AAF as it is capable of hitting the bull's eye.

Thus in the present scenario of costly medication, AAF can act as an effective immunosuppressant.

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