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Original Article

ATTENUATING EFFECT OF TRITERPENOID SAPONIN RICH FRACTION OF *ACHYRANTHES ASPERA* LINN. ON ACUTE AND CHRONIC INFLAMMATION IN EXPERIMENTAL RATS

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ABSTRACT

Objective: *Achyranthes aspera* Linn. is used as a traditional remedy for the treatment of various inflammatory conditions in India. The present study was designed to investigate the anti-inflammatory activity of methanolic extract of *Achyranthes aspera* (AA) and its active fraction using bioassay guided fractionation.

Methods: The dry whole plant of AA was extracted with methanol and then fractionated with different polarity of solvents. Bioactive petroleum ether fraction was re-fractionated into triterpenoid saponin rich (TSR) and non-saponin subfractions and tested for anti-inflammatory activity. The activity of TSR subfraction was evaluated against oxidative stress induced by carrageenan in rat paw tissues.

Results: TSR fraction showed significant ($p<0.05$) inhibition of rat paw oedema volume. Moreover, TSR fraction was also found to attenuate carrageenan induced oxidative damage by improving antioxidant enzymes levels. Furthermore, TSR fraction inhibited both heat and hypotonicity induced haemolysis of erythrocytes *in vitro*. In addition, TSR fraction significantly ($p<0.05$) reduced the granuloma formation in cotton pellet-induced granuloma in rats.

Conclusions: The present study indicates TSR is a potential therapeutic for the treatment of inflammation-associated disorders.

Keywords: *Achyranthes aspera,* Anti-inflammatory, Antioxidant, Triterpenoid saponins.

INTRODUCTION

Inflammation is the host defence response to infection, injury or irritation. It is a complex process involving various mediators, such as prostaglandins, leukotrienes, platelet activating factor (PAF), pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and the reactive free radical nitric oxide (NO) contribute to the development of inflammatory diseases [1,2]. The majority of anti-inflammatory drugs have potent activity, but also they have various and severe adverse effects. Therefore, agents of natural origin are required to study promising medicinal plants so as to develop new and safe drugs.

Achyranthes aspera Linn. (amaranthaceae) commonly known as 'Apamarga' in Sanskrit. It is a small herb found all over India possessing valuable medicinal properties useful in inflammation and rheumatism. *Achyranthes aspera* (AA) has been reported for its anti-inflammatory and anti-arthritic activity, antinociceptive, anti-fungal, immunomodulatory antifertility and abortifacient, antiurolithiatic and anxiolytic activity [3-9]. Previous phytochemical studies have reported that AA is comprised of a mixture of different compounds, such as achyranthine, D-glucuronic Acid, β-D-galactopyranosyl ester of D-glucuronic Acid, oleonolic acid glycosides, ecdysterone, 20-hydroxyecdysone, quercetin-3-O-β-D-galactoside [10-12]. Preliminary study has confirmed the anti-inflammatory activity of methanolic extract of AA in carrageenan-induced rat paw edema [3]. Hence, the present study focuses on the evaluation of the *in vivo* and *in vitro* anti-inflammatory effects of bioactive saponin rich fraction AA through bioassay-guided fractionation techniques.

MATERIALS AND METHODS

Plant material

Whole plant of *Achyranthes aspera* was collected during the month of September 2012 from Thane District, Maharashtra, India. The plant material was authenticated by Prof. Ganesh Iyer, Ramnarain Ruia College, Matunga (East), Mumbai. A voucher specimen (2012/01) of the plant was deposited at the Herbarium of the Department of Pharmaceutical Science and Technology, Institute of Chemical Technology, Mumbai, India for future reference.

Chemicals and Instruments

Indomethacin and λ-carrageenan were obtained from Sigma–Aldrich (St Louis, MO, USA). All other chemicals used in the experiments were of analytical grade. Rats paw volume was measured on Ugo Basile 7140 Plethysmometer.

Extraction and fractionation

Air-dried and powdered whole plant (3.5 kg) of AA was extracted with Methanol in soxhlet apparatus for 72 h. Solvent was removed under reduced pressure yield 418 g of green gummy residue. The extract (400 g) was dissolved in water and partitioned with petroleum ether, chloroform, ethyl acetate and *n*-butanol, successively using a separating funnel. The partitioned fractions were further concentrated to give PAA (petroleum ether, 86 g), CAA (Chloroform, 63 g), EAA (Ethyl acetate, 80 g), BAA (*n*-butanol, 63 g) and AAA (aqueous residue, 91 g). PAA fraction showed significant inhibitory activity against carrageenan-induced paw edema than other fractions. Hence, PAA fraction was partitioned with methanol. Methanolic fraction was further taken up in water and re-extracted with diethyl ether until all chlorophyll pigments were removed. Then aqueous phase partitioned with *n*-butanol. The *n*-butanol fraction yield a saponin mixture (5.60 g) and aqueous residue yield non-saponin mixture (4.12 g). (fig. 1).

Quantification of total triterpenoid content

Total triterpene content was measured according to colorimetric assay using Shimadzu UV–visible spectrophotometer in pet ether fraction, which described by Chen *et al.* (2007) [13]. Briefly, the fraction (0.5 ml) was mixed with the vanillin–glacial acetic acid (5%, w/v, 0.5 ml) and 1 ml perchloric acid. The absorbance was measured at 550 nm. The analysis was performed in triplicate and total triterpenoids content was expressed as oleanolic acid equivalents (µg/mg of fraction) through oleanolic acid standard calibration curve.

Animals

Sprague-Dawley rats aged 7-8 weeks (180-220 g) were procured from Animals house, Haffkine Bio-Pharmaceutical Corporation Ltd., Mumbai. The animals were housed in standard polypropylene cages and maintained under controlled room temperature (22±2 °C) and humidity (55±5%) with 12h light/dark cycle. All the rats were provided with standard pellet diet *ad libitum* and had free access to water. The Animal Ethics Committees of the Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Mumbai approved all experimental protocols in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines (ICT/IAEC/2013/P37 and ICT/IAEC/2014/P15).

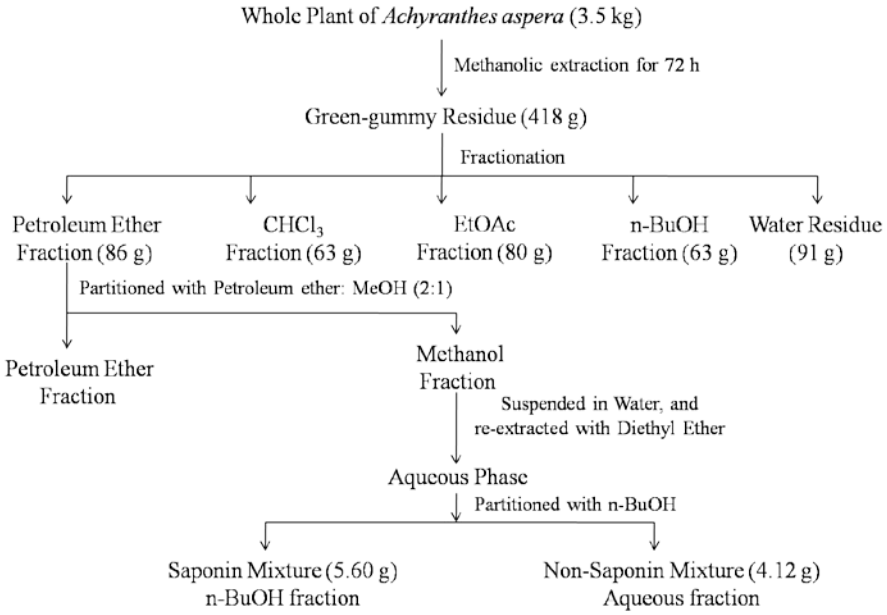

Fig. 1: Schematic representation of extraction and fractionation of *Achyranthes aspera* Linn

Preparation of test samples for bioassay

Test samples were given orally to test animals after suspending in 0.5% sodium carboxymethyl cellulose (CMC). The control group animals received appropriate volumes of dosing vehicle except the drug treatment. Indomethacin (10 mg/kg) was used as a reference drug.

Carrageenan-induced paw oedema in rat [14]

Carrageenan induced rat paw oedema model was used as screening platform for activity guided fractionation procedure for anti-inflammatory activity. One hour after the oral administration of a test sample or dosing vehicle, each rat was injected with 0.1 ml of 1% suspension of λ-carrageenan into subplantar tissue of the left hind paw. Paw volumes were measured at 0, 1, 2, 3 and 4 h after carrageenan injection with a plethysmometer and the volume of oedema at each time point was calculated by subtracting initial paw volume (0 h). The animals were sacrificed and left hind paw were dissected and stored at-70 °C for estimation of antioxidant parameters.

Biochemical estimation

Tissue homogenates were prepared according to the modified method of Anderson (1970) [15]. Paw oedema tissues of each rat treated with 2 ml containing 0.05M Tris buffer, 0.15M NaCl and 0.1 % (v/v) Triton X-100. The mixtures were homogenised on ice. Homogenates were filtered and centrifuged by using a refrigerated centrifuge (15 min, 6000 rpm) at 4 °C. Then, these supernatants were used for the determination of the superoxide dismutase (SOD) [16], catalase (CAT) [17], reduced glutathione (GSH) [18], myeloperoxidase (MPO) activity [19, 20].

Membrane stabilization assay [21]

Preparation of erythrocyte suspension

Fresh human whole blood (6 ml) of was collected in heparinised tubes. The tubes were centrifuged at 3000 rpm for 5 min, and washed three times with equal volume of normal saline. The volume of blood reconstituted as a 40% v/v suspension with 10 mM sodium phosphate buffer (pH 7.4).

Heat-induced haemolysis

Erythrocyte suspension (50 µl) added in isotonic buffer solution containing methanolic extract (200 and 400 µg/ml), TSR fraction (50 and 100 µg/ml) and hydrocortisone (100 µg/ml) of each concentration. Triplicate of tubes were incubated at 54 °C for 20 min. The other triplicates of tubes were maintained at 0–4 °C in a freezer for 20 min. After incubation reaction mixture was centrifuge at 1300 x g for 3 min. Absorbance of the supernatant measured spectrophotometrically at 540 nm. The percent inhibition of haemolysis was calculated.

Hypotonicity-induced haemolysis

Erythrocyte suspension (50 µl) added in hypotonic (distilled water) solution (triplicate) and isotonic solution (triplicate) containing 50 and 100 µg/ml of TSR and hydrocortisone (100 µg/ml) while the control sample was mixed with drug free solution. The mixtures were incubated for 1 h at room temperature (28 °C). After incubation, the reaction mixture was centrifuged for 3 min at 1300 x g and the absorbance (OD) of the supernatant measured spectrophotometrically at 540 nm. The percent inhibition of haemolysis was calculated.

$$\text{Inhibition of haemolysis (\%)} = 100^{*}\left[1 - \frac{OD_2 - OD_1}{OD_3 - OD_1}\right]$$

OD_1 = absorbance of test sample (unheated/isotonic)

OD_2 = absorbance of test sample (heated/hypotonic)

OD_3 = absorbance of control sample (heated/hypotonic)

Cotton pellet induced granuloma

This experiment was done according to winter and Porter (1957) [22]. Sterile cotton pellets weighing 20 mg each was implanted (s. c.)

in the inter scapular area. PAA fraction (200 and 400 mg/kg) and TSR fraction (50 and 100 mg/kg) were administered for 7 days consecutively starting from the day of cotton pellet insertion. The control group received normal saline 1 ml/kg, *p. o.* On an eighth day, the animals were sacrificed and the pellets were dissected out from the surrounding tissues and dried in a hot-air oven at 60 °C till a constant weight was obtained. The difference between the initial and the final dry weights of cotton pellets was considered as the weight of granulomatous tissue produced.

Statistical analysis

The results were expressed as mean±S. E. M. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test for comparison between control and test groups. '*p*' value<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The use of *A. aspera* in traditional Indian medicine in the treatment of various inflammatory disorders implies that active compounds from the plant might be potential anti-inflammation therapeutic agents. Henceforth, the methanolic extract and fractions of AA were screened for anti-inflammatory potential in the carrageenan-induced rat paw oedema model. The experimental results were listed in table 1. The methanol extract AA (200 and 400 mg/kg) effectively inhibits rat paw oedema induced by carrageenan. Fractions of methanolic extracts of AA did not show any remarkable effect against carrageenan-induced paw oedema, except petroleum ether fraction, which was significantly active at 200 and 400 mg/kg doses from 1-4 h. Furthermore, phytochemical analysis of petroleum ether fraction demonstrated that triterpenoids were the major components. The amount of triterpenoids in PAA was 111.67 µg/mg equivalents to oleanolic acid. In order to identify active fraction, fractionation procedure followed to isolate triterpenoid saponins as mentioned in fig. 1. Henceforth, the effect of triterpenoid Saponin rich (TSR) and non-saponin subfractions was investigated in rat paw edema model. Results of the present study was confirmed that, the TSR subfraction showed maximum inhibition 52.09% and 55.67% of paw oedema at dose of 50 and 100 mg/kg at 4 h post-carrageenan administration respectively, while non-saponin subfraction were found to possess weak inhibitory activity against carrageenan-induced oedema model (table 2).

Table 1: Anti-inflammatory effect of methanolic extract and its fractions of *Achyranthes aspera* on carrageenan-induced paw oedema in rats

Treatment	Dose (mg/kg)	1 h	2 h	3 h	4 h
Carr	10 ml/kg Vehicle	0.178±0.017	0.407±0.032	0.622±0.042	0.993±0.029
Indo	10	0.097±0.007* (45.79)	0.223±0.028*** (45.08)	0.293±0.026*** (52.82)	0.335±0.033*** (66.28)
MAA	100	0.158±0.021 (11.21)	0.328±0.037 (19.26)	0.527±0.042 (15.28)	0.812±0.055* (18.29)
	200	0.118±0.023 (33.64)	0.272±0.015** (33.20)	0.393±0.023*** (36.73)	0.532±0.030*** (46.48)
	400	0.107±0.015 (40.19)	0.253±0.015** (37.70)	0.332±0.014*** (46.65)	0.437±0.022*** (56.04)
PAA	100	0.160±0.024 (10.28)	0.347±0.025 (14.75)	0.528±0.021 (15.01)	0.798±0.030* (19.63)
	200	0.115±0.009 (35.51)	0.252±0.022*** (38.11)	0.372±0.041*** (40.21)	0.490±0.031*** (50.67)
	400	0.108±0.011 (39.25)	0.240±0.023*** (40.98)	0.342±0.032*** (45.04)	0.417±0.025*** (58.05)
CAA	100	0.173±0.022 (2.80)	0.405±0.024 (0.41)	0.620±0.025 (0.27)	0.942±0.030 (5.20)
	200	0.152±0.017 (14.95)	0.358±0.021 (11.89)	0.532±0.025 (14.48)	0.868±0.049 (12.58)
	400	0.167±0.01 (6.54)	0.360±0.018 (1.48)	0.567±0.027 (8.85)	0.900±0.047 (9.40)
EAA	100	0.172±0.013 (3.74)	0.402±0.021 (1.23)	0.603±0.019 (2.95)	0.957±0.038 (3.69)
	200	0.173±0.022 (2.80)	0.390±0.027 (4.10)	0.620±0.041 (0.27)	0.967±0.054 (2.68)
	400	0.175±0.027 (1.87)	0.403±0.018 (0.82)	0.610±0.025 (1.88)	0.977±0.030 (1.68)
BAA	100	0.177±0.017 (0.93)	0.402±0.029 (1.23)	0.618±0.016 (0.54)	0.983±0.045 (1.01)
	200	0.173±0.019 (2.80)	0.405±0.032 (0.41)	0.617±0.024 (0.80)	0.990±0.034 (0.34)
	400	0.163±0.022 (8.41)	0.382±0.035 (6.15)	0.558±0.038 (10.19)	0.873±0.044 (12.08)
AAA	100	0.177±0.011 (0.93)	0.383±0.040 (5.74)	0.615±0.038 (1.07)	0.987±0.061 (0.67)
	200	0.172±0.018 (3.74)	0.392±0.013 (3.69)	0.583±0.013 (6.17)	0.948±0.025 (4.53)
	400	0.175±0.016 (1.87)	0.388±0.025 (4.51)	0.595±0.021 (4.29)	0.935±0.048 (5.87)

Values expressed as mean±SEM (n=6); Carr: 1% Carrageenan (0.1 ml); Indo: Indomethacin; MAA: Methanolic extract of *Achyranthes aspera*; PAA: petroleum ether fraction; CAA: chloroform fraction; EAA: ethyl acetate fraction; BAA: *n*-butanol; and AAA: aqueous residue of *Achyranthes aspera*. *p<0.05, **p<0.01, ***p<0.001 compared with control; In parentheses: % inhibition.

Table 2: Anti-inflammatory effect of triterpenoid saponin rich and non-saponin subfractions from PAA fraction on carrageenan-induced paw oedema in rats

Treatment	Dose (mg/kg)	1 h	2 h	3 h	4 h
Carr	10 ml/kg Vehicle	0.292±0.017	0.537±0.044	0.787±0.027	1.117±0.045
Indo	10	0.197±0.012* (32.57)	0.320±0.023*** (40.37)	0.375±0.025*** (52.33)	0.357±0.017*** (68.06)
TSR fr.	50	0.213±0.009* (26.86)	0.337±0.017*** (37.27)	0.448±0.027*** (43.01)	0.535±0.024*** (52.09)
	100	0.192±0.025** (34.29)	0.293±0.029*** (45.34)	0.392±0.026*** (50.21)	0.495±0.025*** (55.67)
NSR fr.	50	0.253±0.027 (13.14)	0.450±0.036 (16.15)	0.643±0.045* (18.22)	0.962±0.038** (13.88)
	100	0.257±0.026(12.00)	0.458±0.034 (14.60)	0.635±0.049* (19.28)	0.947±0.038** (15.22)

Values expressed as mean±SEM (n=6); Carr: 1% Carrageenan (0.1 ml); Indo: Indomethacin, TSR fr.: Triterpenoid saponin rich fraction, NSR fr.: Non-saponin fraction; *p<0.05, **p<0.01, ***p<0.001 compared with control; In parentheses: % inhibition.

Carrageenan-induced oedema has been linked to inflammation mediators like prostaglandins (PGs), leukotriene (LTs) and free radicals such as hydrogen peroxide (H_2O_2), superoxide, and hydroxyl radical, as well as to the release of other neutrophil derived mediators like myeloperoxidase [23]. These reactive oxygen species (ROS) play an important role in inflammatory conditions. ROS causes cellular injury due to impairment of enzymatic and non enzymatic antioxidant defence system [24]. Henceforth, the effect of antioxidant parameters such as SOD, CAT, GSH and MPO were investigated in oedematous paw tissues induced by carrageenan. SOD destroys the highly reactive molecule O_2^- by converting it into less reactive H_2O_2 and that can be destroyed by catalase reaction. Catalase converts H_2O_2 to water and non-reactive oxygen species thus prevents generation of hydroxyl radical, while GSH serve as an electron donor for glutathione peroxidase the formation of conjugates with some harmful endogenous compounds [23]. SOD, CAT and GSH levels were significantly increased after treatment with 50 and 100 mg/kg of TSR sub-fraction, when compared to carrageenan control (table 3). Our results have confirmed that, TSR fraction inhibits lipid peroxidation by increasing the activity of enzymatic antioxidants.

Table 3: Effects of triterpenoid saponin rich fraction on anti-oxidative enzymes activities in carrageenan-induced paw oedema (4 h) in rats

Treatment	Dose	SOD	CAT	GSH	MPO
Control	10 ml/kg Vehicle	63.59±2.526	27.44±0.772	12.97±0.697	11.10±2.159
Carr.	10 ml/kg Vehicle	13.08±1.382###	12.28±0.545###	08.17±0.490###	110.90±8.744###
Indo	10 mg/kg	28.20±1.181***	19.72±0.596***	10.77±0.496**	57.10±3.995***
TSR fr.	50 mg/kg	37.88±1.407***	22.66±0.321***	10.88±0.458**	83.50±1.581**
	100 mg/kg	42.57±1.132***	25.29±0.242***	12.50±0.493***	64.30±6.829***

Values expressed as mean±SEM (n=6); SOD = U/min/mg of tissue; CAT: U/min/mg of tissue; GSH: µg/mg of tissue; MPO: U/min/mg of tissue; Carr: 1% Carrageenan (0.1 ml); Indo: Indomethacin; TSR fr.: Triterpenoid saponin rich fraction; *p<0.05, **p<0.01, ***p<0.001 compared with carrageenan; # indicates carrageenan group was compared with the control.

MPO is components of azurophilic granules of neutrophils. Therefore, MPO activity is directly proportional to the neutrophil concentration on the inflamed tissue [19]. Tissue myeloperoxidase is specific marker for acute inflammation and reflect polymorphonuclear cell infiltration of parenchyma [23]. In view of these finding, the MPO levels were significantly (p<0.01) lower in TSR fraction (50 and 100 mg/kg) when compared with carrageenan treated group (table 3).

To confirm the action of methanolic extract and triterpenoid rich fraction of AA against inflammation, experiment was performed on human erythrocyte membrane. It was found to inhibit the haemolysis of erythrocyte induced by both heat and hypotonic solution. Methanolic extract (200 and 400 µg/ml) and TSR fraction (50 and 100 µg/ml) dose-dependently inhibited heat and hypotonicity induced haemolysis of human erythrocytes *in vitro.* However, TSR fraction showed slightly weak membrane stabilization activity in comparison with methanolic extract of AA (fig. 2). It is speculated that methanolic extract might contain other active component(s), which could be responsible for elevated membrane stabilization activity. Membrane stabilizing activity of AA possibly by increasing the surface area/volume ratio of the cells, either by an expansion of membrane or shrinkage of the cell and an interaction with membrane proteins [25]. In heat induce haemolysis, interaction of fraction with membrane protein may causes cytoprotection, while hypotonicity induced lyses involves process that prevent migration of intracellular components towards outside of cells which occurs due to osmotic loss of intracellular electrolyte and fluid components [21].

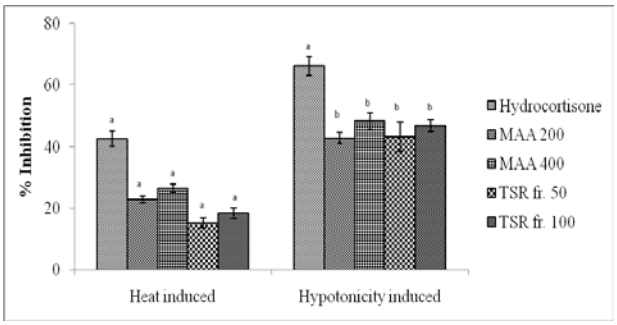

Fig. 2: Effect of methanolic extract and saponin rich fraction of *A. aspera* on heat and hypotonicity induced haemolysis. MAA 200 and 400: Methanolic extract of *Achyranthes aspera* (200 and 400 µg/ml), TSR fr. 50 and 100: Triterpenoid saponin rich fraction of 50 and 100 µg/ml. Values are mean±SEM (n=3); [a]p<0.001, [b] p<0.01 vs. control (no inhibition)

Table 4: Effect of methanolic extract and saponin rich fraction of *A. aspera* on cotton pellets induced granuloma in rats

Treatment	Transudative phase		Proliferative phase	
	Cotton pellet wet weight (mg)	(% inhibition)	Cotton pellet dry weight (mg)	(% inhibition)
Control	236.14±6.760	-	87.17±1.701	-
Indo (1 mg/kg)	52.33±3.169[a]	77.84±1.342	31.48±1.058[a]	63.88±1.214
MAA 200	123.83±3.497[a]	47.57±1.481	58.78±2.331[a]	32.56±2.674
MAA 400	107.00±5.196[a]	54.69±2.200	48.73±2.015[a]	44.09±2.311
TSR fr. 50	130.17±4.438[a]	44.88±1.879	35.88±2.232[a]	58.83±2.561
TSR fr. 100	108.67±9.344[a]	53.99±3.957	32.83±1.969[a]	62.33±2.259

Values expressed as mean±SEM (n=6); [a]p<0.001 compared with control; Indo: Indomethacin; TSR fr. 50 and 100: Triterpenoid saponin rich fraction (50 and 100 mg/kg); MAA 200 and 400: methanolic extract of *Achyranthes aspera* (200 and 400 mg/kg).

To determine the chronic activity of the plant, the methanolic extract and TSR were studied using cotton pellet induced granuloma in rats. The granuloma formation is the main feature of proliferative components chronic inflammatory reaction. The dry weight of cotton pellet correlate with the amount of granulomatous tissue. Proliferative chronic inflammation includes increases in the number of fibroblasts, the infiltration of neutrophils and mononuclear cells, and synthesis of collagen and mucopolysaccharides during granuloma formation [26, 27].

The effect of methanolic extract and triterpenoid enrich fraction on cotton pellet-induced granuloma in albino rats is shown in table 4. The oral treatment with 50 and 100 mg/kg of TSR led to 58.83% and 62.33% reduction of granulomatous tissue formation. These data indicate the inhibition of an important event during the proliferative phase of the inflammatory response. Triterpenoid saponins were reported to inhibit release of inflammatory mediators like cytokines, iNOS and NF-κB and cycloxygenase-2 (COX-2) enzymes [28]. The potent activity of AA may be attributed by the cumulative effect of these major triterpenoid saponins compound.

CONCLUSION

In connection with the role of triterpenoid saponin of *A. aspera,* results of the present study have clearly demonstrated that the AA possesses significant anti-inflammatory activities which support its traditional utilization in India. Further work is currently in progress to identify, and characterise the active compound(s) from the triterpenoid saponins of *achyranthes aspera*.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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