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An Experimental Evaluation of *Ageratum conyzoides* on Membrane Stabilization and Protein Denaturation during Acute Inflammation and Arthritis

ARVIND KUMAR[1], SOURAV GHOSH[2]* and VAISHALI[1]

[1]Department of Pharmaceutical Chemistry, [2]Department of Pharmacology,
S.D. College of Pharmacy & Vocational Studies, Muzaffarnagar (India).
*Corresponding author: E-mail: souravcology@gmail.com

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ABSTRACT

Methanolic leaves extract and isolated flavonoides of *Ageratum conyzoides* was prepared and tested for *in-vitro* anti-arthritic activity. The in-vitro anti-arthritic activity was studied using the membrane stabilization and protein denaturation model. The activity was expressed as percentage inhibition of heamolysis and protein denaturation. The experiment data was expressed as mean ± SEM, the significance of difference among the various treated groups and control group were analysed by the means of one –way ANNOVA followed by dunnett t-test.

The phytochemical screening revealed that presence of flavonoides in large amount. In membrane stabilizing activity different concentration (100, 250 µg/ml) of extract and isolated compound showed significant inhibitory activity (47.39%,54.79% and 63.69% respectively) and in protein denaturation activity, the same extracts and isolated compound have remarkable inhibitory activity on protein denaturation (45.65%, 56.42% and 76.46% respectively) when compared with the control.

Key words: *Ageratum conyzoides,* Flavonoides, Heamolysis, Protein denaturation, Anti-arthritic.

INTRODUCTION

Inflammation is the complex process which is frequently associated with pain and involved occurrences such as increased vascular permeability, protein denaturation and membrane alteration. Inflammatory diseases including different types of rheumatic diseases are a major cause of morbidity of the working force throughout world. This has been called the 'King of Human Miseries'[1]. Although rheumatism is one of the oldest known diseases of the mankind and affects a large percentage of population of the world, no substantial progress was seen till the synthesis of aspirin in 1899 by the German Company Bayer, the hint of which also was obtained from a plant, the Willow bark (Salix alba) used worldwide in folk medicine for the relief of aches, fever and rheumatic pain. Since then many compounds were introduced as a result of laboratory search for drugs with anti-inflammatory activity. Though many of them produced a dramatic symptomatic improvement in rheumatic processes, did not arrest the progress of the diseases process and all of them shared the common side effect i.e., gastro-intestinal irritations[2].

In India, many Ayurvedic practitioners are using various indigenous plants for the treatment of different types of arthritic conditions. Although the application of these medicaments has a sound tradition and a rational background according to the Indian system of medicine, perhaps it is essential

to investigate the rationality of their use in modern scientific terms. The scientific studies to work out the actual efficacy and other limitations to these drugs would definitely widen their scope for future use if they come out to be really effective. This is particularly important, firstly due to the gravity of the problem of rheumatism and arthritis and secondly due to the absence of the right type of drug of synthetic origin for its treatment. The presently available drugs provide only symptomatic relief and are not free from side effects. The target should be to discover newer drugs from plant kingdom which may provide therapeutic cure and would be free from undesirable effects as well as economical, which would be accepted by the developing nations like India. [3]

MATERIAL AND METHODS

Plant material

The leaves of *Ageratum conyzoides* Linn was collected from local area of muzaffarnagar, Uttar Pradesh, India in the month of February, 2011 and authenticated by Botanical Survey of India. (Vide no. CNH/I-I/ (292)/2011/Tech.II/333).

Preparation of extracts

Leaf was shade dried at room temperature and powdered to #40 mesh particle size. The powder (250 g) of crude plant was defatted with petroleum-ether and subjected to extraction with methanol by using Soxhlet apparatus. The extract was filtered and evaporated at 40°C under vacuum and the residue was freeze-dried.

Phytochemical tests[4]

Azaretum conyzoides leaf extract was separately tested for the presence of tannins, alkaloids, carbohydrates, steroids, flavonoids.

Test for alkaloids

1. Mayer's test – 2-3 ml. of solution of extract was added with few drops of Mayer's reagent.
2. Dragendroff's test - 2-3 ml. of solution of extract was added with few drops of Dragendroff's reagent.
3. Hagger's test - 2-3 ml. of solution of extract was added with few drops of Hagger's reagent.

Test for tannins

2-3 ml. of solution of extract was added with few drops of 5% $FeCl_3$ solution.

Test for flavonoids

1. 2-3 ml. of solution of extract was added with 95% ethanol and neutral $FeCl_3$ solution.
2. 2-3 ml. of solution of extract was added with 95% ethanol and lead acetate solution.

Test for carbohydrate

Molish test

2-3 ml. of solution of extract was added with few drops of alpha napthol solution in alcohole, shaked well and add conc.H_2SO_4 from sides of the test tube.

Test for steroid

Salkowski reaction

2-3 ml. of solution of extract was added with 2 ml. of chloroform and 2 ml. of conc. H_2SO_4.

Isolation of flavonoides

Methanol part of extract (5 gm) was successively extracted with Ethyl acetate, and then the Ethyl acetate extract was concentrated to dryness. The Ethyl acetate extract was subjected to a column of silica gel (60-120 mesh; 3 cm dia, X 60 cm length) being eluted a gradient of Pet ether Ethyl acetate with increasing polarity. 10 main fraction were collected and individual fraction were tested for presence of the active bi-flavonoid compounds. Thus from fraction of ether/ Ethyl acetate (10:90), compound I was separated by using preparative TLC using Hexane/ Ethyl acetate in ratio of 8:2 system as eluent.

There were two different spots on the TLC plate, when illuminated with UV light with R_f value of compound 0.28 respectively from point of origin of sample. The compound with R_f value of 0.28, showed relatively higher concentration of flavonoid tests, (Shinoda and NaOH). Compound labeled as AZ-1 (R_f 0.28). The isolated compound was identified after analyzing spectra obtained from IR Spectra.

Preparation of test sample

Sample solutions for *in- vitro* studies were prepared by dissolving 10 mg of dried extract of

Azaretum conyzoides leaf in 0.5 ml water and with phosphate buffer salt solution. According to concentration range from 50 µg/ml, 100 µg/ml and 250 µg/ml and also by dissolving 5 mg of isolated compound of leaf extract in 0.25 ml water and with phosphate buffer salt solution according to concentration 100 µg/ml.

***In-vitro* anti-arthritic study**

Membrane stabilizing activity

The test was followed by the method described by shinde *et al* (1999) [5] with some modifications. Whole human blood obtains from a healthy human volunteer and transferred to heparinised centrifuge tube. The blood was washed three times with isotonic buffer solution (154 mM NaCl) in 10 mM sodium phosphate buffer solution (pH-7.4) for 10 mins at 3000g. The test sample considered to stock erythrocyte (RBC) suspension(0.5 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffer solution (pH-7.4) containing the leaf extract and isolated compound solution or indomethacine (0.1 mg/ml). The control sample considered of 0.5 ml of RBC suspension mixed with hypotonic buffer saline solution alone. The mixture was incubated for 10 mins at room temperature and centrifuge for 10 mins at 3000g and the absorbance of supernatant was measured at 540 nm. Each experiment was carried out triplicate and the average was taken. The percentage inhibition of heamolysis or membrane stabilization was calculated by following equation.

% inhibition of heamolysis = 100 X $(A_1 - A_2/A_1)$

Where

A_1 = Absorption of hypotonic buffer solution alone.

A_2 = Absorption of test sample in hypotonic solution.

Effect on protein denaturation

Test solution (extract and isolated compound) containing different concentration of plant extract or indomethacine (100µg/ml) was mixed with 1 ml of egg albumin solution (1mM) and incubated at 27±1°C for 15 mins. Denaturation was induced by keeping the reaction mixture at 70°C in a water bath for 10 mins. After cooling the turbidity was measured spectrophotometrically at 660nm[6,7]. Percentage inhibition of denaturation was calculated from control where no drug was added. Each experiment was carried out in triplicate and the average was taken.

Percentage inhibition = (Abs control – Abs sample) X 100/ Abs control

Statistical analysis

The experiment data was expressed as mean ± SEM, the significance of difference among the various treated groups and control group were analyzed by the means of one –way ANNOVA followed by dunnett t-test.

RESULTS

Phytochemical screening

The results of the test for chemical constituents are as follows:

Table 1: Preliminary phytochemical investigation

Test	Extract	Result	
		(+)	(-)
Test for alkaloid			
1. Mayer's test	Methanolic extract of leaf	(+) (+) (+)	
2. Dragendroff's test	Methanolic extract of leaf	(+) (+) (+)	
3. Hagger's test	Methanolic extract of leaf	(+) (+)	
Test for tannin (Extract with 5% $FeCl_3$ solution)	Methanolic extract of leaf	(+) (+) (+)	
Test for favonoid	Methanolic extract of leaf	(+) (+) (+)	
Extract of alcohol + neutral $FeCl_3$ solution)			
Test for carbohydrate (Molish test)	Methanolic extract of leaf	(+)	
Test for steroid (Salkowaski test)	Methanolic extract of leaf	(+) (+) (+)	

Table 2: Effect of methanol extract and isolated compound (flavonoides) of *Azaretum conyzoides* leaves on membrane stabilizing activity

Samples	Concentration	Absorption at 540 nm	% of Inhibition
Hypotonic solution	50mM	0.730 ± 0.06	-
Methanolic extracts	50 µg/ml	0.480 ± 0.05	34.24
	100 µg/ml	0.384 ± 0.37*	47.39
	250 µg/ml	0.330 ± 0.24**	54.79
Isolated compound	100 µg/ml	0.265 ± 0.16**	63.69
Indomethacine	100 µg/ml	0.224 ± 0.29 **	69.31

Each value represents the mean ± SEM; n=3, *p<0.05 and **p<0.01 when compared with blank

Table 3: Effect of methanol extract and isolated compound (flavonoides) of *Azaretum conyzoides* leaves on protein denaturation

Samples	Concentration	% of Inhibition of protein denauration
Hypotonic solution	50mM	-
Methanolic extracts	50 µg/ml	24.68
	100 µg/ml	45.65
	250 µg/ml	56.42
Isolated compound	100 µg/ml	76.46
Indomethacine	100 µg/ml	82.67

Preliminary phytochemical investigation reveals the presence of flavonoides. Methanolic extract have showed significant activity in the some of the parameters at higher concentrations (Table 1).

Isolation of flavonoides

Compound AZ-1

Brownish yellow crystal, mp. 225^{0}C, R_f value 0.28, FT-IR (Jasco-5300) (KBR) V max/cm: 3414 (-OH), 1651 (Unsaturated –C=O), 2928 (C-Me), 1072 cm^{-1} glycosidic (C-O) groups that are found in flavonoids[8-9].

Membrane stabilizing activity

Different concentration (100, 250 µg/ml) of extract showed significant membrane stabilizing activity and isolated compound and indomethacine have significant inhibitory activity (63.69% and 69.31% respectively) which is shown in table 2.

Protein denaturation activity

Different concentrations (100, 250 µg/ml) of extracts and isolated compound have remarkable inhibitory activity on protein denaturation when compared with the control, which is shown in table 3.

DISCUSSION

Inflammation is the response of living tissue injury. It involves a complex array of enzyme activation, mediator release, extravastation of fluid, cell migration, tissue breakdown and repair[10].

The vitality of cells depends on the integrity of their membrane, exposure of RBC to injurious substance such as hypotonic medium results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin[11,12]. The haemolytic effect of hypotonic solution is related to exessesive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical induces lipid peroxidation. It is therefore

expected that compound with membrane stabilizing properties, should offer significant protection of cell membrane against injurious substance[13,14,15]. Compounds with membrane stabilizing properties are well known for their ability to interfere with the release of phospholipases that trigger the formation of inflammatory mediators.[16] The extract and isolated compound has shown significant membrane stabilizing activity, which suggests that its anti arthritic activity observed in this study, may be related to the inhibition of release of phospholipases that trigger the formation of inflammatory mediators.

Denaturation of proteins is well documentated cause of inflammation and rheumatoid arthritis. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induce protein denaturation[17]. Ability of *Azaretum conyzoides* extracts and isolated compound to bring down thermal denaturation of protein is possible contribution factor for its anti-inflammatory activity.

The *in-vitro* anti-arthritic activity of *Azaretum conyzoides* found may be due to the presence of flavonoides.

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