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Antiarthritic efficacy of *Clematis orientalis*

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Abstract

Clematis orientalis has been used traditionally to cure rheumatism, but its efficacy has not been scientifically explored in treating rheumatoid arthritis. The present study was designed to investigate the antiarthritic efficacy of *C. orientalis* using blockade of heat-induced protein alterations and HRBC membrane stabilization methods along with formaldehyde induced arthritis in rats. Aqueous ethanol (30:70) extract and various fractions (hexane, *n*-butanol and aqueous) produced concentration-dependent and significant ($p<0.001$) inhibition of protein (egg albumin and bovine serum albumin) denaturation and red blood cell membrane stabilization at 50-6400 ug/mL concentration. Similarly in formaldehyde induced arthritis plant extract and fractions revealed the considerable reduction in paw swelling and its diameter at doses of 50, 100 and 200 mg/kg with maximum ($p<0.001$) effect at the highest dose. Thus present findings imply that aqueous ethanol extract as well as different fractions of *C. orientalis* has noteworthy antiarthritic potential supporting its folkloric use in inflammatory arthritic conditions.

Introduction

Arthritis is a chronic inflammatory disorder affecting primarily joints and it affects about 1-2% of the populace worldwide. Rheumatoid arthritis is an unremitting multisystem disease accompanied by immune hyperactivity, persistent synovitis, and synovial hyperplasia along with deposition of autoantibodies to immunoglobulins leading to articular cartilage annihilation and resorption of osseous matter.

Moreover, current therapeutic modalities are not curative but provide symptomatic relief reducing pain, inflammation, articular damage, functional impairment and systemic complications. Some therapies including NSAIDs, glucocorticoids, DMARDs, biologic response modifiers are used in clinical practices (Subramoniam et al., 2013). Notwithstanding the substantial progress in current therapy of joint problems, exploration for newer drugs continues because the existing synthetic drugs are devoid of satisfactory treatment. Allopathic medications are associated with several side effects like gastric disturbances, cardiovascular risks and kidney injury. Current therapeutic modalities are allied with considerable toxic profile leading to the inclination of public attention towards herbal remedies (Baranwal et al., 2012).

In accordance with World Health Organization (WHO), it has been anticipated that more than 80% of the world's populace relies upon traditional herbal medicines for basic health care needs. Seeing that in most cultural societies conventional herbal remedies like *Vatari Guggulu* (Patel and Pundarikakshudu, 2016), *Berberis orthobotrys* (Alamgeer et al., 2017b), *Rhododendron molle* (Fang et al. 2017) and *Trichilia monadelpha* (Ben et al., 2017) and *Ephedra gerardiana* (Uttra and Alamgeer, 2017) are racially and psychologically more acceptable than modern allopathic treatment because of their safest approach to treat ailments (Ahmad et al., 2012). Since

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the discovery of first anti-inflammatory and analgesic drug aspirin is an evidence for use of phytochemicals in painful inflammatory conditions. Moreover, natural products like medicinal herbs proffer a great source of bioactive compounds that might serve as lead compounds for the discovery of novel and safer drugs for the treatment of inflammatory diseases.

Clematis orientalis Linn commonly known as Bailari cheiti, belongs to *Ranunculaceae* found wildly in Leepa Valley, Muzaffarabad, Pakistan. Its leaves, root, stem bark and flower are used therapeutically. Leaves are used in the form of a paste in combination with the resin of pine on wounds to cure promptly, while root extract is used as a vasodilator in hypertension. Moreover, stem gist is used in syphilis and pimple treatment and flowers are dried and taken with honey and desi ghee to cure rheumatism (Ishtiaq et al., 2012). The present study was designed to pharmacologically validate its use in rheumatoid arthritis using different methods.

Materials and Methods

Drugs and chemicals

Formaldehyde (VWR, International Ltd, England), diclofenac sodium (Sigma-Aldrich, USA), aspirin (Uni-Chem, Germany), bovine serum albumin (Sigma-Aldrich, USA), egg albumin from fresh hen's egg, *n*-butanol (Sigma-Aldrich, USA), sodium chloride (Sigma -Aldrich, USA), disodium hydrogen phosphate (Merck, Germany), potassium dihydrogen phosphate (Riedel-de -Haen, USA), sodium hydroxide (Sigma-Aldrich, USA) and hydrochloric acid (Riedel-de-Haen, USA) were used.

Plant material

Flowers of *C. orientalis* were collected from Gilgit Baltistan and were authenticated by a botanist through macroscopic and microscopic examination. A voucher specimen was deposited in the herbarium of Department of Pharmacology, College of Pharmacy.

Extraction and fractionation

The aqueous ethanolic extract was prepared by cold maceration technique. Air dried flowers were grounded to a fine powder and soaked in aqueous-ethanol (30:70) mixture. Soaking and filtration process was repeated 3 times more. After removal of solvent under pressure in a rotary evaporator, the residue was dried with 12.6% yield from 5 kg powdered plant material (Alamgeer et al., 2015). Different fractions were prepared using extract (120 g) by sequential solvent partition using various organic solvents hexane, dichloromethane, ethyl acetate and butanol so as to concentrate the activities in any one of the resultant composites. Fractions were obtained with following yields as hexane (10.7%), dichloromethane (0.9%) ethyl acetate (2.3%), butanol (24.2%) and aqueous (60.1%). Among the fractions, hexane, butanol and aqueous fractions got in adequate quantities to conduct all investigational procedures (*in vitro* and *in vivo* biological activities).

Animals

Sprague-Dawley rats (200-300 g) had been used in this study. All the rats were kept under standard laboratory conditions of 23 to 25°C, relative humidity 55% with 12 hours light/dark cycle. They were fed with stock rodent diet and tap water.

Experimental protocol

Inhibition of protein denaturation method using Bovine serum albumin.

The reaction mix comprising of 5% aqueous solution of bovine serum albumin (0.5 mL) and 70% ethanolic extract/fractions of *C. orientalis* (0.1 mL) and the standard drug aspirin was prepared at different concentrations. 1N HCl was employed to maintain 6.3 pH. Afterwards, samples were incubated at 37°C for 20 min followed by heating at 57°C for 30 min. Subsequent to refrigeration the samples, 2.5 mL buffer (pH 6.3) was added to each test tube. For control test tube dist. H_2O was used rather than aqueous ethanolic extract/ fractions of the plant while product control did not contain bovine serum albumin. Optical density was taken using spectrophotometer at 660 nm. Blockade of protein distortion was deliberated as follows:

$$\%\text{Age inhibition} = \frac{100 - (\text{Abs. of Ts} - \text{Abs. of PC})}{\text{Abs. of TC}} \times 100$$

(Alamgeer et al., 2017a)

Inhibition of protein denaturation method using fresh egg albumin

In this method reaction mixture (5 mL) contained 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate buffered saline (PBS 6.4) and 2 mL of different concentrations of 70% ethanolic extract of plant/ fractions. Distilled water served as control. The reaction mixture was incubated at 37 ± 2°C in an incubator for 15 min followed by heating at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm. Standard drug diclofenac sodium at same concentrations served as reference control and treated similarly for the determination of absorbance. Following formula has been used to compute protection against pathological alterations of egg albumin:

$$\%\text{Inhibition} = 100 \text{ X } [Vt / Vc\text{-}1]$$

Where, Vt = absorbance of the test sample, Vc = absorbance of control (Uttra and Alamgeer, 2017).

Human red blood cell (HRBC) membrane stabilization method

Preparation of reagents

Alsever's solution was prepared by dissolving 2 g dextrose, 0.8 g sodium citrate, 0.05 g citric acid and 0.4 g sodium chloride in distilled water. The final volume was made up to 100 mL with distilled water. Hypotonic saline was prepared by dissolving 0.4 g of sodium chloride in 100 mL of distilled water. While isotonic saline was prepared by dissolving 0.9 g of sodium chloride in 100 mL of distilled water. Buffer of pH 7.4, 0.2 M was prepared using 2.4 g disodium hydrogen phosphate, 0.2 g of potassium dihydrogen phosphate and 8 g of sodium chloride in 100 mL of distilled water (Gautam et al., 2011).

Preparation of suspension (10% v/v) of human red blood cell (HRBC)

10% v/v suspension of RBCs was prepared using blood from a normal healthy participant with isosaline for use in the experiment by following method as described by (Alamgeer et al., 2015).

Assay of membrane stabilizing activity

Assay mixtures containing 1 mL of PBS, hypotonic saline (2 mL) and 0.5 mL of HRBC suspension and 0.5 mL of different concentrations of ethanol extract of the plant, fractions, standard drug were separately mixed. 1 mL of phosphate buffer, 2 mL of hypotonic saline, 0.5 mL of ethanolic (70%) extract at different concentrations and 0.5 mL of 10% erythrocytes suspension were used as a test solution. 1 mL phosphate buffer, 2 mL water and 0.5 mL of RBCs was used as test control. 1 mL phosphate buffer, 2 mL hypotonic saline, 0.5 mL standard drug solution of varying concentration and 0.5 mL of cell suspension were taken as a standard solution (diclofenac sodium). After that resultant solution heated in an incubator at 37°C for half an hour followed by centrifugation at 3,000 rpm. The optical density of upper layer was assessed at 560 nm. Percentage of HRBC membrane stabilization or protection against lysis has been analyzed using a formula as described by Alamgeer et al. (2017b).

Formaldehyde-induced arthritis in rats

Animals have been divided into different groups with 5 rats in each group. Group I was given distilled water (3 mL/kg; PO) and labeled as a negative control. Group II was treated with piroxicam at 10 mg/kg. Group III, IV and V were treated with crude extract at 50, 100 and 200 mg/kg dose respectively. Group VI, VII and VIII was administered hexane fraction at 50, 100 and 200 mg/kg in that order. Similarly, Group IX, X and XI were given butanol fraction at same doses as given above. Likewise, Group XII, XIII and XIV were treated with aqueous fraction at 50, 100 and 200 mg/kg correspondingly. All treatments were given orally for 10 days respectively. On day 1, 30 min after drug administration, formaldehyde (0.1 mL of 2% solution) was administered subcutaneously in rat left hind paw of all groups. The injection was given again on the 3rd day. The antiarthritic effect was appraised by measuring paw diameter and paw volume of the treated and control rats at alternative days during a period of 10 days by means of digital vernier caliper and digital plethysmometer accordingly and blockade of paw edema was calculated as described by Alamgeer et al. (2017b).

Statistical analysis

Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison tests using the GraphPad Prism. The minimal level of significance was considered as $p<0.05$.

Results

Aqueous ethanol extract and its fractions produced dose-dependent inhibition of protein denaturation. The crude extract exhibited significant ($p<0.001$) inhibition 4073.6% of egg albumin denaturation at 6400 ug/mL. Hexane, butanol and aqueous fraction showed substantial ($p<0.001$) protection 2402.1, 2236.1 and 2702.1% against protein denaturation at 6400 ug/mL respectively. Diclofenac sodium produced 717.4% inhibition of protein denaturation. These findings depict that crude extract and fractions possess greater potential to inhibit protein denaturation than diclofenac sodium as given in Table I.

Moreover, hydroalcoholic extract showed significant ($p<0.001$) and dose reliant blockade of heat-induced conformational changes in BSA as 91.9% at 6400 ug/mL while hexane, butanol along with aqueous fraction revealed notable ($p<0.001$) protection as 92.9, 94.1 and 91.5% against BSA denaturation at highest concentration in that order as shown in Table I. Aspirin showed 80.6% inhibition of protein denaturation at the same concentration.

In membrane stabilization method diclofenac sodium exhibited 70.5% protection against red blood cell lysis. Ethanolic extract showed noteworthy ($p<0.001$) as 73.7% HRBC membrane stabilization whereas hexane, butanol and aqueous fraction significantly ($p<0.001$) protected the red blood cells against hypotonicity-induced lysis and demonstrated 76.5, 76 and 78.7% membrane stabilization correspondingly as illustrated in Table I .

In formaldehyde-induced arthritis hydroethanolic extract and various fractions reduced paw edema in dose-dependent manner. At 200 mg/kg oral dose hydroalcoholic extract showed significant ($p<0.001$) decrease in volume of inflammatory exudate of rat paw as 78.5

Table I

Effect of *Clematis orientalis* on inhibition of protein denaturation and HRBC membrane stabilization

Treatment Groups	Percentage protection against denaturation of egg albumin concentration (ug/mL)							Percentage protection against protein bovine serum albumin denaturation concentration (ug/mL)							Percentage protection against membrane lysis concentration (ug/mL)						
	50	100	200	400	800	1600	3200	50	100	200	400	800	1600	3200	50	100	200	400	800	1600	3200
Diclofenac sodium	66.7 (5.2)	67.4 (4.2)	69.4 (1.4)	77.1 (2.4)	88.1 (1.9)	182.6 (2.5)	370.8 (2.1)								20.3 (0.1)	21.8 (0.2)	22.6 (0.2)	24.6 (0.2)	26.7 (0.1)	31.4 (0.2)	49.5 (0.1)
Aspirin								62.4 (0.6)	64.7 (0.5)	66.6 (0.4)	69.3 (0.5)	72.6 (0.4)	74.7 (0.4)	77.6 (0.5)							
Aqueous ethanol extract	136.1 (5.0)[a]	167.4 (3.0)[c]	197.9 (4.3)[c]	250.9 (11.4)[c]	379.2 (3.6)[c]	893.1 (5.9)[c]	2533.3 (4.3)[c]	67.7 (2.1)[a]	70.7 (0.5)[b]	72.7 (2.3)[b]	77.9 (0.4)[c]	79.3 (0.7)[b]	83.4 (0.2)[c]	88.8 (2.0)[c]	25.8 (0.8)[c]	30.5 (0.1)[c]	36.2 (0.2)[c]	39.5 (0.1)[c]	44.8 (0.1)[c]	56.0 (0.3)[c]	60.5 (0.2)[c]
Hexane fraction	126.4 (5.0)[ns]	136.1 (1.4)[a]	141.7 (2.4)[a]	161.7 (3.0)[b]	320.1 (6.2)[c]	744.4 (6.1)[c]	1207.6 (64.1)[c]	67.5 (1.3)[a]	70.2 (0.5)[a]	72.4 (0.6)[b]	76.8 (2.8)[c]	79.5 (3.2)[c]	84.4 (0.7)[c]	90.8 (0.4)[c]	31.6 (0.9)[c]	35.2 (0.2)[c]	40.5 (0.3[c]	45.6 (0.2)[c]	51.6 (0.1)[c]	58.1 (0.2)[c]	65.1 (0.1)[c]
n-Butanol fraction	125.7 (2.8)[ns]	136.1 (1.4)[a]	139.6 (4.3)[a]	147.9 (3.6)[a]	238.9 (3.0)[c]	447.9 (6.4)[c]	952.1 (6.0)[c]	68.8 (0.5)[b]	70.6 (0.5)[b]	74.6 (1.6)[c]	77.6 (0.1)[c]	82.4 (0.4)[c]	86.4 (0.5)[c]	90.8 (0.1)[c]	33.2 (1.3)[c]	38.8 (0.2)[c]	44.3 (0.2)[c]	51.1 (0.2)[c]	54.7 (0.2)[c]	63.7 (0.2)[c]	70.3 (0.1)[c]
Aqueous fraction	141.7 (8.4)[b]	154.9 (4.9)[b]	165.9 (3.7)[c]	191.7 (13.6)[c]	263.9 (1.8)[c]	590. (5.7)[c]	1157.6 (4.2)[c]	68.9 (1.6)[b]	70.9 (0.6)[b]	75.0 (0.4)[c]	79.0 (0.8)[c]	83.1 (0.2)[c]	86.1 (0.3)[c]	89.4 (0.1)[c]	37.5 (0.3)[c]	45.4 (0.2)[c]	48.0 (0.1)[c]	54.9 (0.1)[c]	59.0 (0.2)[c]	66.5 (0.2)[c]	72.8 (0.2)[c]

Data is given as mean ± SEM; n = 3; Two-way ANOVA, $p<0.05$ was considered as significant as compared to standard control where [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$, ns = non-significant

Table II

Effect of crude extract and different fractions of *C. orientalis* on paw volume in formaldehyde induced arthritis

Treatment groups	2nd day	4th day	6th day	8th day	10th day
	Increase in paw volume (mL)				
Arthritic control (3 mL/kg)	1.5 ± 0.1	1.8 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.1 ± 0.0
Piroxicam (10 mg/kg)	1.2 ± 0.1^b	1.0 ± 0.1^c	0.9 ± 0.1^c	0.7 ± 0.0^c	0.5 ± 0.0^c
Aqueous ethanol extract (50 mg/kg)	1.2 ± 0.1^b	1.3 ± 0.1^c	0.8 ± 0.1^c	0.7 ± 0.1^c	0.6 ± 0.0^c
Aqueous ethanol extract (100 mg/kg)	1.1 ± 0.1^c	1.2 ± 0.0^c	0.8 ± 0.0^c	0.6 ± 0.0^c	0.6 ± 0.0^c
Aqueous ethanol extract (200 mg/kg)	0.9 ± 0.0^c	1.1 ± 0.0^c	0.7 ± 0.0^c	0.5 ± 0.0^c	0.4 ± 0.0^c
Hexane fraction (50 mg/kg)	1.2 ± 0.1^{ns}	1.4 ± 0.1^c	1.2 ± 0.1^c	1.0 ± 0.1^c	0.9 ± 0.1^c
Hexane fraction (100 mg/kg)	1.1 ± 0.0^b	1.3 ± 0.0^c	1.0 ± 0.1^c	0.9 ± 0.1^c	0.8 ± 0.1^c
Hexane fraction (200 mg/kg)	1.0 ± 0.1^c	1.2 ± 0.0^c	0.9 ± 0.1^c	0.8 ± 0.1^c	0.7 ± 0.0^c
Butanol fraction (50 mg/kg)	1.1 ± 0.1^c	1.3 ± 0.1^c	1.0 ± 0.1^c	0.9 ± 0.1^c	0.8 ± 0.0^c
Butanol fraction (100 mg/kg)	0.9 ± 0.1^c	1.2 ± 0.1^c	0.8 ± 0.1^c	0.7 ± 0.1^c	0.6 ± 0.1^c
Butanol fraction (200 mg/kg)	0.9 ± 0.1^c	1.1 ± 0.0^c	0.7 ± 0.0^c	0.6 ± 0.0^c	0.5 ± 0.0^c
Aqueous fraction (50 mg/kg)	1.0 ± 0.1^c	1.2 ± 0.1^c	0.9 ± 0.1^c	0.8 ± 0.0^c	0.7 ± 0.0^c
Aqueous fraction (100 mg/kg)	0.9 ± 0.0^c	1.1 ± 0.0^c	0.8 ± 0.1^c	0.7 ± 0.0^c	0.7 ± 0.0^c
Aqueous fraction (200 mg/kg)	0.8 ± 0.0^c	1.1 ± 0.1^c	0.7 ± 0.0^c	0.6 ± 0.0^c	0.5 ± 0.0^c

Data is represented as mean ± SEM (n = 5) analyzed by Two-way ANOVA and $p<0.05$ was considered as significant as compared to arthritic control. Where, $^a p<0.05$, $^b p<0.01$, $^c p<0.001$, ns = non-significant

and 74.9% decline in diameter as compared to arthritic control. While, hexane, butanol and aqueous fraction showed 64.2, 71.8 and 75.6% inhibition of paw swelling at maximum dose correspondingly as depicted in Table II. Likewise, hexane, butanol and aqueous fractions substantially decreased foot thickness as 60.7, 70.6 and 72.7% at the highest dose. Furthermore, piroxicam showed 71.9% decrease in paw edema along with 74.9% reduction in paw thickness at 10 mg/kg as demonstrated in Table III.

Discussion

Findings of present investigation have revealed that aqueous ethanolic extract and various fractions of *C. orientalis* dose-dependently exert antiarthritic effects.

Denaturation of tissue proteins is a well-recognized cause of various inflammatory arthritic diseases and generation of autoantigens in various rheumatic diseases might be owing to *in vivo* protein denaturation. It has been stated that alterations in hydrophobic, electrostatic, hydrogen and disulfide bonding is a probable mechanism in protein denaturation (Alamgeer et al., 2017b; Gupta et al., 2013).

Moreover, it has been documented that numerous non-steroidal anti-inflammatory drugs including diclofenac sodium, salicylic acid, indomethacin and flufenamic acid inhibit malformations of bovine albumin mediated by detrimental mileu (Alamgeer et al., 2017b). Altogether present findings suggest that possible antiarthritic effect of *C. orientalis* might be owing to inhibition of protein denaturation.

Aqueous ethanolic extract and its fractions averted the protein (egg albumin and bovine serum albumin) alterations more noticeably than standard drugs. The cell membrane of erythrocyte resembles lipid bilayer of neutrophilic lysosomes and maintenance of lysosomal membrane against hypotonicity induced lysis proposes another mechanism of antiarthritic effect. As, hyposaline causes massive accumulation of fluid resulting in cell rupture and damage to lysosomal membrane initiate discharge of phospholipase A2 that hydrolyze membrane phospholipids resulting in a cascade of inflammatory mediators (Umukoro and Ashorobi, 2006; Aitadafoun et al., 1996). Consequently, stability of phospholipid sheath prevents cell rupture and resultant discharge of cellular contents, which consequently alleviate tissue destruction and provocative reactions (Okoli et al., 2008).

Thus, data obtained imply that ethanolic extract, hexane, butanolic and aqueous portions of *C. orientalis* extract have the potential to exert concentration-dependent protection of lysosomal membrane rupture compared with standard diclofenac sodium. Albeit, precise action for maintaining constancy of RBCs lipid-bilayer has not been identified but the direct interaction of phytochemicals with membrane proteins, which make ion channels, seems nearly all probable (Umukoro and Ashorobi, 2006). In formaldehyde induced arthritis *C. orientalis* appreciably and dose-dependently decreased edematous swelling all through the study

Table III

Effect of crude extract and different fractions of *C. orientalis* on paw diameter in formaldehyde-induced arthritis

Treatment groups	2nd day	4th day	6th day	8th day	10th day
	Increase in paw diameter (mm)				
Arthritic control (3 mL/kg)	6.4 ± 0.0	8.4 ± 0.0	10.5 ± 0.0	11.1 ± 0.1	12.7 ± 0.1
Piroxicam (10 mg/kg)	4.8 ± 0.1[c]	5.7 ± 0.3[c]	5.2 ± 0.1[c]	4.1 ± 0.0[c]	3.7 ± 0.1[c]
Aqueous ethanol extract (50 mg/kg)	5.2 ± 0.1[c]	5.6 ± 0.2[c]	5.1 ± 0.1[c]	4.8 ± 0.0[c]	4.7 ± 0.1[c]
Aqueous ethanol extract 100 mg/kg	4.7 ± 0.1[c]	5.3 ± 0.0[c]	4.8 ± 0.0[c]	4.4 ± 0.3[c]	4.3 ± 0.5[c]
Aqueous ethanol extract 200 mg/kg	4.4 ± 0.0[c]	4.7 ± 0.1[c]	3.9 ± 0.0[c]	3.4 ± 0.0[c]	3.2 ± 0.0[c]
Hexane fraction 50 mg/kg	5.6 ± 0.3[a]	6.8 ± 0.4[c]	6.2 ± 0.3[c]	6.0 ± 0.3[c]	5.6 ± 0.3[c]
Hexane fraction 100 mg/kg	5.0 ± 0.2[c]	6.2 ± 0.1[c]	5.9 ± 0.1[c]	5.3 ± 0.1[c]	5.1 ± 0.1[c]
Hexane fraction 200 mg/kg	5.1 ± 0.2[c]	5.8 ± 0.1[c]	5.5 ± 0.0[c]	4.9 ± 0.2[c]	5.0 ± 0.1[c]
Butanol Fraction 50mg/kg	5.5 ± 0.1[a]	5.8 ± 0.3[c]	5.0 ± 0.2[c]	4.9 ± 0.0[c]	4.8 ± 0.2[c]
Butanol fraction 100 mg/kg	4.9 ± 0.2[c]	5.2 ± 0.1[c]	4.7 ± 0.3[c]	4.3 ± 0.3[c]	4.1 ± 0.3[c]
Butanol fraction 200 mg/kg	4.8 ± 0.1[c]	5.0 ± 0.1[c]	4.4 ± 0.1[c]	4.1 ± 0.1[c]	3.7 ± 0.1[c]
Aqueous fraction 50 mg/kg	5.2 ± 0.2[c]	5.7±0.2[c]	4.8 ± 0.1[c]	4.6 ± 0.2[c]	4.2 ± 0.1[c]
Aqueous fraction 100 mg/kg	4.8 ± 0.0[c]	5.0 ± 0.1[c]	4.7 ± 0.0[c]	4.2 ± 0.2[c]	3.8 ± 0.2[c]
Aqueous fraction 200 mg	4.6 ± 0.2[c]	4.8 ± 0.1[c]	4.0 ± 0.1[c]	3.9 ± 0.1[c]	3.4 ± 0.0[c]

Data is denoted as mean ± SEM (n = 5); by Two-way ANOVA. $p<0.05$ was considered as significant as compared to arthritic control. Where, [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$, ns = non-significant

with maximum effect at highest dose that is 200 mg/kg.

Formaldehyde-induced arthritis is generally used as a model to induce acute non-immunological arthritis for investigating a newer antiarthritic drug (Greenwald, 1991). Previously it has been reported that inflammation of rat paws involve swelling of fibrous joint capsule subsequent to formaldehyde injection (Rakesh and Prashant, 2012). In addition, formaldehyde produces local inflammation accompanied by distinct biphasic pain explicitly an early neurogenic pain after that inflammatory algesia (Owoyele et al., 2011). It is noteworthy to state that while inhibiting paw swelling hydroalcoholic extract and its fractions also have the potential of acting both centrally as well peripherally in alleviating biphasic pain possibly by inhibiting cyclooxygenase as well as lipoxygenase pathway with reference to aspirin. Moreover, in preceding studies, it has been documented that formaldehyde causes the denaturation of proteins at the site of injection thus instigating immunological reaction against besmirched products (as antigens) thus, provoking joint edema (Gardner, 1960).

Results of *in vitro* methods of protein denaturation support anti-arthritic effect of *C. orientalis* by neutralizing the autoantigens produced during *in vivo* denaturation. In earlier studies it has been recognized that formaldehyde causes synovial fibroblast overgrowth leading to synovial hyperplasia, pannus formation, discharge of various factors that prop up inflammation, neovascularization, cartilage and joint tissue obliteration as seen in rheumatoid arthritis (Saleem et al., 2011). Thus, *C. orientalis* has potential antiarthritic activity as affirmed by results of formaldehyde induced arthritis. Beforehand, it has been well acknowledged that various clematis species are rich in a variety of bioactive compounds mainly saponins, flavonoids, lignans, anemonin, protoanemonin, ranunculin and essential oils responsible for their antimicrobial, anti-inflammatory, antiarthritic, anti-cancer, antimalarial, hepatoprotective and cardioprotective effects.

Likewise, these phytochemicals exert antiarthritic effects by inhibiting formation of reactive oxygen species, activities of cyclooxygenase-1, cyclooxygenase-2 and 5-lipoxygense and on various immunomodulatory molecules like cytokines and chemokines (Jin, 2012). Thus, antiarthritic activity of *C. orientalis* might be linked to the presence of such biologically active principles.

Conclusion

C. orientalis possess substantial antiarthritic potential as avowed by aforementioned results.

Ethical Issue

Animals were treated according to standard procedures guided by National Research Council 1996 (NRC, 1996). Institutional Animal Ethical Committee at College of Pharmacy approved all experimental procedures (Approval No. 41B66 IEC UOS).

Conflict of Interest

The authors have no conflicts of interest.

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