ORIGINAL ARTICLE

Standardization protocol development of hydroalcoholic extract of fruits of *Citrullus Colocynthis* against anti-arthritic activity

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Abstract

Aim: So, in the present study, it was targeted to develop standardization protocol of hydroalcoholic extract of fruits of *Citrullus colocynthis* against the anti-arthritic activity. **Materials and Methods:** Hydroalcoholic extract of fruit was prepared, and the physical, phytochemical, and chromatographic evaluation of extract were carried out then after pharmacological evaluation for the anti-arthritic activity was done with paw edema method and membrane markers (aspartate transaminase [AST] and alanine transaminase [ALT]). **Results and Discussion:** The percentage yield of extract was found to be 2.915%. The phytochemical investigations and high performance thin layer chromatography (HPTLC) analysis indicate the presence of Quercetin, a flavonoid which may be responsible for its anti-arthritic activity. Evaluation of paw volume and membrane markers (AST and ALT) showed significant results against anti-arthritic activity **Conclusion:** The biological activity concluded that *C. colocynthis* fruit's hydroalcoholic extract has shown significant anti-arthritic activity which may be due to some sort of phytochemicals such as flavonoids.

Key words: Arthritis, *Citrullus colocynthis*, high performance thin layer chromatography, membrane markers

INTRODUCTION

Nature has gifted us a vast number of plants having different biological activities to cure all ailments of mankind. The Indian subcontinent is enriched by a variety of flora - both aromatic and medicinal plants. There are estimated 250,000 species of higher plants and in total around 30 million species are present.[1] The WHO has identified 3,000 plants from the forests of India and other tropical countries, which can be used as medicine. Since, time immemorial herbal medicines have been exclusively used by men for the treatment of myriad illness. Herbal medicines are more effective to preventive rather than cure a disease. Lower cost and reduced side effects as compared to conventional medicines have increased great reliability on holistic medicine.[2]

Citrullus colocynthis is among one of these herbal medicines which have been extensively used for its curative properties. It is used as a laxative, purgative, and in abortion. It also has antibacterial,[3] anti-diabetic,[4] analgesic, and anti-inflammatory action.[5] The plant also shows growth inhibitory effect in cancer.[6] Moreover, it has been evident from the recent studies that extract of fruits of *C. colocynthis* has been found to shown anti-arthritic activity. Rheumatoid arthritis is an autoimmune disease that causes chronic inflammation of the joints. The joint inflammation of rheumatoid arthritis causes swelling, pain, stiffness, and redness in the joints. Chronic inflammation leads to the destruction of the cartilage, bone, and ligaments causing deformity of the joints. So, in the present study, it was aimed to develop a standardization protocol for the anti-arthritic activity of hydroalcoholic extract which contains quercetin and cucurbitacin glycoside[7] and responsible for the various biologic activities of fruits of *C. colocynthis*.[8-11]

MATERIALS AND METHODS

Materials *C. colocynthis*

The plant material (fruits) of was collected from Bikaner during the month of September-October, 2010. The plant

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material was identified and authenticated by Dr. Rajesh Garg, (Lecturer, B.R. Nahata College of pharmacy, Mandsaur, M.P.) and specimen number is BRNCP/C/014/2011/*C. colocynthis*. All the reagents and chemicals were of analytical grade.

Methods

Preparation of extract

The dried sample (300 mg) was soxhlet extracted in 80% methanol for 24 h. The extract was concentrated and re-concentrated in petroleum ether (40-60°C) (fraction I), ethyl ether (Fraction II), and ethyl acetate (Fraction III) in succession. Each of the steps was repeated three times to ensure complete extraction in each case. Fraction I was rejected since it was rich in fatty substances. Fraction II was analyzed for the free flavonoids in the sample. Fraction III of the test sample was hydrolyzed by refluxing with 7% H2SO4 (10 ml/g residue) for 5 h. The mixture was filtered and the filtrate extracted with ethyl acetate in separating funnel. The ethyl acetate layer was washed with distilled water until neutrality and dried. The residue was taken up in small volumes of ethanol separately and then subjected to various tests.[11,12]

The percentage yield of extract was calculated using following formula:

$$\text{Percentage yield} = \frac{\text{Weight of extract}}{\text{Weight of powder material}} \times 100$$

Evaluation of extract

The physical, phytochemical, and chromatographic evaluation of extract was carried out. Physical evaluation involves the determination of ash value, extractive value, and moisture content.[13] The phytochemical evaluations were employed for the detection of alkaloids, glycosides, flavonoids, saponins, tannins, sterols, and tri-terpenoids, etc. The extract was subjected to chromatographic evaluation for identification of the flavonoids, particularly for the quercetin.[14] The experimental conditions for high performance thin layer chromatography (HPTLC) were shown in Table 1 as follows:

Table 1: Chromatographic conditions for quercetin

Sample name	Hydroalcoholic fruit extract of *C. colocynthis*
Standard compound	Quercetin
Stationary phase	HPTLC plates silica gel 60 F 254, 5×10 cm
Mobile phase	Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26)
Sample solvent	Methanol
Sample volume	2 µl
Solvent front	8 cm

C. colocynthis: Citrullus colocynthis

Pharmacological investigations

Animals

Albino rats of Wistar strain weighing around 120-180 g were used. All animals were housed in polypropylene cages in a temperature controlled animal house room at 24 ± 1°C temperature, 60 ± 5% relative humidity and 12 h light and 12 h dark cycles. The animals were fed with pelleted feed with standard rat diet and tap water throughout the experiment. These animals were used for the anti-arthritic activity.

Induction of arthritis in rats

Rheumatoid arthritis was induced by synthetic adjuvant oil, pristine. 2 weeks after a single intradermal injection of 150 µm (0.15 ml) of pristine in right hind paw; the rats were developed arthritis. A glass syringe (1 ml) with the locking hubs and a 26 G needle was used for injection. The swelling in hind paws was periodically examined in each paw from the ankle using a plethysmometer.[8]

Experimental setup

Before any treatment rats were divided into four groups, each group contains six animals as followed for biological analysis.

Group I-Normal control: Rats were injected intradermally 0.9%, 0.1 ml saline.

Group II-Arthritic control: 0.15 ml of pristine was injected into right hind paw of the rats on day zero.

Group III-Treatment group: Pristane-induced arthritic rats were administered with fruit extract dose 100 mg/kg body weight/rat/day by oral administration

Group IV-Standard group: 0.7 mg/kg body weight/rat/day dexamethasone by intraperitoneal injection.[8,9]

Evaluation of arthritis

Inflammation parameters - measurement of paw volume

Anti-inflammatory effect of the drug was evaluated by measurement of physical changes in the right hind paw of rats with the help of mercury plethysmometer, equipped with accurate measurement of the rats paw swelling through dislocation of fluid volume. Paw volume was examined after every 3-4 days. Paw volume was measured on day zero and repeated on days 5, 10, 14, 20, and 24. The change in volume of affected paw was evaluated on before the induction of arthritis ($V_{b.i.}$), 14 days after induction (V_{14}) and 24 days after induction (V_{24}), and paw volume index was calculated by the following formula.[8]

Paw volume index (%) = $(V_{24}) - (V_{14}) \times 100/(V_{b.i.}) - (V_{14})$

Here $V_{b.i.}$ = Paw volume before arthritis induction

V_{24} = Paw volume after 14 days

V_{14} = Paw volume after 24 days

Biochemical assay

Aspartate transaminase (AST) and alanine transaminase (ALT)

1.0 ml of buffered substrate was incubated for 10 min at 37°C. Then, 0.2 ml of enzyme was added. The tubes were incubated for 1 h (AST) and 30 min (ALT) at 37°C. To the control tubes, the enzyme was added after arresting the reaction with 1.0 ml of DNPH, and the tubes were kept at RT for 30 min. Then, 5.0 ml of 0.4 N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The color developed was read at 540 nm. The enzyme activity was expressed as micromoles of pyruvate liberated per mg of protein per minute.[10]

RESULTS AND DISCUSSION

Percentage Yield of Extract

Table 2 shows a considerable yield of plant material in hydroalcoholic solution, and physical evaluation results are mentioned in Table 3.

Physical Evaluation

Physiscal evaluations were done for ash value, extractive value and moisture content and results were shown in Table 3.

Phytochemical Investigation

In plant extract, the phytochemical investigation was performed and extract shown a positive test for the majority of chemical constituents such as alkaloids, glycosides, flavonoids, tannins, sterols, and tri-terpenoids except saponins.

Chromatographic Evaluation of Extract

The chromatographic evaluation shown that the plant fruit material possibly contain the compound quercetin, which was confirmed by comparison between the HPTLC analysis of the standard quercetin (R_f = 0.64) and the fruit extract (R_f = 0.65) using mobile phase as ethyl acetate, formic acid, glacial acetic acid, and water shown in Table 4.

Pharmacological Investigations

Evaluation of paw volume

The arthritic rats showed tissue swelling around the joints during the development of arthritis which was considered as edema of the particular tissue. Reduction of paw swelling in the extract treated rats may be due to immunological protection rendered by the plant extract as depicted in Table 5.

Table 2: Yield of plant material

Weight of plant material initially	Weight of extract	Percentage yield of extract
330 g	9.62 g	2.915

Table 3: Percentage yield of plant material

Physical parameter	Percentage yield (% w/w)
Ash value	
Total ash	7.87
Acid insoluble ash	2.98
Water soluble ash	4.89
Extractive value	
In alcohol	15.3
In water	11.8
Moisture content	4.5

Table 4: Chromatographic solvent system (mobile phase)

Solvent system	R_f value standard	R_f value extract	Detection wavelength
Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26)	0.64	0.65	254 nm

Evaluation of Membrane Markers (AST and ALT)

Increased activities of AST and ALT were observed in the arthritic rats. Reduction in levels of AST and ALT in the extract treated rats may be due to immunological protection rendered by the plant extract as mentioned in Table 6.

Values are expressed as mean ± SEM, n = 6 rats in each group.

ns = not significant, One way ANOVA followed by Dunnet's test

*p<0.05, *p<0.01 when compared with arthritic control, ***p<0.001 when compared with normal control.

Yield of the extract was found to be 2.195% as shown in Table 2. For physical value for the ash value. Physical evaluation under which total ash value was 7.87%, acid soluble ash 2.98%, and water soluble ash was 4.89, and extractive value in alcohol and water was found to be 15.3 and 11.8, respectively. Moisture content was found to be 4.5% as shown in Table 3. Phytochemical investigation shows the presence of a majority of chemical constituents such as alkaloids, glycosides, flavonoids, tannins, sterols, and tri-terpenoids except saponins.

Table 5: Evaluation data of paw volumes

Group	Paw volume of rats on different days in ml					
	Day 0	Day 5	Day 10	Day 14	Day 20	Day 24
Normal control	0.50±0.02	0.58±0.02	0.62±0.03	0.58±0.03	0.55±0.03	0.48±0.02
Arthritic control	0.62±0.03	0.79±0.03	0.94±0.02	1.05±0.03	1.10±0.02	1.17±0.02
Treatment group	0.60±0.03	0.90±0.03	0.84±0.02	0.88±0.03	0.88±0.03	0.90±0.03
Standard group	0.60±0.03	0.94±0.04	0.80±0.02	0.76±0.03	0.72±0.02	0.71±0.03

Values are expressed as mean±SEM, *n*=6 rats in each group, ns: Not significant, one-way ANOVA followed by Dunnet's test, **P*<0.05 when compared with arthritic control, SEM: Standard error mean

Table 6: Evaluation data of membrane markers

Groups	Treatment	Dose (mg/kg)	AST	ALT
Normal control	Vehicle		2.77±0.12***	2.33±0.10***
Arthritic control	Vehicle		6.07±0.14	5.69±0.10
Treatment group	Extract	100 mg/kg	3.52±0.13*	3.40±0.06*
Standard group	Dexamethasone	0.7 mg/kg	3.00±0.10**	3.00±0.09**

Values are expressed as mean±SEM, *n*=6 rats in each group, ns=Not significant, One way ANOVA followed by Dunnet's test, **P*<0.05, **P*<0.01 when compared with arthritic control, ****P*<0.001 when compared with normal control, SEM: Standard error mean, AST: Aspartate transaminase, ALT: Alanine transaminase

HPTLC analysis of the standard quercetin (R_f = 0.64) and the fruit extract (R_f = 0.65) using mobile phase as ethyl acetate, formic acid, glacial acetic acid, and water shown in Table 4 same value which indicates the presence of quercetin in the extract. The arthritic rats showed tissue swelling around the joints during the development of arthritis which was considered as edema of the particular tissue. Reduction of paw swelling after treatment with extract treated rats may be due to immunological protection rendered by the plant extract as depicted in Table 5 and because of the presence of glycosides quercetin and showing reduction more as compared to extract. Similarly, increased activities of AST and ALT were observed in the arthritic rats. Reduction in levels of AST and ALT in the extract treated rats may be due to immunological protection rendered by the plant extract as mentioned in Table 6. So, in future extension of the study can give a promising herbal product for arthritis.

CONCLUSION

In the present research work, extraction of plant material of *C. colocynthis* was done. The qualitative tests show the presence of a variety of phyto-constituents in *C. colocynthis*. Various physical constant has also been performed to indicate any type of substitution or adulteration in plant raw material. HPTLC technique was employed to identify the flavonoid particularly the quercetin. The biological activity concluded that *C. colocynthis* fruit's hydroalcoholic extract has shown significant anti-arthritic activity which may be due to some sort of phytochemicals such as flavonoids.

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