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Pharmacognostical, Phytochemical And Invitro, Invivo Anti-Inflammatory Potential Of Ethanolic Extract Of Sarcostemma Brevistigma Stems

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Abstract

A multimillion-dollar boom is achieved every year by the ethnopharmaceutical companies, creating awareness around the globe to use herbal medicines to stay and live a healthy life. Nearly, two-thirds of the plants were discovered for herbal remedies, and some plants are even endangered to get extinct from the globe for their repeated utility. *Sarcostemma brevistigma* is one among the undiscovered medicinal plants which belongs to the family *Asclepiadaceae* found distributed in Indian states of Bihar, Bengal, Konkan, Tamil Nadu, and Kerala. The objective of this study was to investigate pharmacognostical, phytochemical features and *invitro, invivo* anti-inflammatory activity of ethanolic extracts of *Sarcostemma brevistigma* stems by using human red blood cells (HRBC) membrane stabilization assay, protein denaturation method and formalin-induced paw edema model. The different pharmacognostical parameters were evaluated as per standard protocols with some modifications. Qualitative analysis of various phytochemical constituents was determined by the well-known test protocol available in the literature. Phytochemical analysis revealed the presence of alkaloids, glycosides, steroids and triterpenoids, tannins, proteins and amino acid and carbohydrate. The ethanolic extract of the stem of *Sarcostemma brevistigma* were studied for in vitro anti-inflammatory activity by HRBC membrane stabilization method. Three different concentrations of extract 200µg/ml, 400µg/ml and 600µg/ml were used. Among which extract at concentration 600µg/ml showed 58.20% Inhibition of HRBC in hypotonic solution. All the results were compared with standard diclofenac sodium which showed 68.82% Inhibition at concentration 300µg/ml. The inhibitory concentration (IC_{50}) of *Sarcostemma brevistigma* (Stem) in Protein denaturation is found to be 600µg/ml in comparison with diclofenac sodium 300µg/ml. Percentage inhibition of edema by extract containing ethanolic extract in rat's left hind paw was observed to be 16.92±0.12% at 1 hr. and 26.93±0.16% at 4 hr. All the results were compared with standard voveran emulgel gel. The skin irritation test was conducted for a period of seven days. The results indicated that the control preparation, extract and marketed products did not cause any skin reaction. It can be assured that ethanolic extract of

Sarcostemma brevistigma and the excipients did not cause any skin irritation and can be used in the gel formulation. *Sarcostemma brevistigma* extract showed appreciable anti-inflammatory property. Further analysis is to be carried out to isolate active chemical constituent responsible for anti-inflammatory activity and its mechanism involved.

Keywords: ***Sarcostemma brevistigma, Pharmacognostical, Phytochemical features and Invitro, invivo anti-inflammatory activity, Formalin-induced paw edema model***

Introduction

Inflammation is defined as a reaction that produces redness, warmth, oedema, and soreness as a result of infectious, chemical and physical agents such as microorganisms, toxins, radiations, bruises and caustic chemicals [1, 2]. The inflammatory response is a protective process aims to restrict the harmful agents. Another aim is to remove damaged cells to reach healing of the affected tissues or organs [3]. Inflammatory process starts by various chemical mediators which are released from macrophages and neutrophils which are responsible for the initiation, progression, regulation, and eventual resolution of the acute stage of inflammation. Monocytes play a main role in the clearing of cell debris. If the resolution is not occur in the acute stage, a chronic stage will develop [4]. Chronic inflammatory illnesses have been recently considered as the most cause of death worldwide, with more than half of all deaths being attributed to diseases related to inflammation such as ischemic heart disease, chronic kidney disease, cancer, diabetes and neurodegenerative and autoimmune conditions [5]. The main types of anti-inflammatory medications are the steroidal and non-steroidal drugs. Corticosteroids (steroidal drugs) are used to treat asthma and autoimmune inflammatory response. In addition, nonsteroidal drugs are used for mild to moderate pain and as antipyretic through the inhibition of cyclooxygenase enzyme [6]. Nevertheless, non-steroidal anti-inflammatory drugs (NSAIDs) have many side effects, such as cardiovascular risk and gastric irritations [7]. Therefore, extensive research was conducted on different plant species and their active compounds, which could constitute a source of new compounds which have anti-inflammatory property with fewer side effects and lower cost [2, 8]. According to the World Health Organization, approximately 80% of the world population still uses plant-based drugs which include the medicinal use of plants as anti-nociceptive drugs in traditional treatment [9]. *Sarcostemma brevistigma* is generally known as moon plant, soma (Sanskrit) and somlata (Hindi) is a leafless sprawling shrub which belongs to the family Asclepiadaceae [10]. The plant contains chemical constituents like malic acid, succinic acid, reducing sugars, traces of tannin, alpha- and beta-amyrins, lupeol and lupeol acetate, and beta-sitosterol [11]. Esculentin, pregnane triglycosides, and cardenolide tetraglycosides have been isolated from the root. The phytochemical studies indicated the presence of bergenin, brevine, brevinine, sarcogenin, sarcobiose, and flavonoids in the aerial parts of the plant extract [12]. The objective of this study was to investigate pharmacognostical, phytochemical features and *invitro*, *invivo* anti-inflammatory activity of ethanolic extracts of *Sarcostemma brevistigma* stems by using human red blood cells (HRBC) membrane stabilization assay, protein denaturation method and formalin-induced paw edema model.

Materials and methods

Plant material

The plant *Sarcostemma brevistigma* was collected from Jabalpur and was authenticated by Dr. S. N. Dwivedi, Head & Prof., Department of Botany, Janata PG College, APS, University, Rewa, M.P. and Voucher specimen No. VG/MGU/3210 was deposited in our department. Plant material (stems) selected for the study were washed thoroughly under running tap water and then were rinsed in distilled water; they were allowed to dry for some time at room temperature. Then the plant material was shade dried without any contamination for about 3 to 4 weeks. Dried plant material was grinded using electronic grinder. Powdered plant material was observed for their colour, odour, taste and texture. Dried plant material was packed in air tight container and stored for Phytochemical and biological studies.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Physicochemical parameters
Physicochemical parameters such as loss on drying, total ash value, acid insoluble ash value, water soluble ash value and foaming index were determined using standard procedures [13, 14].

Determination of ash values
The determination of ash values is meant for detecting low-grade products, exhausted drugs and sandy or earthy matter. It can also be utilized as a mean of detecting the chemical constituents by making use of water-soluble ash and acid insoluble ash.

Total ash value
Accurately about 3gms of air dried powder was weighed in a tared silica crucible and incinerated at a temperature not exceeding 450^0C until free from carbon, cooled and weighed and then the percentage of total ash with reference to the air dried powdered drug was calculated. The percentage of total ash with reference to the air-dried drug was calculated.

Acid insoluble ash
The ash obtained in the above method was boiled for 5 minutes with 25ml of dilute HCl. The residue was collected on ash less filter paper and washed with hot water, ignited and weighed. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

Water soluble ash
The ash obtained in total ash was boiled for 5 minutes with 25 ml of water. The insoluble matter was collected on an ash less filter paper, washed with hot water and ignited to constant weight at a low temperature. The weight of insoluble matter was subtracted from the weight of the ash. The difference in weights represents the water soluble ash. The percentage of water soluble ash with reference to the air dried drug was calculated.

Determination of moisture content (Loss on drying)
About 10 g of drug (without preliminary drying) after accurately weighing was placed in a tared evaporating dish and kept in oven at 105^0 C for 5 hours and weigh. The percentage loss on drying with reference to the air dried drug was calculated.

Determination of foreign organic matter
Accurately weighed 100 g of the drug sample was spread in a thin layer. The foreign matter was detected by inspection with the unaided eye by the use of a lens (6X). The foreign matter was separated and weighed and the percentage present was calculated.

Determination of swelling index
Swelling index is determined for the presence of mucilage. Accurately weighed 1 g of the powdered plant part was placed in 150 ml measuring cylinder. To this 50 ml of distilled water was added and kept aside for 24 hours with occasional shaking. The volume occupied by the seeds after 24 hours of wetting was measured.

Preparation of extracts
The dried powder stem of plant was extracted with various solvents. Aqueous extract was prepared by cold maceration process. Ethanolic, chloroform, and petroleum ether extract were obtained using Soxhlet apparatus. About 250 gm of dried powder stem of plant was subjected to soxhlation. It was first defatted with petroleum ether then exhaustively extracted with solvent in a Soxhlet apparatus for 36 hours. The temperature was maintained at 40-50 degree centigrade. The solvents were removed by distillation under reduced pressure and the resulting semisolid mass was vacuum dried using rotary flash evaporator to obtain the extract [15].

Phytochemical screening of the extract
Various phytoconstituents, including alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins, proteins, amino acids, and flavonoids were analysed qualitatively in the *Sarcostemma brevistigma* extract [16, 17].

Assessment of *in vitro* anti-inflammatory activity

Human red blood cells (HRBC) membrane stabilization assay

Ethanolic extract of *Sarcostemma brevistigma* were investigated for *In-vitro* anti-inflammatory activity by human red blood cell membrane stabilization method. Four different concentrations of extracts: 1mg/ml, 2mg/ml, 4mg/ml and 6mg/ml were used for anti-inflammatory study.

Preparation of Suspension (10% v/v) of Human Red Blood cell

The blood sample was collected from healthy human volunteer who has not taken any NSAID for 2 weeks prior to the experiment and transferred to heparinized centrifuge tube. Blood samples were centrifuged at 3000 rpm at room temperature for 15 min. The supernatant (plasma and leucocytes) were carefully removed while the packed red blood cells were washed with fresh normal saline (0.85% w/v NaCl). The process of washing and centrifugation was repeated five times until the supernatant was clear. Then, Human erythrocytes suspension (10% v/v) was prepared [18].

Assay of Membrane stabilizing activity

The HRBC membrane stabilizing activity assay was carried out as reported by Oyedapo et al.,[18] using 10% (v/v) using 10% (v/v) Human erythrocyte suspension while diclofenac sodium was used as standard drugs. The assay mixtures consisted of 2 ml of hyposaline (0.25% w/v) sodium chloride, 1.0 ml of 0.15 M sodium phosphate buffer, pH 7.4, 0.5 ml of 10% (v/v) human erythrocyte suspension, 1.0 ml of drugs (standard and extracts) and final reaction mixtures were made up to 4.5 ml with isosaline. Drug was omitted in the blood control, while the drug control did not contain the erythrocyte suspension. The reaction mixtures were incubated 30 mints at 37°C and centrifuged 20 mints at 3000 rpm. The absorbance of the supernatant solution was measured by spectrophotometrically at 560 nm. Each experiment was carried out in triplicate and the average was taken. The percentage inhibition of membrane stabilization or haemolysis was calculated using the following equation.

% Inhibition of haemolysis = 100 x (A_1-A_2/A_1)

Where: A_1 = Absorption of hypotonic buffered saline solution alone, A_2 = Absorption of test sample in hypotonic solution.

Inhibition of protein denaturation

1. The Test solution (0.5ml) consists of 0.45 ml bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of Plant extracts (10, 20, 30, 40, and 50 µg/ml concentration.)
2. Test control solution (0.5ml) consist of 0.45ml of bovine serum albumin (5%W/V aqueous solution) and 0 .05ml of distilled water.
3. Product control (0.5ml) consists of 0.45ml of distilled water and 0.05 ml of Plant extract (10, 20, 30, 40, and 50 µg/ml concentration).
4. Standard solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5%w/v aqueous solution) and 0.05ml 0f Diclofenac sodium (250mcg/ml).

All the above solutions were adjusted to pH 6.3 using 1N HCl. The samples were incubated at 37°c for 20minutes and the temperature was increased to keep the samples at 57°c for 3minutes. After cooling, add 2.5 ml of phosphate buffer to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 416nm [19]. The control represents 100% protein denaturation. The results were compared with the standard drug diclofenac sodium treated sample

In-vivo anti-inflammatory study

Animals

Healthy young adult albino (100-120 gm) of either sex and of approximate same age were used throughout the study were housed under standard laboratory conditions in polyacrylic cages, and were provided with pelleted food and water ad libitum. The animals were acclimatized to the laboratory condition for 1 week before starting the experiment. Animal studies were approved by Institutional Animal Ethics Committee (IAEC) of SRK University, Bhopal, M.P. and carried out in accordance with the Guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)

Formalin induced paw edema model

In-vivo anti-inflammatory study of *Sarcostemma brevistigma* plant extract was conducted by formalin induced paw edema model using 12 albino rats and divided into three groups of four animals on each. In all groups, acute inflammation was induced by sub-planter injection of 0.1 ml of freshly prepared 1 % suspension of

sterilized formalin in normal saline in left hind paw of the rats. The medicated formulations (0.3g) or base or standard were applied topically to the planter surface of hind paw with gentle rubbing with index finger to each rat of respective group one hour before and one hour after the formalin challenge. The paw edema volume was measured using plethysmometer at every 30 mint intervals for 4 hour after injection of formalin. The average paw edema volume of all the groups were calculated and compared with that of control. The percent inhibition of edema was calculated by using following formula [20].

% Edema inhibition = (1-Vt/Vc) 100

Where, Vt = Mean edema volume of test, Vc = Mean edema volume of control

Statistical Analysis

The statistical analysis of various studies were carried out using analysis of variance (ANOVA) followed by Dunnett's 't' test and standard deviation, $p<0.05$ was accounted significant.

Results and discussions

The dried parts of plants were subjected to standard procedure for the determination of various physicochemical parameters-ash values (total ash, acid insoluble ash and water soluble ash) and loss on drying were determined Table 1. The dried powder of plant was extracted with various solvents i.e., water, ethanolic, chloroform, and petroleum ether. The solvents were removed by distillation under reduced pressure and the resulting semisolid mass was vacuum dried using rotary flash evaporator to obtain the extract. The percentage yields of various extract was presented in Table 2. The various extracts obtained were subjected to preliminary phytochemical screening. The extraction was carried out with water, ethanol, chloroform, and petroleum ether the extract were screened for the presence of various medicinally active constituents. Aqueous extract shows presence of alkaloids, carbohydrates, glycosides, tannins, protein, amino acids, and steroids. Ethanolic extract shows presence of alkaloids, carbohydrates, glycosides, tannins, protein, amino acids, and steroids. Chloroform extract shows presence of carbohydrates, tannins while petroleum ether extract shows presence of alkaloids, carbohydrates, glycosides, protein and amino acids, steroids Table 3. During inflammation, lysosomal hydrolytic enzymes are released into the sites which cause damages of the surrounding organelles and tissues with attendance of variety of disorders. Various methods were employed to screen and study drugs, chemicals, herbal preparations that exhibit anti-inflammatory properties or potentials. These techniques include uncoupling of oxidative phosphorylation (ATP biogenesis linked to respiration), inhibition of denaturation of protein, erythrocyte membrane stabilization, lysosomal membrane stabilization, fibrinolytic assays and platelet aggregation. In the present study, stabilization of erythrocyte membranes exposed to both heat and hypotonic induced lyses was employed due to its simplicity and reproducibility [21]. The ethanolic extract of the stem of *Sarcostemma brevistigma* were studied for in vitro anti-inflammatory activity by HRBC membrane stabilization method. Four different concentrations of extract 200µg/ml, 400µg/ml and 600µg/ml were used. Among which extract at concentration 600µg/ml showed 58.20% Inhibition of HRBC in hypotonic solution. All the results were compared with standard diclofenac sodium which showed 68.82% Inhibition at concentration 300µg/ml Table 4. The activity may be due to the presence of one or more phytochemical constituents present in the extract. The ethanolic extract of the stem of *Sarcostemma brevistigma* were studied for in vitro anti-inflammatory activity by protein denaturation method. The protein used in this study is bovine serum albumin. Denaturation of protein is carried out by heating. The aim of this activity is to inhibit denaturation and to exhibit protective effect against rheumatoid arthritis. The inhibitory concentration (IC_{50}) of *Sarcostemma brevistigma* (Stem) in protein denaturation is found to be 600µg/ml in comparison with diclofenac sodium 300µg/ml. The inhibition of protein denaturation by *Sarcostemma brevistigma* may be due to the presence of phenolic compounds, flavonoids or tannins Table 5. Percentage inhibition of edema by extract containing ethanolic extract in rat's left hind paw was observed to be$16.92\pm0.12\%$ at 1 hr. and $26.93\pm0.16\%$ at 4 hr. All the results were compared with standard voveran emulgel gel Table 6. The result revealed that extract containing ethanolic extract of *Sarcostemma brevistigma* has anti-inflammatory action.

Table 1: Physico-chemical analysis of *Sarcostemma brevistigma*

Sr. No.	**Parameters**	**Values (%)**
1.	Total ash	7.2
2.	Loss on drying at110°C	1.7

3.	Water soluble ash	1.8
4.	Acid insoluble ash	1.6

Table 2: Extractive value of different extract of *Sarcostemma brevistigma*

S/No.	Type of Extract	% Yield (w/w)	Color of Extract
1.	Aqueous extract	4.12	Light Green
2.	Ethanolic extract	15.32	Dark Green
3.	Chloroform extract	6.65	Light Green
4.	Pet. ether extract	7.54	Brownish Green

Table 3: Preliminary phytochemical screening of different extract of Sarcostemma brevistigma

S/No.	Constituents	Test	AE	EE	CE	PE
1.	Alkaloids	Mayer's test	-	+	-	-
		Dragendroff' test	+	+	-	-
		Hager's test	-	-	-	-
		Wagner's test	-	-	-	-
2.	Carbohydrates	Molisch's test	+	+	+	-
		Fehling's test	+	+	-	-
3.	Glycosides	Brontrager's test	-	-	-	-
		Legal's test	+	+	-	-
4.	Fixed oil and fats	Spot test	-	-	-	-
		Soap formation test	-	-	-	-
5.	Tannins	$FeCl_3$	-	+	-	-
		Vanillin hydrochloride	+	+	+	-
		Alkaline reagent	-	+	-	-
6.	Protein and amino acid	Million's test	+	+	-	-
		Ninhydrin test	+		-	-
		Biuret test	-	-	-	+
7.	Flavanoids	With NaOH	-	-	-	-
		Shinoda test	-	-	-	-
		With H_2SO_4	-	-	-	-
8.	Steroids and triterpenoids	Libermann's Burchard test	-	-	-	-
		Salkowski's test	+	+	-	+
9.	Mucilage and gum	With 90% alcohol	-	-	-	-
10.	Waxes	With alc. KOH	-	-	-	+

AE: Aqueous Extract; EE: Ethanolic Extract; CE: Chloroform Extract; PE: Petroleum ether Extract, (+ Present, - Absent)

Table 4: In-vitro anti-inflammatory activity of extract of Sarcostemma brevistigma by membrane stabilization method

Treatment	Con. (µg/ml)	Absorbance (560nm)	% of Inhibition
Control	-	0.250±0.21	-
***S. brevistigma* Extract**	200	0.181±0.23[a]	30.32
	400	0.148±0.37 [b]	44.05
	600	0.113 ±0.12 [b]	58.20
Standered drug (Diclofenac sodium)	300	0.069±0.26 [b]	69.82

Values are expressed as X (Mean) ±SEM, n=3. (One way ANOVA followed by Student t-test). Statistically significance of [a]P < 0.05, [b]P<0.01, [c]P<0.001 and [d]NS in comparison to respective control.

Table 5: Effect of plant extract and diclofenac sodium on inhibition of protein denaturation

Treatment	Con (µg/ml)	% of Inhibition
Control	-	-
***S. brevistigma* Extract**	200	58.5±0.342
	400	65.0±0.231
	600	71.2±0.326
(Diclofenac sodium)	300	81.43 ±0.231

*Mean of three readings ± SEM

Table 6: Percentage inhibition of edema

Group	Percentage inhibition of edema			
	1 hr	2hr	3hr	4hr
Control	-	-	-	-
Ethanolic extract of *S. brevistigma*	16.92±0.12	19.67±0.14	23.26±0.14	26.93±0.16
Standard Drug (Voveran Emulgel)	41.32±0.20	48.54±0.16	51.53±0.19	55.51±0.32

Conclusion

The study also provides a strong evidence for the use of the stems *Sarcostemma brevistigma* in folkloric treatment as anti-inflammatory agent. The plant therefore could be regarded as a natural source of membrane stabilizers and was capable of providing an alternative remedy for the management and treatment of inflammatory related disorders and diseases. However, the isolation of compounds responsible for anti-inflammatory effect is subject of further investigation.

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