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Research

Phytochemical Profiling, GC-MS Analysis and *In Vitro* Anti-Inflammatory Activity Of *Alternanthera Philoxeroides Leaves*

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Abstract

Ethanolic extract of whole plant of *Alternanthera Philoxeroides* (Family: *Amaranthaceae*) was assessed for its anti-inflammatory activity by in vitro methods. *In vitro* anti-inflammatory activity was evaluated using albumin denaturation assay activity at different concentrations. Diclofenac sodium was used as standard drugs. The results showed that ethanolic extraction of *Alternanthera Philoxeroides* (EEAP) at a concentration range of 100-1000µg/ml significantly ($p<0.01$) protects the heat induced protein denaturation. EEAP showed significant ($p<0.01$). The results obtained in the present study indicate that ethanolic extracts of *Alternanthera Philoxeroides* can be a potential source of anti-inflammatory agents.

Keywords: *Alternanthera Philoxeroides, In vitro* anti-inflammatory, albumin denaturation assay.

INTRODUCTION

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as: the increase of vascular permeability, increase of protein denaturation and membrane alteration. When cells in the body are damaged by microbes, physical agents or chemical agents, the injury is in the form stress. Inflammation of tissue is due to response to stress. It is a defensive response that is characterized by redness, pain, heat, and swelling and loss of function in the injured area. Loss of function occurs depends on the site and extent of injury. Since inflammation is one of the body's nonspecific internal systems of defense, the response of a tissue to an accidental cut is similar to the response that results from other types of tissue damage, caused by burns due to heat, radiation, bacterial or viral invasion [1]. When tissue cells become injured they release kinins, prostroglandins and histamine. These work collectively to cause increased vasodilation (widening of blood capillaries) and permeability of the capillaries. This leads to increased blood flow to the injured site. These

substances also act as chemical messengers that attract some of the body's natural defense cells a mechanism known as chemotaxis. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. Several experimental protocols of inflammation are used for evaluating the potency of drugs. The management of inflammation related diseases is a real issue in the rural community; the population in these areas uses many alternative drugs such as substances produced from medicinal plants. Enicostemma axillare is a perennial herb found throughout India and common in coastal areas. It is also called as Vellarugu in Tamil, Chota chirayata in Hindi, Mamejavo in Gujarati and Nagajivha in Bengal. The plant is used in folk medicine to treat diabetes mellitus, rheumatisum, abdominal ulcers, hernia, swelling, itching and insect poisoning [2], anti-inflammatory [3], hypoglycaemic [4], [5], [6] and anticancer [6] activities have been reported. The whole plant is used in medicine as digestive, anti-inflammatory, liver tonic, antimalarial, antipyretic and as a laxative [7],[8]. According to ayurvedic literature survey, the fresh juice of leaves has been used as a bitter tonic, to control arthritis, in typhoid fever and as cooling agent. The plant is traditionally used in the treatment of hepatic diseases and as a blood purifier. It also acts as ethnomedicine for snakebite [9]. The plant paste is applied on boils. The leaves are fed to cattle to increase appetite.

Alternanthera Philoxeroides can thrive in both dry and aquatic environments and is characterized by whitish, papery flowers along its short stalks, irregular, or sprawling hollow stems, and simple and opposite leaf pattern sprouting from its nodes. The species is dioecious. [failed verification] It is also considered a herbaceous plant due to its short-lived shoot system. It produces horizontal stems, otherwise known as stolons that can sprout up to 10 metres (33 ft) in length and thanks to its hollow stems, floats easily. These results in large clusters of stem amassing and create dense mats along the surface. The plants flowers from December to April and usually grows around 13 millimetres (1⁄2 in) in diameter and tend to be papery and ball-shaped. The weed's intricate root system can either allow them to hang free in the water to absorb nutrients or directly penetrate the soil/sediment or pull their nutrients from below. This plant has traditionally been used in India as a remedy for anaemia, for the treatment of diarrhoea and dysentery in Bangladesh, and to treat certain blood conditions, fever, post-natal depression, wounds and to stimulate milk secretion in Thailand. The study was aimed to investigate the phytochemical profiling, GC-MS Analysis and *in vitro* anti-inflammatory activity of *Alternanthera Philoxeroides Leaves.*

MATERIALS AND METHODS

Collection and Authentication of plant

Alternanthera Philoxeroides was collected from the local market of Hyderabad, Telangana. The plant was identified, confirmed and authenticated by L. Rasingam**,** (Scientist 'E' & HOD) Ministry of Environment, forest & Climate Change/Botanical survey of India (BSI), Hyderabad, Telangana.

Chemicals Used

95% ethanol, Molisch reagent, Fehling's A, Fehling's B, Benedict's solution, dil. HCl, conc. H_2SO_4, ruthenium red, aqueous KOH, acetic anhydride, chloroform, Dragendroff's reagent, Mayer's reagent, Hager's reagent, Wagner's reagent, ammonium hydroxide, sodium hydroxide, lead acetate solution, Bromine water, Dil. Iodine solution, Dil. HNO_3, Sudan Red III, egg albumin, Diclofenac Sod., phosphate buffer, 1N HCl, Dimethyl formamide.

Preparation of ethanolic extracts

The Leaves of *Alternanthera Philoxeroides* were collected thoroughly washed and shade dried. The dried leaves were made into coarse powder with the help of mortar and pestle. About 300 g of coarse powder of plant material was weighed and subjected to extraction using Ethanol (99%) as the solvent by cold maceration process for a week with an occasional stirring. The plant material soaked in the solvent was separated with the help of muslin cloth, filtered with the help of funnel and filter paper and the solvent was kept undisturbed in a beaker for evaporation at room temperature to obtain a mass of extract. This was transferred into a petri plate and was stored into a desiccator until its use. The extract obtained was dark black in color and was suspended in distilled water using carboxy methyl cellulose as a suspending agent for oral administration to experimental animals.

Preliminary Phytochemical screening

The ethanolic extract of *A. Philoxeroides* plant leaves was subjected to preliminary screening of phytochemical constituents. Phytochemical analysis was carried out for alkaloids, tannins, fixed oils, steroids,

gums and mucilage, carbohydrates, glycosides, reduced sugars and flavonoids were performed as described by the authors [10].

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was carried out in a combined 7890A gas chromatograph system (GCMSQP2010, SHIMADZU) and mass spectrophotometer, fitted with a HP-5 MS fused silica column (5% phenyl methyl siloxane 30.0m ×250µm, film thickness 0.25µm), interfaced with 5675C Inert MSD with Triple-Axis detector. Helium gas was used as carrier gas and was adjusted to column velocity flow of 1.0ml/min. Other GC-MS conditions are ion-source temperature, 250°C; interface temperature, 300°C; pressure, 16.2 psi; out time, 1.8 mm; and 1µl injector in split mode with split ratio 1:50 with injection temperature of 300 °C. The column temperature started at 36°C for 5 min and changed to 150 V at the rate of 4 °C/min. The temperature was raised to 250°C at the rate of 20°C/min and held for 5 min. The total elution was 37 min. The relative percent amount of each component was calculated by comparing its average peak area to total areas. MS solution software provided by supplier was used to control the system and to acquire the data.

Identification of compounds

Identification of components was achieved based on their retention indices and interpretation of mass spectrum was conducted using the database of National Institute of Standards and Technology (NSIT). The database consists of more than 62,000 patterns of known compounds. The spectra of the unknown components of *Alternanthera Philoxeroides* fraction obtained were compared with the standard mass spectra of known components stored in NIST library (NISTII).

Anti-Inflammatory activity

Anti-inflammatory activity was determined using Albumin denaturation Method

Albumin Denaturation method

The following procedure was followed for evaluating the percentage inhibition of protein denaturation [11,12].

Preparation of Phosphate buffer (pH 6.4)

Dissolve 2.5 g of disodium hydrogen phosphate, 2.5 g of sodium dihydrogen phosphate and 8.2 g of sodium chloride in 950 ml of water. Adjust the pH of the solution to 6.4 with 1 M sodium hydroxide or 1 M hydrochloric acid, if necessary. Dilute to 1000 ml with water.

Control solution (50ml)

2ml of egg albumin, 28 ml of phosphate buffer (pH 6.4) and 20ml distilled water.

Standard solution (50ml)

2ml of egg albumin 28ml of phosphate buffer and various concentrations of standard/reference drug (Diclofenac Sod.). Concentrations of 100, 200, 400, 800, and 1000µg/ml.

Test solution (50ml)

2ml of egg albumin, 28ml of phosphate buffer and various concentrations of extract (*Alternanthera Philoxeroides* ethanolic extract. Concentrations of 100, 200, 400, 800, and 1000µg/ml.

All of the above solutions were adjusted to pH using a small amount of 1N HCl. The samples were incubated at 37ºC for 15mins and heated at 70ºC. Diclofenac Sodium was used as standard drug. The absorbance was values were measured at 276nm. After cooling the absorbance of the above solutions percentage inhibition of protein denaturation was calculated by using the given formula. The experiment was performed in triplicate.[6]

Percentage inhibition = [Vt / Vc -1] x 100

Where,

Vt = Absorbance of test sample.

Vc = Absorbance of control.

RESULTS AND DISCUSSIONS

Preliminary Phytochemical Screenings

The preliminary phytochemical investigation of Ethanolic extract of *Alternanthera Philoxeroides* leaves revealed that the ethanolic extract contains carbohydrates, flavonoids, alkaloids, tannins, steroids, fats and oils. Results were showed in Table 1.

Table 1: Preliminary Phytochemical evaluation of *Alternanthera Philoxeroides*

Phytochemical Compounds	Tests performed	Results
Carbohydrates	Molisch test	+
	Fehling's test	+
	Benedict's test	+
Alkaloids	Dragendroff's test	+
	Mayer's test	+
	Hager's test	+
	Wagner's test	+
Steroids	Salkowski's reaction	+
	Liebermann-Burchard reaction	+
	Liebermann's reaction	+
Mucilage	Swelling test	+
	Test with Ruthenium Red	+
Flavonoids	Shinoda Test	+
	Lead acetate test	+
	Test with NaOH	+
Tannins	Lead acetate test	+
	Bromine water test	+
	Dil. Iodine solution test	+
Fats and Oils	Solubility test	+
	Tincture alkana test	+
	Filter paper test	+

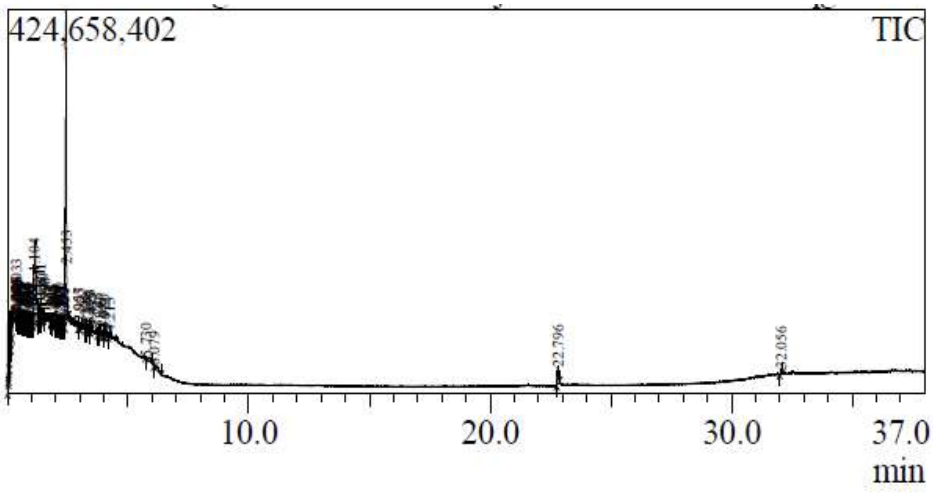
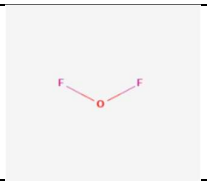
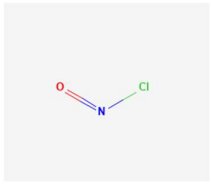
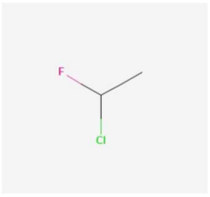
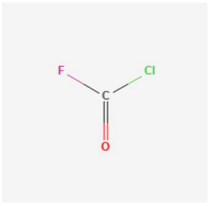
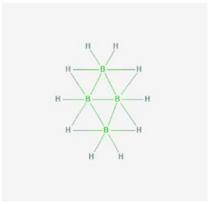
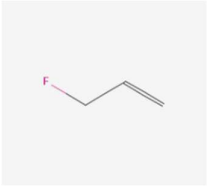
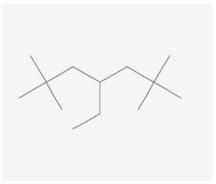
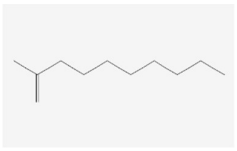
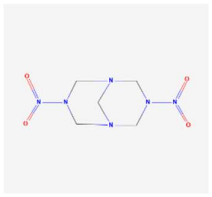
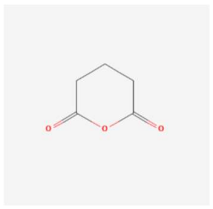
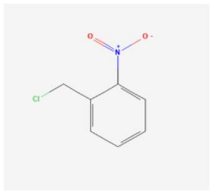
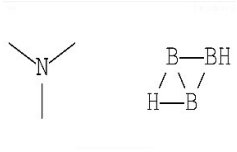
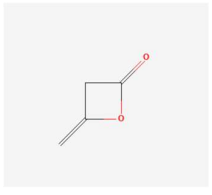
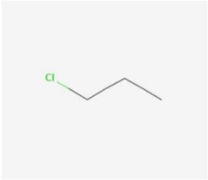
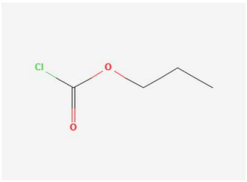
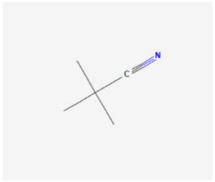
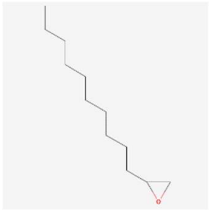
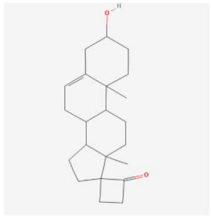


Fig 1: GC-MS chromatogram of Ethanolic extract of *Alternanthera Philoxeroides* (EEAP)

Table 2: Bioactive compounds found in Ethanolic extract of *Alternanthera Philoxeroides* (EEAP)

S.NO	Retention Time	% Area	Name of the compound	Molecular Formula	Molecular Weight	Structure
1.	0.033	6.59	2-Oxetanone, 4,4-dimethyl-	$C_5H_8O_2$	100	
2.	4.213	0.18	Difluorinemonoxide	F_2O	54	
3.	2.957	0.41	Nitrosyl chloride	ClNO	65	
4.	0.860	0.87	Ethane, 1-chloro-1-fluoro-	C_2H_4ClF	82	
5.	0.765	0.11	Carbonic chloride fluoride	CClFO	82	
6.	1.104	20.93	Tetraborane (10)	B_4H_{10}	54	
7.	1.321	5.22	1-Propene,3-fluoro-	C_3H_5F	60	

8.	1.371	2.12	Heptane,4-ethyl-2,2,6,6-tetramethyl-	$C_{13}H_{28}$	184	
9.	1.480	2.05	1-Decene,2-methyl-	$C_{11}H_{22}$	154	
10.	3.849	0.26	N, N-Dinitro-1,3,5,7-tetrazabicyclo[3,3,1]nonane	$C_5H_{10}N_6O_4$	208	
11.	2.224	0.18	2H-Pyran-2,6(3H)-dione,dihydro-	C5H6O3	114	
12.	2.453	13.90	Benzene,1-(chloromethyl)-2-nitro-	$C_7H_6ClNO_2$	171	
13.	2.995	0.63	Methanamine, N,N-dimethyl-, compd.withtriborane(7) (1:1)	$C_3H_{16}B_3N$	99	
14.	3.980	0.32	2-Oxetanone, 4-methylene-	$C_4H_4O_2$	84	

15.	4.022	0.20	Propane,1-chloro-	C_3H_7Cl	78	
16.	5.730	1.99	Carbonochloridic acid, propyl ester	$C_4H_7ClO_2$	122	
17.	6.079	1.64	Propanenitrile, 2,2-dimethyl-	C_5H_9N	83	
18.	22.796	2.90	Oxirane, decyl-	$C_{12}H_{24}O$	184	
19.	32.056	0.88	Spiro [androst-5-ene-17, 1'-cyclobutan]-2'-one, 3-hydroxy-, (3.beta, 17.beta.)-	$C_{22}H_{32}O_2$	328	

The chromatogram of GC-MS displayed in Figure 1 whereas the chemical constituents with their Retention Time (RT), atomic equation, Molecular weight (MW) and Area (%) within the EEAP is displayed in Table 2. The following bioactive compounds were present in the Ethanolic extract of *Alternanthera Philoxeroides* Tetraborane (10), Heptane,4-ethyl-2,2,6,6-tetramethyl-‘ N, N-Dinitro-1,3,5,7-tetrazabicyclo[3,3,1] nonane, Spiro[androst-5-ene-17,1'-cyclobutan]-2'-one,3-hydroxy-,(3.beta,17.beta.)-

Table 3: Percentage Inhibition of Ethanolic extract of *Alternanthera Philoxeroides* by Albumin Denaturation Method

Concentrations (µg/ml)	% Inhibition Standard	%Inhibition Test
2	17.4 ± 0.2	35.4 ± 0.3
4	19.6 ± 0.1	38.05 ± 0.15
6	42.8 ± 0.1	52.2 ± 0.2
8	58.2 ± 0.3	64.3 ± 0.4
10	68.1 ± 0.2	73.8 ± 0.1

Values are expressed as mean ± Standard deviation from Albumin Denaturation Method

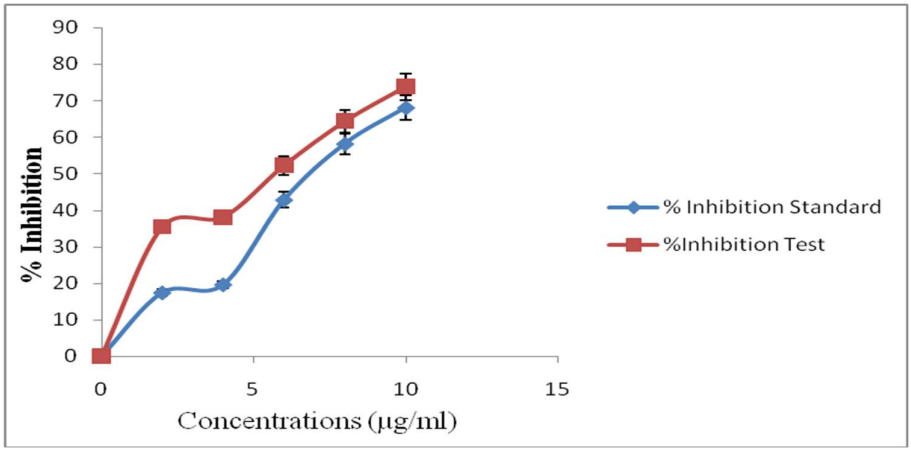


Fig 2: Percentage Inhibition of Ethanolic extract of *Alternanthera Philoxeroides* by Albumin Denaturation Method

DISCUSSION

Medicinal plants are an affluent source of phytochemical compounds that can play a vital role to treat several chronic diseases [13]. An extensive number of potent biomolecules come from a diverse number of medicinal plants in recent times [14]. Scientists believed that these potent chemical constituents obtained from nature are used for treating many disorders with fewer side effects [15]. These potent compounds are highly capable of inhibiting the harmless act of a multiple number of chronic diseases [16]. Few chronic diseases are so critical and there are no specific drugs for those diseases [17]. In such cases, medicinal plants should be applied and they give an effective result in pharmacologically and phytochemically [18].

Herbal medicine is the use of plant extracts to treat various types of diseases. Medicinal plants exist in many local varieties depending upon the regional flora. Many modern drugs were originally extracted from plant sources, they are now made synthetically, and many other drugs are descended from plant extracts. The investigation is based on the need for anti-inflammatory activity from natural sources with potent activity and lesser side effects as substitutes for chemical therapeutics. Protein denaturation is a process in which protein lose their tertiary structure andsecondary structure by application of external stress or compound such as strong acid or base a concentration inorganic salt , an organic solvent or heat most biological protein lose their biological function when denaturized. Denaturation of protein is a well-documented cause of inflammation. As a part of the investigation on the mechanism of the anti-inflammatory activity, ability of plant extract to inhibit protein denaturation was studied. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation. Denaturation of protein is a well-documented cause of inflammation in condition like rheumatoid arthritis. The lysosomal enzyme released during inflammation produce a various disorders. The extracellular activity of these enzymes are said to be related to acute to chronic inflammation. The non-steroidal drugs act either by inhibiting the lysosomal enzymes or by stabilizing lysosomal membranes. Ethanolic extract of *Alternanthera Philoxeroides* was studied for In-vitro anti-inflammatory activity by Albumin denaturation methods. The preliminary phytochemical investigation revealed that the ethanolic extract contains carbohydrates, flavonoids, alkaloids, tannins, steroids, fats and oils. These phytochemicals have various health benefits such as anti-oxidant, anti-microbial, anti-inflammatory, cancer preventive, anti-diabetic and anti-hypertensive effects.

The GC–MS investigation of *Alternanthera Philoxeroides* extract revealed the presence of 19 phytochemical compounds, they are 2-Oxetanone, 4,4-dimethyl-, Difluorinemonoxide, Nitrosyl chloride, Ethane, 1-chloro-1-fluoro-, Carbonic chloride fluoride, Tetraborane (10), 1-Propene,3-fluoro-, Heptane,4-ethyl-2,2,6,6-tetramethyl-, 1-Decene,2-methyl-, N, N-Dinitro-1,3,5,7-tetrazabicyclo[3,3,1]nonane, 2H-Pyran-2,6(3H)-dione,dihydro-, Benzene,1-(chloromethyl)-2-nitro-Methanamine, N,N-dimethyl-, compd.withtriborane(7) (1:1), 2-Oxetanone, 4-methylene-, Propane,1-chloro-, Carbonochloridic acid, propyl ester, Propanenitrile, 2,2-dimethyl-, Oxirane, decyl-, Spiro [androst-5-ene-17, 1'-cyclobutan]-2'-one, 3-hydroxy-, (3.beta, 17.beta.)-, The secondary metabolites and other chemical constituents of medicinal plants account for their medicinal values. Results obtained from the study demonstrate that ethanolic extract inhibited protein denaturation in concentration dependent manner. At highest concentration the extract produced 73 ± 0.1% % inhibition of protein in Albumin denaturation method.

CONCLUSION

Phytochemical investigation of ethanolic extraction of *Alternanthera Philoxeroides* reveled carbohydrates, flavonoids, alkaloids, tannins, steroids, fats and oils. GC–MS analysis identified various bioactive compounds, *in vitro* anti-inflammatory activity of the ethanolic extraction of *Alternanthera Philoxeroides* inhibited protein denaturation in concentration dependent manner. The bioactive compounds are responsible for the different therapeutic and pharmacological properties of *Alternanthera Philoxeroides*.

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