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PRELIMINARY PHYTOCHEMICAL SCREENING OF ETHANOLIC EXTRACT OF *ASTERCANTHA LONGIFOLIA* (HYGROPHYLLA SCHULLI) ROOTS ALONG WITH VARIOUS ACTIVITIES

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ABSTRACT

Hygrophila spinosa T Ander is a promising medicinal plant with great economic potential. Its medical values are also appreciated in the ancient medical literature. The present work was carried out to investigate the Antioxidant, Antibacterial, Cytotoxic and Anti-inflammatory activities of Astercantha Longifolia (Hygrophylla Schulli). Disc diffusion assay, DPPH assay, brine shrimp assay and rat paw edema test were performed to check the activities respectively. The TLC Fingerprinting of ethanolic root extract shows presence of compound whose structure was established as Stigmast-5-en-3 β-0l which is responsible for anti-inflammatory activity and its activity was checked using carraageenan induced rat paw edema model. The Extract produced significant antibacterial activity against Bacillus subtillus, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli with zone of inhibition of 20, 16, 18 and 18 mm respectively. The extract showed a significant free radical scavenging activity with an IC50 of 88.06µg/mL. The extract showed significant lethality to brine shrimp lethality using Vincristine sulfate as standard with LC50 value of 56.37 µg/mL.

KEYWORDS: Astercantha Longifolia, Hygrophylla Schulli, Antioxidant, Antibacterial, Cytotoxicity and Anti-inflammatory activity.

1. INTRODUCTION

In recent years there is progressive increase in the screening and research on medicinal plants with therapeutic activity but only few of them are included in health care system after clinical research. *Astercantha Longifolia* belonging to the family Acanthaceae, generally known for its medicinal properties, it grows widely throughout India, and in large parts of Africa and Indo-china also in Srilanka, Burma, Malaysia and Nepal. It is a common plant of water bodies, that can grow in polluted water, forms large stands and easily colonizes in waterlogged areas, there are no threats to this species are known, although it is extracted for medicinal use, in India.[1,2] It is a spiny, stout, annual herb, leaves sub sessile, oblong-lanceolate or linear lanceolate, spines yellowish brown, 2-3 cm long, flower yellowish brown, fruit two celled, linear oblong, compressed about 8 cm long, pointed, 4-8 seed ovate, flat or compressed, 0.2-0.25 cm long and 0.1-0.15cm wide, hairy but appearing smooth; get coated with mucilage immediately after soaking in water, light brown in color, and taste slightly bitter.[3,4]

The whole plant including, roots, seeds, and ashes are extensively used as traditional medicine for various ailments like rheumatism, inflammation, jaundice, hepatic obstruction, pain, UTI, oedema and gout. It is classified in ayurvedic system as mathuravipaka, seethaveeryam and used for the treatment of diabetes (premeham), dysentery (athisaram) etc.[5,6,7]

2. MATERIALS AND METHODS

2.1 Plant material

The roots of *A. Longifolia* were collected in the month of November to December from the jungle area of Gondia District, Maharashtra, India. The plant specimen was mounted on herbarium sheet and the sample was identified and authenticated by the expert Dr. N. D. Dongarwar, HOD, Department of Botany, University Campus, R.T.M. Nagpur University, Nagpur and notated as identification voucher No, HS-ACC/ 17.08.2016/9985. The test microorganisms used in this study Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus, aureus and Escherichia coli were obtained from Rajiv Gandhi Biotechnology Centre RTM Nagpur University, Nagpur.

2.2 Preparation of Extract

The coarsely dried powdered (500 g) of roots was extracted with petroleum ether 60:80 by using Soxhlet apparatus until it completely defatted. Raffinate of plant material was removed

from assembly, dried and further extracted with ethanol. The extract was filtered through Whatman paper (No. 44), concentrated over thermostat water bath and kept in airtight container and preserve in vacuum desiccators until further use.

2.3 Preliminary phytochemical analysis

For the detection of major chemical groups, the extract was subjected to preliminary phytochemical screening. In each test 10% (w/v) solution of the extract was taken or as mentioned in the standard procedure.

2.4 Animals

Sprague dawley Rats of either sex (170-200 g body weight) were collected from animal resources. Mice of random sex (Swiss-webstar strain, 19-40 g body weight) were collected with prior approval from animal ethical committee (Registration no. 853/AC/04/CPCSEA/Dec2008) of Smt. Kishoritai Bhoyar College of Pharmacy, Kamptee and were used for the experiments. The animals were kept at animal house for adaptation after collection under standard laboratory conditions (relative humidity 55%-65%, room temperature (25±2) °C and 12 h light: dark cycle) and feed with standard diets in large spacious polypropylene cages and supplied with water and libitum for period of 14 days, the animals were fasted overnight prior to performing the experiments.

2.5 Chemicals

Carrageenan, gum acacia, diclofenac sodium was purchased from loba chemie and all other chemicals were of analytical grade.

2.6 Antibacterial assay

i. Disc diffusion assay[8]

The obtained strains of microorganism were inoculated in conical flask containing 100 mL nutrient broth. These conical flasks were incubated at 37^0C for 24 h and it was referred as seeded broth. Nutrient agar medium was taken in petri plate and inoculated with the test organisms using cotton swabs from the seeded broth. The cork borer was drilled to prepare well in it. Sterile discs of 6mm had been impregnated with 20µl of test extract onto the upper layer of the seeded agar plate. The plates were incubated overnight at 37^0C for 48 hr. The antibacterial activity was recorded by measuring the width of the clear inhibition zone around the discs using Vernier caliper scale (mm). streptomycin (100µg/mL) and dimethylsulfoxide (DMSO) was used as standards and negative control respectively.

ii. Broth dilution method[9]

A set of 12 test tube were taken and in each test tube 5mL of nutrient broth was added. In the first tube (UT) inoculum was not added which is to determine the sterility of medium used as negative control. Another test tube was inoculated and labelled as positive control to check the suitability of the medium for growth of the test microorganism and viability of inoculums. Remaining 10 test tubes were filled with ethanolic extract of roots at an increasing order of concentration from 50 to 500 µg/mL and volume made up to 10mL with sterile water and then incubated at 37^0C for 48 hr.

2.7 Anti-Oxidant activity[10,11]

Antioxidant activity of test extract was evaluated spectrophotometrically by DDPH (2,2-Diphenyl-1-picryhydrazyl) assay using BHT (Terbutyl-1-hydroxytoluene) as a standard. Ethanolic root extract of H. schulli was dissolved in ethanol to obtain concentrations in the range 0.977-500 µg/mL in 10mL volumetric flask, freshly prepared DPPH solution (2.9mL; 100ugmL in ethanol) and 0.1mL of different range of concentrations of extract were added. The mixture mixed well and allowed to stand in dark for 30 min and absorbance was measured at 517 nm using Shimadzu 1601 UV-Vis spectrophotometer. A control solution consists of 0.1mL of ethanol and 2.9mL of DPPH radical solution. Butylated hydroxyl toluene was used as standard of the same concentration as that of test solution. Percentages scavenging of DPPH pf test extract were calculated by comparing the absorbance between the test mixture and control. Percentage scavenging of DPPH was calculated by using formula.

% scavenging of DPPH= $\frac{A\ control - A\ test}{A\ control} \times 100$

The % inhibition in scavenging of DPPH radical was plotted against concentrations (0.977-500µg/mL).

2.8 Cytotoxicity Screening[12]

Brine shrimp bioassay technique was used to test cytotoxicity of the test extract. Anticancer drug Vincristine Sulfate was used as Positive control, dimethylsulfoxide (DMSO) was used as solvent and ethanolic extract of H. schulli as negative control. In 10 mL of volumetric flask, 4 mg of each test sample was dissolve in DMSO to obtain solutions of varying concentrations (400, 200, loo, 50, 25, 12.5, 6.25, 3.125, 1.5665 and 0.78125 µg/mL) by serial

dilution technique. DMSO solution was applied against artemia saline for 24H to calculate percent mortality.

2.9 Thin Layer Chromatography[13]

The pharmacologically active ethanolic extract obtained from root of *A. longifolia* was subject to thin layer chromatography to find out number of compound present in it. The use of specific visualizing agent such as vanillin sulphuric acid reagent showed the presence of sterol in maximum quantity indicated by pink color spot. Alkaloids were identified as orange zone when Dragendorff's reagent used as a visualizing agent. Tannins were detected as a bluish green zone by using 5% ferric chloride reagent. Abundance of these constituents has been determined by measurement of the zone occupied by the band the intensity of color.

Table 1: Results of TLC of roots extracts of *A. longifolia.*

Extract	Solvent system	Λ	No. of components	Rf value
Ethanolic extract	Toluene: Ethyl acetate: Methanol (5:1:0.5)	254nm	1	0.75
	Toluene: Ethyl acetate:GAA:Methanol (3:4:1:3)	254nm	2	0.45 0.55
	Toluene:Acetone (5:1)	254nm	1	0.88
	Ethyl acetate:Chloroform (9:1)	254nm	3	0.09 0.32 0.41
Petroleum ether	Toluene: Ethanol (8:2)	254 & 366	2	0.43 0.75

2.10 Carrageenan induced rat paw edema[14,15,16,17,18]

The effect of ethanolic extract of *A. Longìfolia* root on acute inflammation, can be studied by using the Carageenan-induced rat paw edema model. Rats were divided into four groups. Each group was consisting of 6 rats. Both hind paws are marked just below the tibia-tarsal junction, to ensure constant paw volume. The Paw volume (both right and left) was measured immediately before the Carrageenan injection (V0, initial paw volume) using Plethysmometer for initial reading. Carrageenan (0.1mL of a 1% suspension in saline) was injected subplantarly into the midline med metatarsal region of left hind paw of the rats of all groups. Thirty minutes prior to the injection of the carrageenan, 10, 100, 200mg/kg were given in separate groups of animals. The paw volumes of both legs of the control, Diclofenac sodium and ethanolic extract treated groups were measured at 1,3 and 5H after induction of

inflammation (vt), comparing with the right paw which is used as reference non-inflamed paw.

The % of inhibition was obtained for each group and at each record, using the following ratio:

$$\textbf{\% inhibition} = \frac{mean\ paw\ volume\ of\ test - mean\ paw\ volume\ of\ control}{mean\ paw\ volume\ of\ control} \times 100$$

Statistical analysis

The statistical analysis of evaluation of the anti-inflammatory activity β-sitosterol from ethanol root extract of *A. longifolia* against the carrageenan induced paw edema is analyzed using ANOVA followed by Dunnett's multiple comparison for the data which are normally distributed using graph pad prism and all the results obtained in the study were compared with the vehicle control group: The values at $p< 0.05$ were considered statistically significant. Each value is the mean ±SD of 3 animals statistically significant from control. *p <0.05, **p< 0.01 and ***p <0.001 value is calculated by comparing with control.

3. RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Screening of Root Extract

Ethanolic extract of roots of *A. longifolia* showed affluent enrich phytochemical profile with the presence of potentially bioactive constituents such as sterols, tannins, alkaloids, glycoside, carbohydrates, amino acid and protein while sterols are present in petroleum ether.

3.2 Antibacterial Activity by disc diffusion method

The test extract showed significant antibacterial activity against Bacillus subtillus with 20mm of zone of inhibition compared to reference standard streptomycin 25mm however, mild to moderate antibacterial activity against Pseudomonas aeruginosa, Staphylococcus, aureus and Escherichia coli are 16, 18 and 18 respectively.

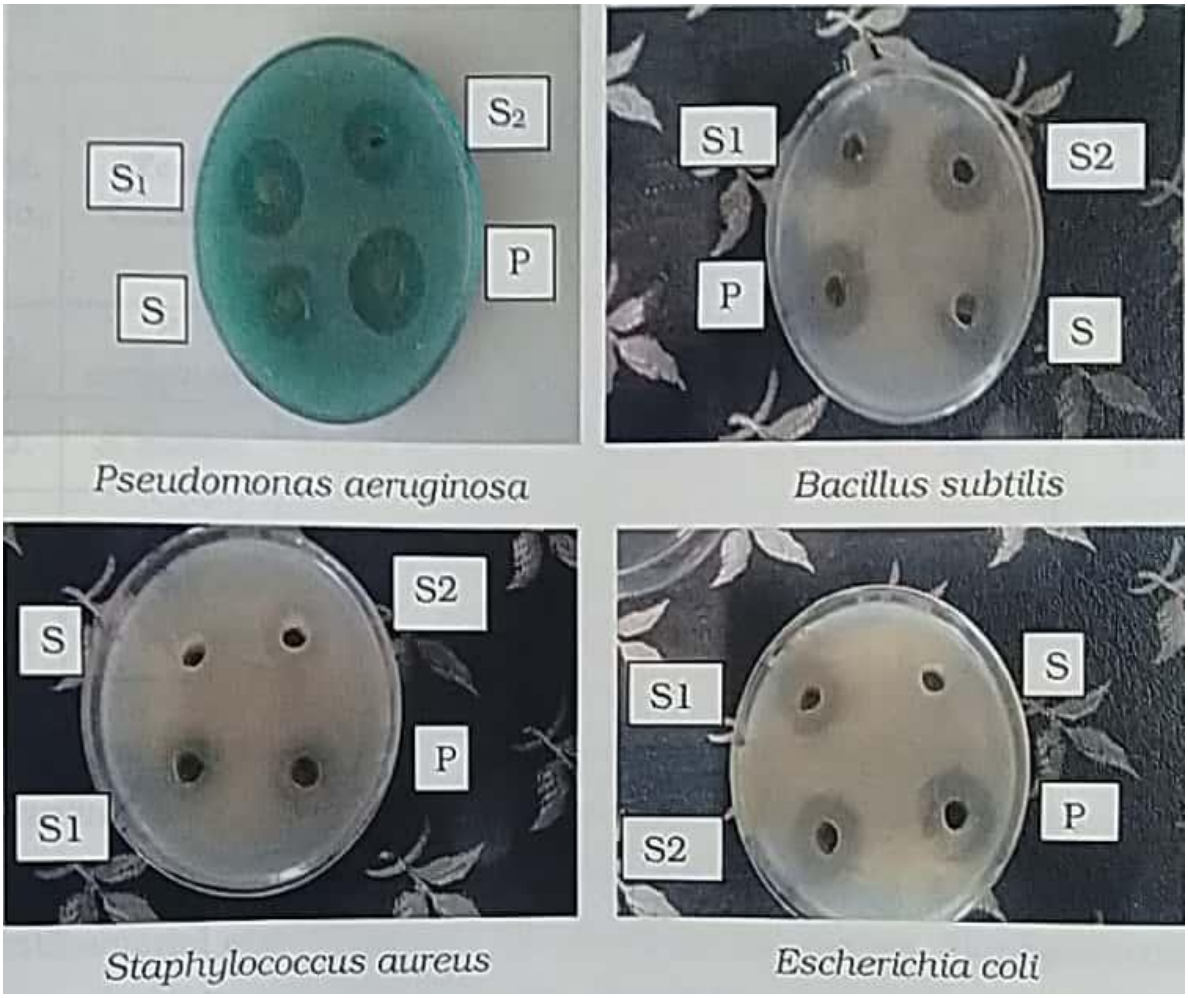

Fig. 1 Antibacterial Activity of Ethanolic Extracts of Roots (200 µg/Ml).

S: DMSO Solvent S1: Sample (Ethanolic Extract)

P: Positive Control (Streptomycin) S2: Sample (Ethanolic Extract)

3.3 Minimum Inhibitory Concentration by broth dilution method

The MIC of antibacterial activity of ethanolic root extract of H. schulli was shown at 50 µg/mL against Bacillus subtillus however, remaining bacterial culture, MIC was found to be at 100 µg/mL of concentration.

Table 2: Measurement of zone of inhibition in antibacterial activity.

Sr. no.	Test culture	Positive control 20 µ for (100 µg/mL)	Ethanol extract 20 µl for (200 µg/mL)	Zone of inhibition in (mm)		
				DMSO	Positive Streptomycin	Plant extract
1	P. Aeruginosa	Streptomycin	Ethanol extract	-	22	16
2	S. aureus	Streptomycin	Ethanol extract	-	24	18
3	E. colli	Streptomycin	Ethanol extract	-	24	18
4	B. subtilis	Streptomycin	Ethanol extract	-	25	20

3.4 Anti-Oxidant Activity

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The MIC of antibacterial activity of ethanolic root extract of H. schulli was shown at 50 µg/mL against Bacillus subtillus however, remaining bacterial culture, MIC was found to be at 100 µg/mL concentration. Antoxidant activity of IC50 determined by using a calibration curve constructed by plotting logarithmic concentration (0.977-500µg/mL) v/s % inhibition in DPPH radical scavenging by test extract which showed significant activity with 88.06µg/mL which is comparable to 61.53µg/mL (BHT)

Table 3: Anti-oxidant activity of ethanolic root extract of H. schulli by DPPH assay.

Sr. No.	Conc. (µg/mL)	Abs		Percentage Inhibition (%)	
		Standard	Extract	Standard	Extract
1	0.977	0.1526	0.1598	32.14	28.94
2	1.953	0.1505	0.1575	33.08	29.96
3	3.906	0.1464	0.1507	34.90	32.99
4	7.813	0.1401	0.1450	37.70	35.52
5	15.625	0.1357	0.1401	39.66	37.70
6	31.5	0.1299	0.1350	42.24	39.97
7	62.5	0.1264	0.1314	43.79	41.57
8	125	0.1159	0.1219	48.46	45.79
9	250	0.0784	0.0899	65.14	60.02
10	500	0.0692	0.0718	69.23	68.07

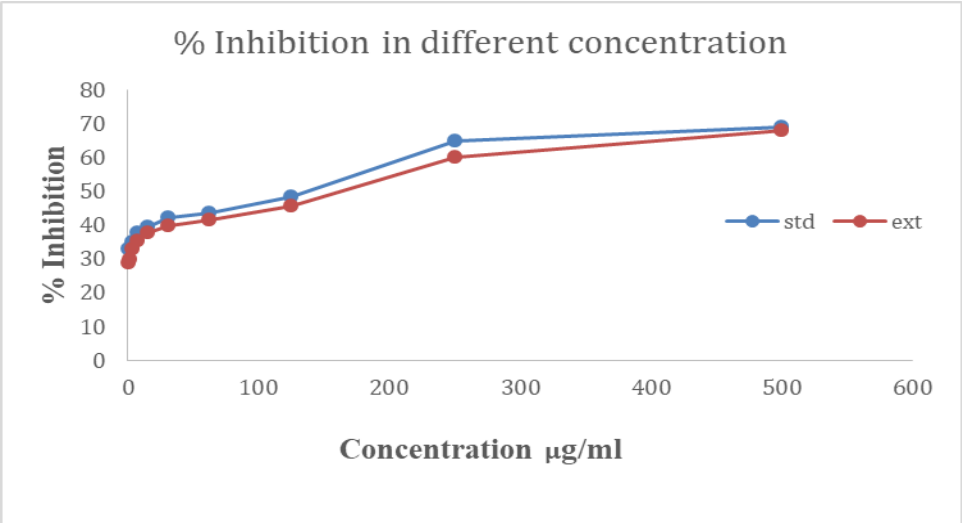


Fig 2. Inhibition in different concentration.

3.5 Cytotoxic Activity

To determine LC50 of cytotoxic activity, a calibration curve was constructed by plotting logarithmic concentrations (0.7812-400 µg/mL) versus percentage mortality by test extract of H. schulli. The ethanolic extract showed significant cytotoxic activity with LC50 of 56.37 µg/mL whereas LC50 of vincristine sulphate was 31.26 µg/mL.

Table 4: Brine shrimp of ethanolic extract of H. Schulli.

Sr. No.	Conc. (µg/mL)	Log Conc.	No. of Brine shrimp added	No. of brine shrimp dead			% Mortality		LC_{50} (µg/mL)	
				Std.	Ext.	DMSO	Std.	Ext.	Std.	Ext.
1	0.7812	-0.1072	25	04	02	0	16	08	31.26	56.37
2	1.5665	0.1949	25	05	03	0	20	12		
3	3.125	0.4948	25	06	04	0	24	16		
4	6.25	0.7958	25	08	07	0	32	28		
5	12.5	1.0969	25	09	08	0	36	32		
6	25	1.3979	25	11	10	0	44	40		
7	50	1.6989	25	13	11	0	52	44		
8	100	2.0000	25	16	14	0	64	56		
9	200	2.3010	25	18	16	0	72	64		
10	400	0.6020	25	21	19	0	84	76		

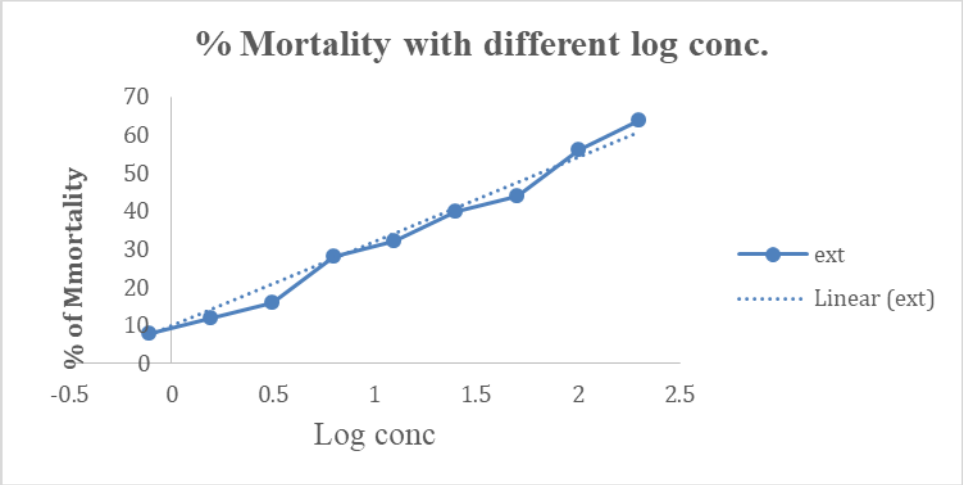


Fig. 3: Graphical representation of brine shrimp bioassay of H. schulli.

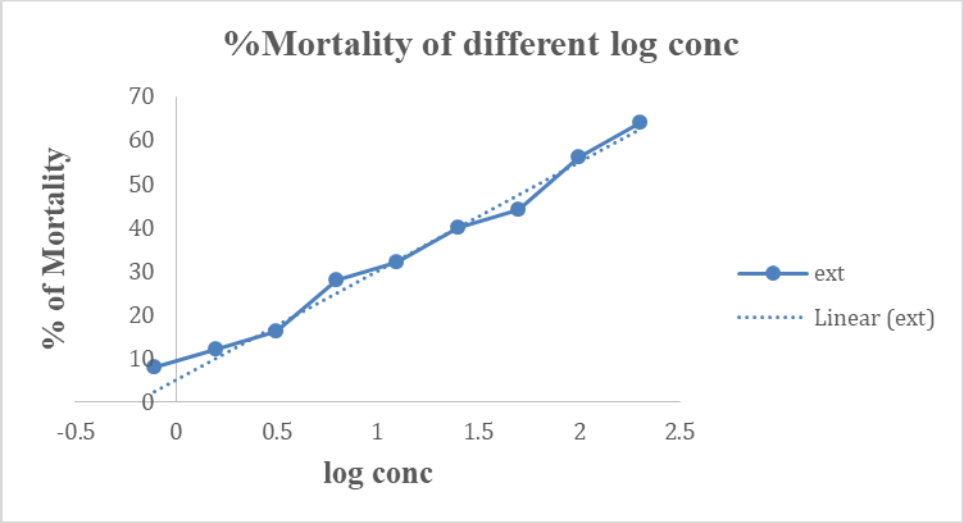


Fig. 4: Graphical representation of brine shrimp bioassay of vincristine sulpate.

3.6 Anti-Inflammatory Activity of root extract by Carrageenan induced rat paw edema model

All the fractions are monitored on TLC to collect the fractions having same Rf value i.e. 0.43. Further fractions are collected and evaporated to get dry residue. The structure of the isolated compound was established i.e. Stigmast-5-en-3 β-0l as shown in **fig. no. 1**. On the basis of elemental analysis and spectrometric evidences (IR, NMR) Then the isolated compound from ethanolic extract of *A. longifolia* roots subjected for the anti-inflammatory activity by using carraageenan induced rat paw edema model and it was found that it showed significant anti-inflammatory activity.

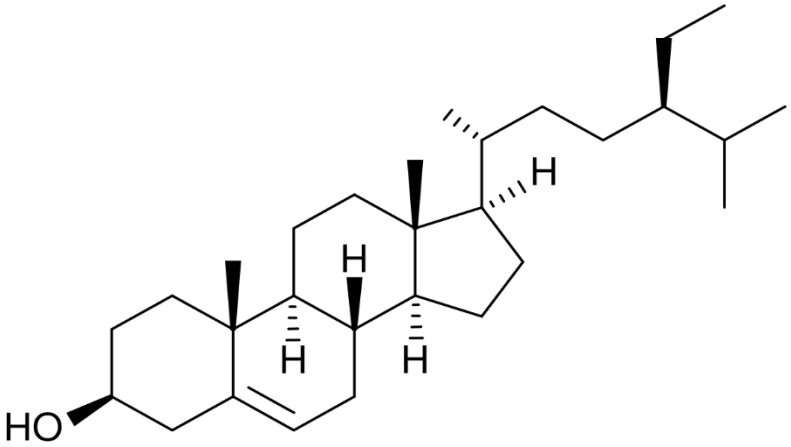
Table 5: Treatment of test materials to carrageenan induced rat paw edema.

Group	Treatment	Dose(mg/kg)
I	Normal saline (Control)	-
II	Diclofenac Sodium (Std)	10
III	β-Sitosterol (Test)	100
IV	β-Sitosterol (Test)	200

Table 6: Percentage inhibition of ethanolic extract for Inflammation in rat paw edema.

Treatment Group	Dose (mg/Kg)	Time (hr)	Paw volume Mean ±SD	Percentage Inhibition
Control	-	1	0.31±0.011	-
		3	0.379±0.006	
		5	0.235±0.025	
Diclofenac sodium (Standard)	10	1	0.213±0.024***	38.63
		3	0.208±0.010***	
		5	0.147±0.020***	
B-Sitosterol	100	1	0.265±0.010*	20.77

(Test)		3	0.258±0.027***	
		5	0.211±0.024ns	
	200	1	0.262±0.019*	30.84
		3	0.212±0.018***	
		5	0.167±0.026**	



Stimast-5-en-3β -ol

Figure 5: Structure of β-sitosterol.

CONCLUSION

A. longifolia (H. Schulli) has a wide array of pharmacological activities and is a potential source of many chemical constituents and widely used for many health problems. In the proposed work the crushed roots of *A. longifolia* extract were extracted with various solvent with increasing polarity, ethanolic extract shows the abundant amount of chemical constituents which may serve as a promising therapeutic agent, so it was subjected for the isolation of phytoconstituents by gradient elution column chromatography. Structure of the isolated compound was identified on the basis of their physical and chemical properties reported in the literature. From the physical, chemical and spectral evidences isolated compound was confirmed as β-sitosterol.

On the basis of phytochemical screening it was found that the plant extract contains a wide range of Phyto-constituents such as sterols, tannins, alkaloids, glycoside, carbohydrates, amino acid and protein which possess good antioxidant and free radical scavenging activity which is believe to be one of the important components for many pharmacological activities. The results also indicated that the isolated compound from Ethanolic extract of *A. longifolia* roots also possess significant cytotoxic and anti-inflammatory activity. This anti-

inflammatory activity may be due to the strong occurrence of polyphenolic compounds such as sterols i.e. β-sitosterol.

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