Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2017; 9(9); 1280-1287

ISSN: 0975-4873

Research Article

Isolation and Characterization of β-Sitosterol from *Justicia gendarussa burm. F.*-An Anti-Inflammatory Compound

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Received: 13th May, 17; Revised 5th Sept, 17, Accepted: 16th Sept, 7; Available Online:25th Sept, 17

ABSTRACT

Justicia gendarussa Burm.f. has become important source of β-sitosterol which is associated with other phenolic,terpenoids,alkaloids and steroids. Plant sterols show anti-inflammatory activity. β-sitosterol is one of phytosterol, in a mouse model of acute inflammation, and β-sitosterol effect on leukocyte recruitment, cytokines levels, and oxidative stress. The anti-inflammatory activities of β-sitosterol were assessed by measuring paw edema induced by different inflammatory agents. It separated from *Justicia gendarussa burm.f.* and characterization of β-sitosterol carried out by IR, NMR, and mass spectrometry. β-sitosterol shows potent as Anti-inflammatory activity by releasing histamine (30.07%), serotin and bradykinin (52.25%), and prostaglandin (69.43%) as compared to standard (Dicolfenac 5mg/kg). Objectives: To isolate, separate and characterization of β-sitosterol and to evaluate the anti-inflammatory activity of β-sitosterol extracted from Chloroform extract of *Justicia Gendarussa Burm.f.*

Keywords: β-sitosterol, *Justicia gendarussa burm.f.* ,Anti-inflammatory activity etc.

INTRODUCTION

Biological screening is necessary to provide a scientific basis for validating the traditional utilization of medicinal plants. A great number of screening programs are going on worldwide for new plant based bioactive molecules. Gas Chromatography (GC) and Mass Spectroscopy (MS) can be used to study Traditional Medicines and characterize the compound of interest.

Studies have shown naturally occurring steroids have anti-inflammatory and redox-protective pharmacological activities. The present study aimed to investigate the anti-inflammatory properties of β-sitosterol, one of phytosterol, in a mouse model of acute inflammation, and β-sitosterol effect on leukocyte recruitment, cytokines levels, and oxidative stress. The anti-inflammatory activities of β-sitosterol were assessed by measuring paw edema induced by different inflammatory agents. Inflammation is a complex biological response of vascularized tissues to harmful stimuli, such as pathogens, damaged cells, or irritants[1]. It is well established that this process involves the local formation of kinins and cytokines that promote vascular endothelial cell activation, followed by leukocyte migration into the inflamed site[2]. Another important component of inflammatory response is oxidative stress leading to the generation of molecules, such as hydrogen peroxide, superoxide anion, and per oxy nitrite, which are produced in response to stimuli and can exacerbate this process[3]. The clinical signs and symptoms of inflammation include edema, fever, erythema, pain, and cell migration (primarily neutrophil migration) into the site of injury[4]. The drugs used to treat these symptoms, such as non steroidal anti-inflammatory drugs (NSAIDs), are not only associated with major adverse effects, such as gastrointestinal ulcers, bleeding, and renal disorders, but also have low therapeutic efficacy[5]. Thus, the search for new products with therapeutic potential for the treatment for inflammation has increased in recent years[6]. Many studies have been conducted as a part of the search for new therapeutic options for inflammation, and classes of secondary metabolites from natural sources, such as lactones[7], alkaloids[8], and terpenoids[9],steroids have attracted the attention of many researchers because of their pharmacological activities. β-Sitosterol is a plant sterol found in the some vegetable oil, nuts. Beta-sitosterol is used for heart disease and high cholesterol. It is also used for boosting the immune system and for preventing cancer, as well as for gallstones, the common cold and flu (influenza), HIV/AIDS, rheumatoid arthritis, tuberculosis, psoriasis, allergies, cervical cancer, fibromyalgia, systemic lupus erythematosus, asthma, hair loss, bronchitis, migraine headache, and chronic fatigue syndrome. β-sitosterol is a plant substance similar to cholesterol. It might help reduce cholesterol levels by limiting the amount of cholesterol that is able to enter the body. It can also bind to the prostate to help reduce swelling (inflammation). The aim of this study was to investigate

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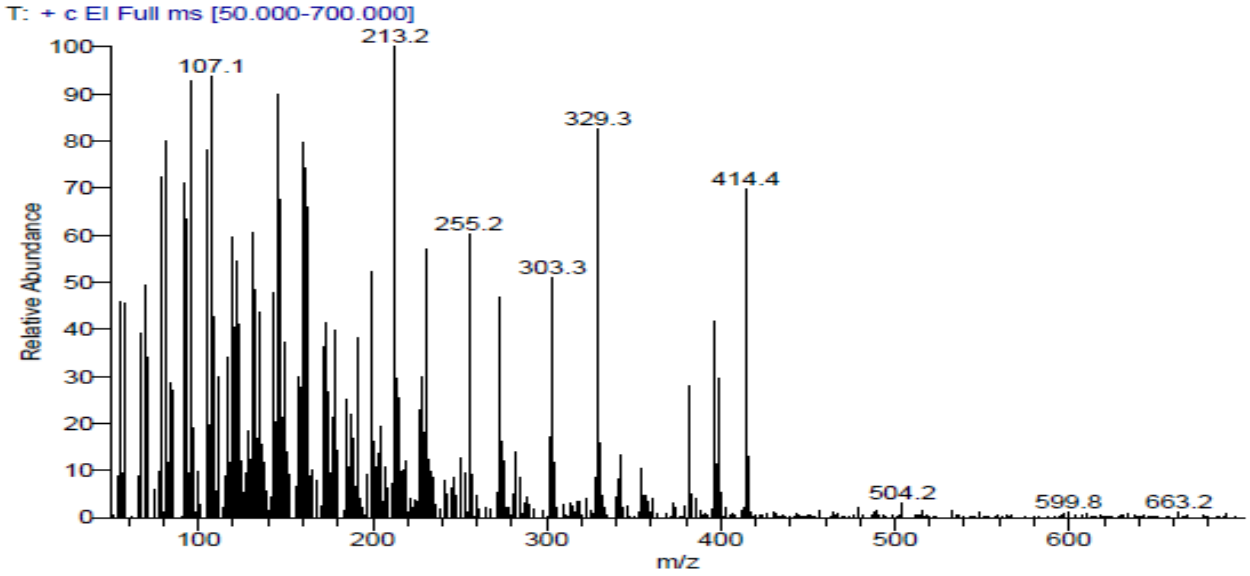


Figure 1: Mass Spectra of β-sitosterol.

Mass fragments (m/z) 414 (M^+), 329, 303, 255, 213,161,145,133, 119, 107, 81, 57, 55

IR spectra of β-sitosterol :

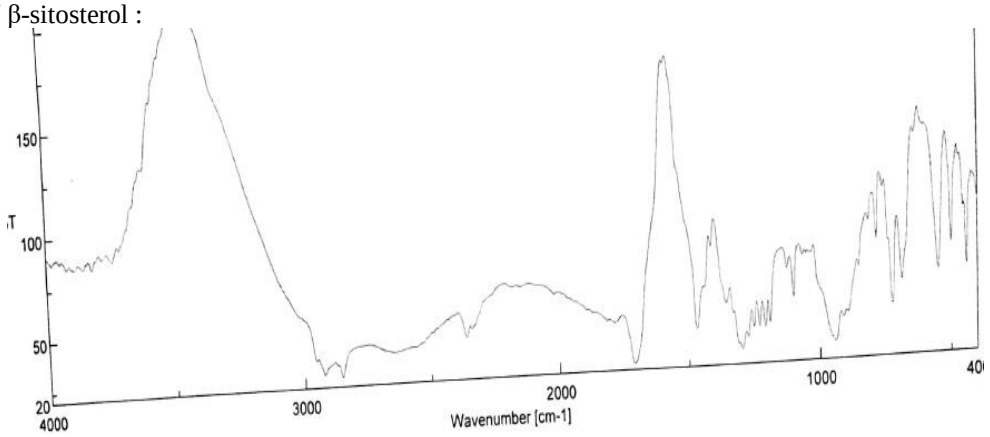


Figure 2: IR spectra of of β-sitosterol.

Table 1: Interpretation of FTIR spectra of compound- 6 (JG06).

Wave number (cm^{-1})	Functional groups
3200-3450 (broad)	OH- stretching
1500	C=C stretching
2950	C-H stretching

the anti-inflammatory properties of β-sitosterol, a plant sterol, in mouse models of acute inflammation. Furthermore, the study investigated the roles of leukocyte recruitment, cytokines, and oxidative stress in β-sitosterol induced effects.

MATERIALS AND METHODS

Plant material

Mature and healthy plants of *Justicia Genandrussa Burm.f.* were collected from Akole, Southern Western Ghats in the district of Ahmednagar, Maharashtra, India. The specimens were identified, comparing the characteristics of floral and vegetative characters in the 'Botanical Survey of India, Pune' (BSI/WRC/Tech./2013/1154). Voucher specimens are documented in the herbarium of, 'Botanical Survey of India', Pune India. The collected Plant leaves were collected dried under shadow below 40^0C.

In beginning of extraction defattation carried out by Soxlet extraction by Pet-ether for defattation. Then cold maceration of marc carried out by ethanol for 7-8 days. Succesive fractionation carried out with chloroform. Compounds are separated by Column chromatography followed by preparative chromatography. Column carried out in dichloromethane. Column monitored by thin layer chromatography carried out in different mobile phases.

GCMS study carried out at CIL, Panjab University Jalandhar, India. The GC - MS analyses were carried out in a Shimadzu GC – MS - QP 2010 gas chromatograph fitted with a DB1 (methylphenylsiloxane, 30 m × 0.25 mm i.d.) capillary column. Carrier gas, helium with a flow rate of 0.7 mL/min; column oven temperature 70 ℃, 5 min in 180 ℃, 180- 260 ℃ at 3 ℃/min, 5 min in 260 ℃, 260-280 ℃ at 0.2 ℃/min, and finally 5 min in 280 ℃; injector temperature, 280 ℃ detector temperature, 290 ℃, Volume injected, 1 µL of TMS ether derivatives in *n*-hexane (2%); Split ratio, 3:0. The MS operating parameters were as

^{1}H NMR:

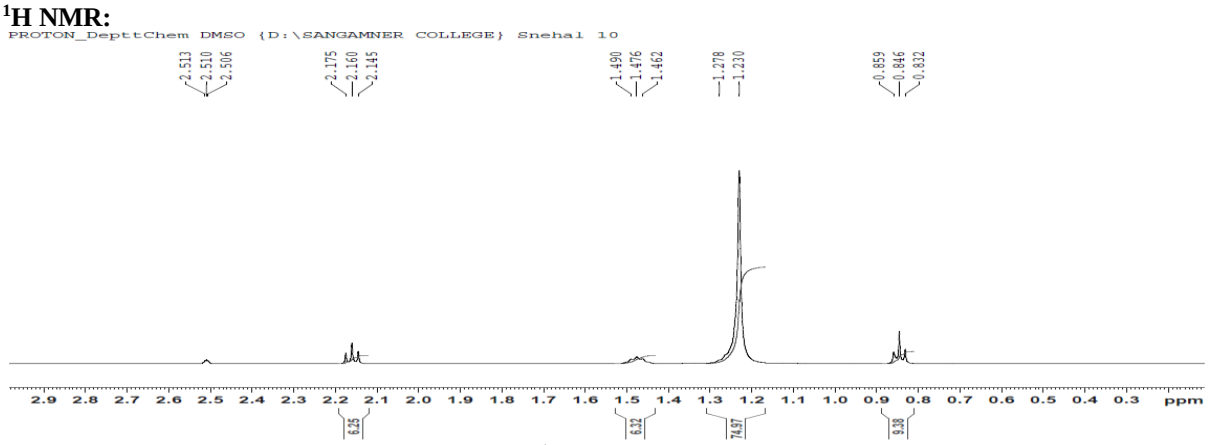


Figure 3:^{1}H NMR of of β-sitosterol.

^{13}C NMR of β-sitosterol:

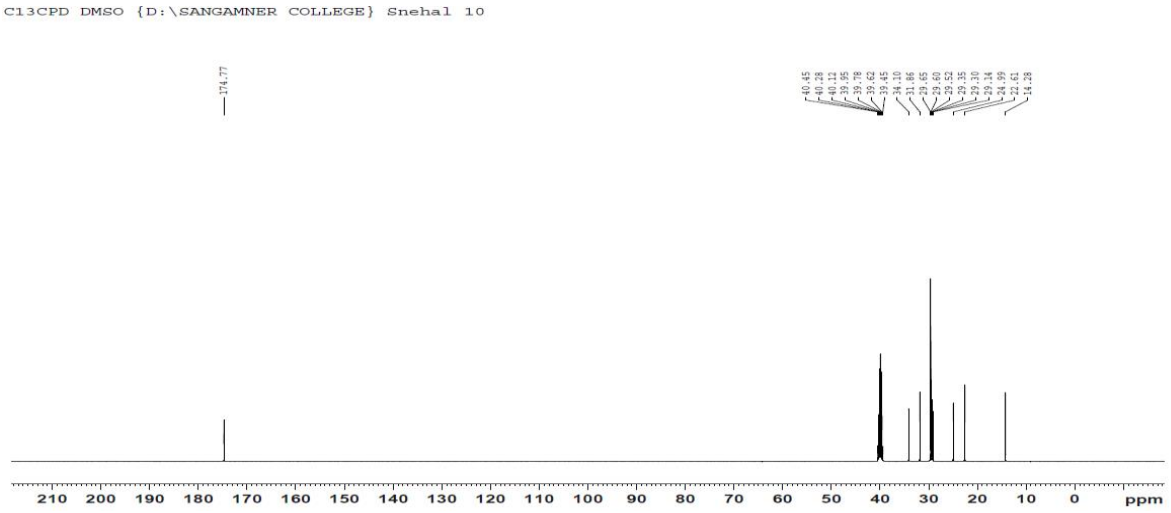
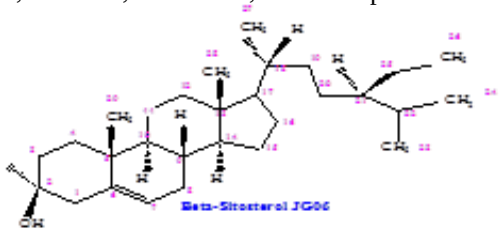


Figure 4: 13C NMR of of β-sitosterol.

The structure of isolated compound confirmed by FTIR,1H NMR, 13C NMR,and mass sperta.



follows: ionization potential 70 eV; ion source temperature 200 ℃; quadrupole 100 ℃, Solvent delay 6.0 min , scan speed 2000 amu/s and scan range 30-600 amu, eV voltage 3000 volts.

Characterization by IR H^{1} NMR, C^{13} NMR

Infra red used for functional group identification of make Brucker at pharmacy college, Amrutwahini, Sangamner. NMR of make Simatzu 500 MHz at Savitribai Phule Pune University, Pune.

Anti-inflammatory Activity

Animal

Wistar rats (125-200 g) were obtained from the Animal House, National Institute of Biosciences, Pune. They were housed at a temperature of 24 ± 2^{0}C, 12-hour light/dark cycles, 35-60% humidity, in polypropylene cages, and fed a standard rodent diet with water. Animals were deprived of food but not water 12 hours before the experiment.

Drugs

Diclofenac (Reckitt Benckiser, Gurgaon, India), and Carrageenan (Sigma Chemicals, St. Louis, MO, USA) were procured from the respective companies and were used in the study.

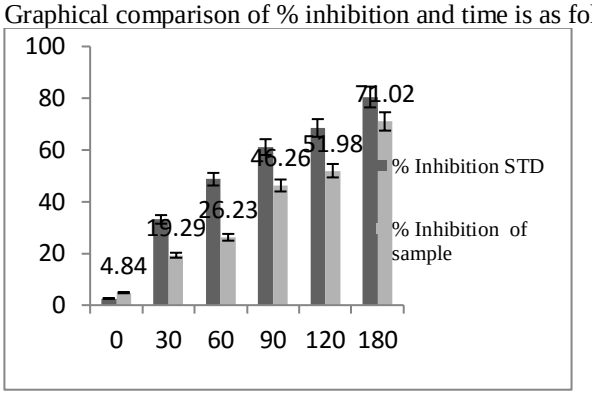
Ethical considerations

Experimental procedures and protocols used in this study were approved by the Amrutwahini College of Pharmacy, Sangamner, Dist Ahmednagar, Maharashtra. and conform to the "Guidelines for care and use of animals in scientific research" (Indian National Science Academy 1998, Revised 2000)(IAEC No.:1153/PO/OC/08/004/CPCSEA). Carrageenan-induced rat paw edema model The rats were divided into three groups (n=5), each receiving distilled water (control), diclofenac 5 mg/kg p.o. (reference standard), and 10, 25,50 mg/kg p.o. dose of the Pet ether extract. Carrageenan (0.1 mL of 1%) was injected into the subplantar tissue of the right hind-paw of each rat. The volume of the Carrageenan injected into the foot was measured at 0, 30, 60, 90, 120, 180,240 and 300 minutes

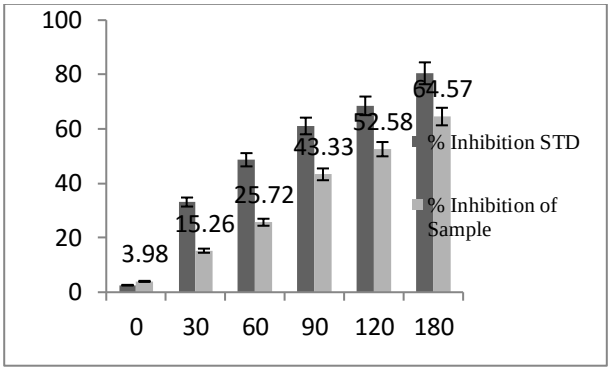
Table 2: The compound was obtained as a white powdered form . Its molecular mass was determined on the basis of mass spectral data at m/z 414 which was in accordance with its molecular formula $C_{29}H_{50}O$.The interpretation of NMR is given below table.

Position	δ_H (ppm, *J*, Hz)	**Position**	δ_H (ppm, *J*, Hz)
1	H_1',H_1'' = 1.230 (d)	16	H_{16}', H_{16}'' = 2.175 (dt)
2	H_2 = 1.278 (tt) OH- = 3.358 (s)	17	H_{17} = 2.160 (t)
3	H_3',H_3'' = 1.462 (dt)	18	H_{18}', H_{18}'' = 1.476(d)
4	H_4',H_4'' =1.490 (t)	19	H_{19}', H_{19}'' = 1.230(dt)
7	H_7' = 4.250 (t)	20	H_{20}', H_{20}'' = 1.462 (dt)
8	H_8',H_8'' = 2.160 (dd)	21	H_{21}', H_{21}'' = 1.230 (dt)
9	H_9',H_9'' = 1.278 (d)	22	H_{22}', H_{22}'' = 0.859 (m)
10	H_{10}' = 1.490 (d)	23,24	H_{23},H_{24} =0.832 (d)
11	H_{11}', H_{11}'' = 1.278 (dt)	25	H_{25}', H_{25}'' = 0.846 (qd)
12	H_{12}', H_{12}'' = 1.278 (t)	26	H_{26}', H_{26}'' = 0.846 (t)
14	H_{14}', H_{14}'' = 2.499 (t)	27	H_{27}', H_{27}'' = 1.476 (d)
15	H_{15}', H_{15}'' = 2.175 (dt)	28	H_{28}', H_{28}'' = 0.859 (s)
11	H_{11}', H_{11}'' = 1.278 (dt)	25	H_{25}', H_{25}'' = 0.846 (qd)
12	H_{12}', H_{12}'' = 1.278 (t)	26	H_{26}', H_{26}'' = 0.846 (t)
14	H_{14}', H_{14}'' = 2.499 (t)	27	H_{27}', H_{27}'' = 1.476 (d)
15	H_{15}', H_{15}'' = 2.175 (dt)	28	H_{28}', H_{28}'' = 0.859 (s)

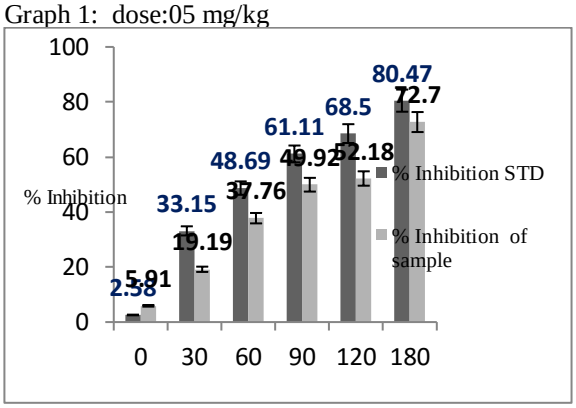
Graphical comparison of % inhibition and time is as follows.



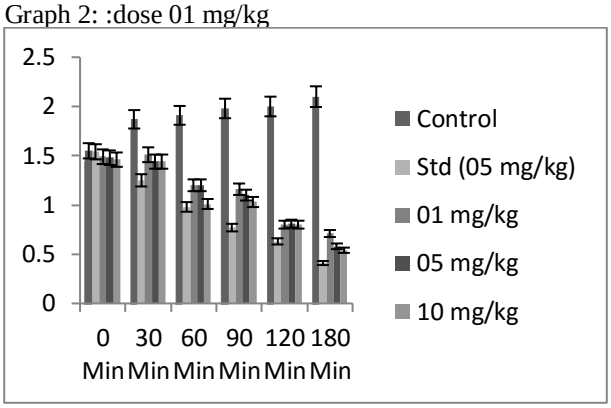
Graph 1: dose:05 mg/kg



Graph 2: :dose 01 mg/kg



Graph 3: dose 10 mg/kg



Graph 4: graph of std, Control and sample

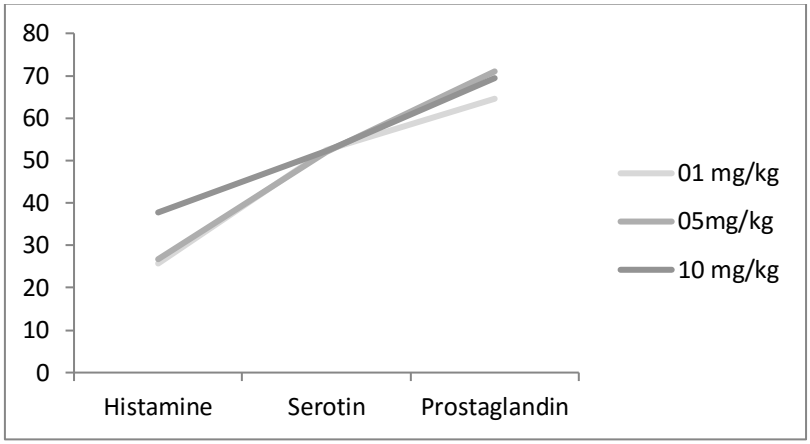
using a plethysmometer (Medicaid System, Mode No.PTH-707 New Delhi, India). The percentage inhibition (PI) of edema at each time interval was calculated

Percentage inhibition of edema = 1- V_t / V_c x 100
Where V_t and V_c are the volumes of edema in control and drug treated rats.

Anti-inflammatory Activity:

Table 3: Data of dose dependant Anti-inflammatory activity of β- Sitosterol (n = 06).

Sample No	Wt of animal in gm	Fraction of Dose	0 Min	30 Min	60 Min	90 Min	120 Min	180 Min	240 Min	300 Min
			Edema	Edema	Edema	Edema	Edema	Edema	Edema	Edema
I	160		1.47	1.45	1.17	1.15	0.83	0.72	0.83	0.92
II	170		1.53	1.47	1.21	1.21	0.89	0.74	0.87	0.95
III	170		1.45	1.54	1.23	1.15	0.74	0.69	0.79	0.89
IV	180	01mg/kg	1.58	1.49	1.19	1.13	0.81	0.72	0.81	0.91
V	160		1.48	1.53	1.25	1.19	0.77	0.67	0.77	0.857
VI	170		1.42	1.57	1.17	1.14	0.74	0.69	0.80	0.90
Mean			1.49	1.51	1.20	1.16	0.80	0.71	0.81	0.90
Std Error of Mean			0.02358	0.0186	0.0133	0.0127	0.0239	0.0105	0.0142	0.0126
Standard Deviation			0.05776	0.0457	0.0326	0.0312	0.0585	0.0258	0.0348	0.0310
% Inhibition			3.98	15.26	**25.72**	43.33	**52.58**	**64.57**	45.53	34.93
I	170		1.49	1.41	1.23	1.11	0.87	0.62	0.79	0.87
II	160	05mg/kg	1.51	1.43	1.17	1.11	0.85	0.58	0.66	0.79
III	150		1.47	1.39	1.21	1.05	0.79	0.54	0.68	0.83
IV	160		1.51	1.41	1.18	1.14	0.74	0.59	0.68	0.79
V	160		1.46	1.46	1.22	1.08	0.81	0.54	0.61	0.77
VI	170		1.41	1.52	1.16	1.12	0.78	0.59	0.72	0.82
Mean			1.48	1.44	1.20	1.10	0.81	0.58	0.69	0.81
Std Error of Mean			0.01544	0.0192	0.0117	0.0130	0.0194	0.0128	0.0247	0.0147
Standard Deviation			0.03782	0.0471	0.0288	0.0318	0.0476	0.0314	0.0606	0.0360
% Inhibition			4.84	19.29	**26.23**	46.26	**51.98**	**71.02**	53.69	41.61
I	170		1.45	1.48	0.98	1.01	0.87	0.63	0.79	0.84
II	160	10mg/kg	1.44	1.41	1.01	0.99	0.79	0.57	0.76	0.91
III	150		1.48	1.49	0.95	1.02	0.75	0.49	0.62	0.78
IV	160		1.45	1.45	1.04	1.06	0.85	0.57	0.73	0.74
V	160		1.41	1.38	1.01	1.02	0.71	0.45	0.59	0.69
VI	170		1.52	1.42	1.06	1.06	0.85	0.55	0.72	0.82
Mean			1.46	1.44	1.01	1.03	0.80	0.54	0.70	0.80
Std Error of Mean			0.01537	0.0174	0.0162	0.0114	0.0261	0.0261	0.0324	0.0316
Standard Deviation			0.03764	0.0426	0.0397	0.0280	0.0640	0.0640	0.0793	0.0776
% Inhibition			5.91	19.19	**37.76**	49.92	**52.18**	**72.70**	52.91	42.69



Graph 5: Release in Histamine (1 hr), Serotonin and bradykinin (2hr) and prostandinin (3 hr).

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by multiple Turkey's comparison tests. A p value < 0.05 was considered statistically significant.

RESULTS

The concentrated extract is injected into the GC/MS instrument (Hewlett Packard 5890 GC/MS with Mass Selective Detector with an HP-1 glass capillary column). The sample is volatilized at the injection port and eluted through a capillary column under increasing temperature. As the sample moves through the column, various components are separated due to their affinity for the stationary phase of the column and can be identified by

Edema in Control:

Table 4: Edema in control (normal saline).

Wt of animal	0 hrs	30 mins	60 mins	90 mins	120 mins	180 mins	240 min	300 min
200	1.51	1.88	1.87	1.96	1.98	2.09	1.97	1.85
180	1.54	1.87	1.92	1.99	2.02	2.11	1.99	1.87
170	1.61	1.81	1.95	2.01	1.94	2.14	2.01	1.91
180	1.56	1.92	1.88	1.98	2.05	2.08	1.95	1.86
170	1.53	1.86	1.89	1.99	2.00	2.05	1.89	1.53
170	1.55	1.85	1.93	1.95	1.99	2.11	2.03	1.89
SD	0.034	0.036	0.031	0.022	0.037	0.031	0.050	0.146
SEM	0.014	0.015	0.013	0.009	0.015	0.013	0.020	0.058
Mean →	1.55	1.87	1.91	1.98	2.00	2.10	1.49	1.39

Paw measurement in Standard (Diclofenac 5 mg/kg route of administration: Peritonial)

Table 5: Paw edema of Standard (Diclofenac 5mg/kg).

Wt of animal	0 hrs	30 mins	60 mins	90 mins	120 mins	180 mins	240 min	300 min
160	1.48	1.21	0.93	0.79	0.61	0.38	0.42	0.52
170	1.59	1.29	1.01	0.83	0.72	0.43	0.48	0.55
160	1.51	1.17	0.94	0.69	0.58	0.45	0.51	0.57
170	1.48	1.23	0.98	0.74	0.65	0.38	0.45	0.53
180	1.54	1.27	0.99	0.77	0.63	0.42	0.49	0.59
190	1.61	1.32	1.03	0.81	0.58	0.41	0.46	0.56
Mean	1.54	1.25	0.98	0.77	0.63	0.41	0.47	0.55
SEM	0.023	0.023	0.016	0.021	0.022	0.011	0.013	0.011
SD	0.055	0.055	0.039	0.051	0.053	0.028	0.032	0.026
% Inhibition	2.58	33.15	48.69	61.11	68.50	80.47	68.46	60.43

Table 6: Effect of a subcutaneous injection of Diclofenac as a standard. Values are the mean ± S.E.M of 6 animal, **P<0.01, ***P<0.001, compared to control (normal saline); P<0.001, compared, Tukey-Kramer test. Compare all pairs of columns, One way analysis of variances.

Treatment (mg/kg)	0 Min	30 Min	60 Min	90 Min	120 Min	180 Min
Control	1.55 ± 0.014	1.87 ± 0.015	1.91 ±0.013	1.98 ± 0.009	2.00 ± 0.015	2.10 ± 0.013
Std (05 mg/kg)	1.54 ± 0.023	1.25 ± 0.023	0.98 ± 0.016	0.77 ± 0.021	0.63 ± 0.022	0.41 ± 0.011
01 mg/kg	1.49 ± 0.024	1.51 ± 0.019	1.2 ± 0.013	1.16 ± 0.013	0.8 ± 0.024	0.71 ± 0.011
05 mg/kg	1.48 ± 0.015	1.44 ± 0.019	1.2 ± 0.012	1.1 ± 0.013	0.81 ± 0.019	0.58 ± 0.013
10 mg/kg	1.46 ± 0.015	1.44 ± 0.017	1.01 ± 0.016	1.03 ± 0.011	0.8 ± 0.026	0.54 ± 0.026

retention time (the time it takes for a compound to pass through the column and gas chromatograph system). Each chemical component in a sample has a distinct retention time measured in minutes, shown in a peak on a graph which measures abundance on the ordinate against retention time on the abscissa. The integrated peak is correlated to the concentration of the chemical. A mass selective detector breaks up each chromatographic component into fragment ions, which are shown by their abundance, with each ion represented as a vertical line in increasing molecular weight. The height of each line corresponds to the abundance of that ion. The resulting mass spectrum is unique to that chemical. This mass spectrum forms a ‘‘fingerprint’’ that can identify the compound by a computer search of mass spectra. A computer search of the mass spectra corresponding to all the chromatographic peaks for a sample should yield a statistical match for different compounds with respect to retention time. They were present two modes of GC/MS were possible with this instrumental method. First, there is a ‘‘Scan’’ mode which looks at all the constituents of a sample, listing whatever chemical components are present.

Compound Identification

Mass spectra were identified by comparison of their mass spectra and retention indices with those published in the literature and contained in the NIST ’98 MS computer library (Wiley). GC/MS analysis was carried out with the assistance of SAIF Panjab University, Jalandhar, India

The chromatogram of the β-sitosterol three important major peaks shows in Fig: 1.

CONCLUSION

β-Sitosterol shows significant Anti-inflammatory activity (72.70%) with respect to standard (Diclofenac 5mg/kg). Histamine release (30.07%) after 1 hr, serotonin and bradykinin release (52.25%) and prostaglandin release (69.43%) indicate remarkable percentage inhibition.

ACKNOWLEDGEMENT

CIL, Panjab University, Savitribai Phule Pune University, Pune, Principal, Amrutwahini pharmacy college,

Sangamner and Principal,S.N.Arts,D.J.M.Commerce and B.N.S.Science college,Sangamner.They made availability of facility for research.

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