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Research Article

Isolation and Characterization of Phytol from *Justicia gendarussa Burm. f.*-An Anti-Inflammatory Compound

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ABSTRACT
Justicia gendarussa burm.f. has become important source of Phytol which is associated with other phenolic,terpenoids,alkaloids. Diterpenes shows anti-inflammatory activity. Phytol is a acyclic diterpene alcohol found as essential oil in aromatic plants.oil is obtained by Keshav-Naren apparatus (patent no:2343/Mum/2013/A).Phytol separated from other oil part by fractional distillation. It separated at 204-208^{0}C.Characterization of Phytol carried out by IR,NMR,and mass spectrometry. Phytol shows potent as Anti-inflammatory activity by releasing histamine (26.92%), serotin and bradykinin (49.90%), and prostaglandin (68.03%) as compared to standard (Dicolfenac 5mg/kg).

Keywords: Keshav-Naren apparatus, *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 diterpenes have anti-inflammatory and redox-protective pharmacological activities. The present study aimed to investigate the anti-inflammatory properties of Phytol, a diterpene alcohol, in a mouse model of acute inflammation, and Phytol effect on release of Histamine (1hr), Serotin and bradykinin (2hr) and Prostaglandin (3hr) leukocyte recruitment, cytokines levels, and oxidative stress. The anti-inflammatory activities of Phytol 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], have attracted the attention of many researchers because of their pharmacological activities. Phytol (Figure 6) is an acyclic diterpene alcohol found in the essential oils of some aromatic plants, such as Cleome serrata[10], and Lantana radula[11]. Various therapeutic activities of Phytol have been reported in previous studies, including its activity against myco bacteria[12], and anticonvulsant[13], antispasmodic[14], and anticancer activities[15]. Some studies have demonstrated promising anti-inflammatory pharmacological activities of diterpenes[16,17], but few have focused on phytol. The aim of this study was to investigate the anti-inflammatory properties of phytol, a diterpene alcohol, in mouse models of acute inflammation. Furthermore, the study investigated the roles of leukocyte recruitment, cytokines, and oxidative stress in Phytol-induced effects.

MATERIAL AND METHODS

Collection of Plant leaves of Justicia Gendarussa Burm.f.

Plant material collection carried out in month of August from Western zone of Sahyadri ranges in Akole tahsil in Ahmednagar district, Maharashtra, India.

Authentification of plant

Authentification of plant carried out at 'Botanical Survey of India, Pune' (BSI/WRC/Tech./2013/1154). Voucher

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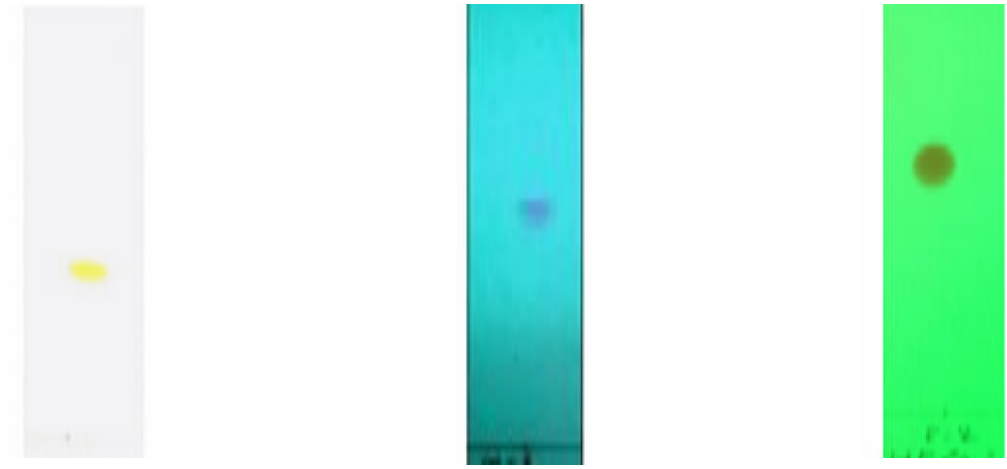


a b c

Figure 1: Demonstration of isolation pattern of pure compounds on TLC plate using iodine (A), UV-detection (B) and spray reagent (C) (Vanillin + H_2SO_4) reagent.

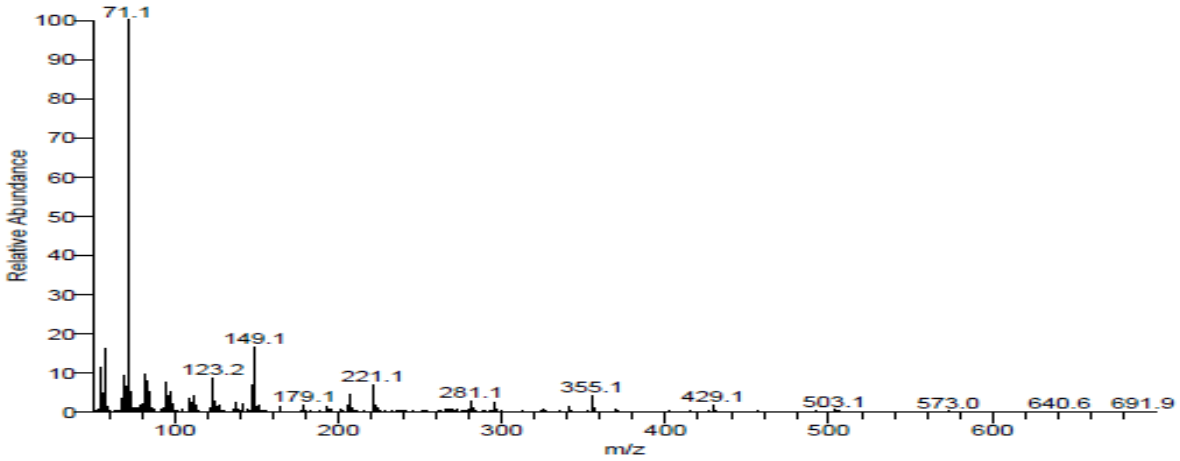


Figure 2: Mass Spectra of Phytol.

specimens are documented in the herbarium of 'Botanical Survey of India', Pune India.

Extraction of plant residue

The collected Plant leaves were collected and performed extraction by patented method entitled "Method for Extraction from plant by Vaporization". The patent no was 2343/MUM/2013 A published on dated 12/07/2013.

Separation of liquid by fractional distillation

Separation of liquids from viscous material of extract isolated from Keshav-Naren apparatus followed by Separation of liquid by fractional distillation .Isolated liquid compounds distillate at various temperature then extract collected at 200- 205^{0}C. Characterization by IR, NMR and mass spectrometry. Its biological efficacy evaluated animal model for Anti-inflammatory activity. Thin layer chromatography carried out in different mobile phases. Single spot observed in EA n-Hexane (2:8).The photograph is as follows

After separation physical properties of Phytol checked are as follows

Colour: Transparent colourless liquid

Odour: Odourless

Boiling point: 202^{0}C -204^{0}C

Density: 845 g/cm^{-3}

GCMS Study

The 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 °C, 5 min in 180 °C, 180- 260 °C at 3 °C/min, 5 min in 260 °C, 260-280 °C at 0.2 °C/min, and finally 5 min in 280 °C; injector temperature, 280 °C detector temperature, 290 °C, Volume injected, 1 µL of TMS ether derivatives in *n*-hexane (2%); Split ratio, 3:0. The MS operating parameters were as follows: ionization potential 70 eV; ion source temperature 200 °C; quadrupole 100 °C, 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 (150-200 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=6), each receiving distilled water (control), diclofenac 5 mg/kg p.o. (reference standard), and 01, 05,10 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

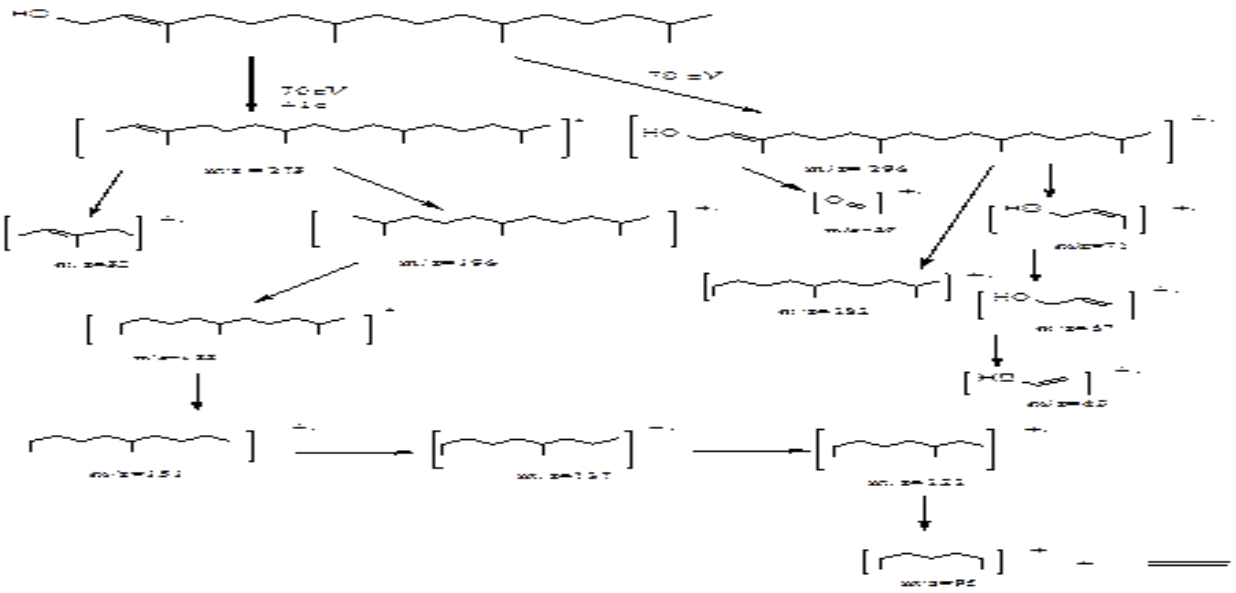


Figure 3: Fragmentation of Mass of Phytol.

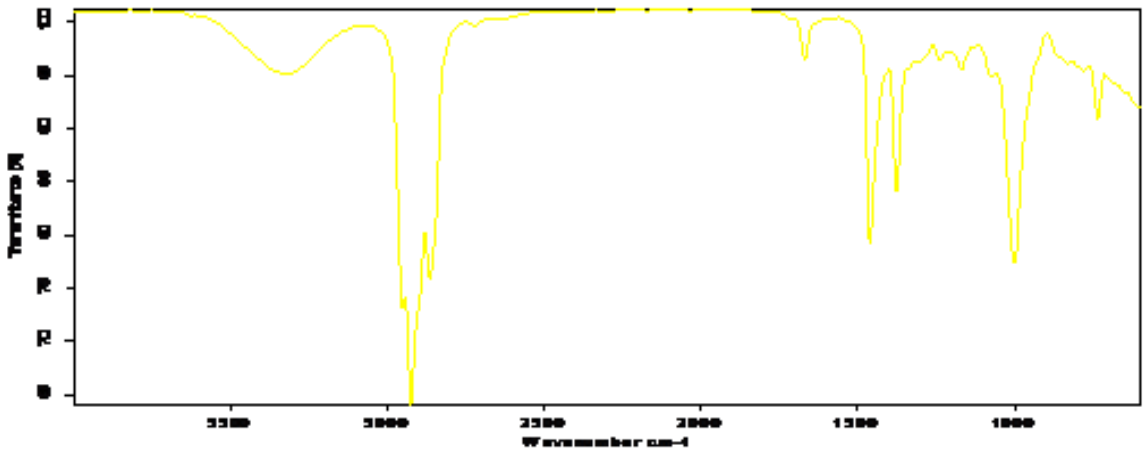


Figure 4:

Table 1: Interpretation of FTIR spectra of compound (JG04) i.e Phytol.

Sr.No.	Wave number (cm-1)	Functional groups
1	2900 cm^{-1}	Alkyl C-H stretching
2	3250 cm^{-1}-3500 cm^{-1}	OH- Stretching (Broad)
3	1450 cm^{-1}	C=C bond (due to α-OH-
4	1005cm^{-1}	C-O stretching

Interpretation of ^{1}H and ^{13}C NMR

Table 2: Interpretation of ^{1}H NMR and ^{13}C NMR.

Position	δ_H (ppm, *J*, Hz)	δc (ppm)	Position	δ_H (ppm, *J*, Hz)	δc (ppm)
-	OH- = 3.913 (s)	-	10	H_{10} = 0.733 (td)	C9 = 37.28 (t)
1	H_1',H_1'' = 1.632 (s)	C1 = 27.60 (t)	11	H_{11}= 1.917 (tt)	C10 = 37.18 (t)
2	H_2 = 4.397(t)	C2 = 126.82(d)	12	H_{12} = 1.220 (td)	C11 = 24.35 (d)
4	H_4 = 1.350 (t)	C3 = 57.98 (q)	13	H_{13} = 1.109 (tt)	C12 = 36.84 (t)
5	H_5 = 1.109 (tt)	C4 = 27.72 (t)	14	H_{14} = 1.046 (td)	C13 = 36.73 (t)
6	H_6 = 1.046 (tq)	C5 = 27.80 (t)	15	H_{15} = 1.904 (tq)	C14 = 36.63 (t)
7	H_7 = 1.934 (tq)	C6 = 27.86 (t)	16	H_{16} = 1.473(d) 4.2 Hz	C15 = 39.5 (d)
8	H_8 = 0.817 (dt)	C7 = 24.69 (d)	17	H_{17} = 1.483 (d)	-
9	H_9 = 0.803 (dd) (2Hz)	C8 = 37.32 (t)	18	H_{18} = 0.832 (d) 4.2 Hz	-
10	H_{10} = 0.733 (td)	C9 = 37.28 (t)	19	H_{19} = 0.832()	-

Anti-inflammatory Activity:

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

Sample no.	Wt of animal in gm	Fraction in Dose	0 min Test Sample	30 min Test Sample	60 min Test Sample	90 Min Test Sample	120 min Test Sample	180 min Test Sample	240 min Test Sample	300 min Test Sample
I	170		1.48	1.53	1.19	1.1	0.87	0.79	0.85	0.97
II	185		1.52	1.58	1.27	1.16	0.92	0.72	0.89	0.99
III	190	**01**	1.49	1.65	1.32	1.13	0.95	0.63	0.78	0.89
IV	170	**mg/kg**	1.51	1.49	1.28	1.04	0.87	0.65	0.79	0.91
V	160		1.49	1.51	1.24	1.19	0.89	0.76	0.87	0.93
VI	180		1.49	1.54	1.25	1.13	0.84	0.69	0.77	0.87
Mean →			1.50	1.55	1.26	1.13	0.89	0.71	0.83	0.93
Std Error of Mean			0.006146	0.02352	0.01778	0.02110	0.01612	0.02539	0.02645	0.02512
Standard Deviation			0.01506	0.05762	0.04355	0.05167	0.03950	0.06218	0.05989	0.06524
% Inhibition			**3.44**	**12.92**	**22.33**	**45.12**	**47.02**	**64.49**	**44.63**	**33.33**
I	150		1.53	1.45	1.21	1.14	0.86	0.65	0.74	0.83
II	180		1.51	1.41	1.22	1.11	0.91	0.71	0.79	0.88
III	160	**05**	1.52	1.39	1.09	1.01	0.78	0.55	0.69	0.81
IV	170	**mg/kg**	1.45	1.47	1.15	1.14	0.82	0.59	0.68	0.78
V	160		1.43	1.52	1.18	1.18	0.89	0.61	0.71	0.83
VI	150		1.49	1.70	1.21	1.05	0.94	0.58	0.73	0.85
Mean →			1.49	1.49	1.18	1.11	0.87	0.62	0.72	0.83
Std Error of Mean			0.01641	0.04597	0.02028	0.02592	0.02418	0.02335	0.01626	0.01390
Standard Deviation			0.04021	0.11260	0.04967	0.06348	0.05922	0.05718	0.03983	0.03406
% Inhibition			**3.98**	**16.29**	**27.37**	**46.10**	**48.41**	**69.10**	**51.45**	**40.29**
I	160		1.39	1.51	1.05	1.05	0.84	0.69	0.74	0.86
II	170		1.45	1.48	1.08	1.01	0.81	0.61	0.76	0.85
III	180	**10**	1.44	1.51	1.13	0.98	0.78	0.68	0.77	0.89
IV	160	**mg/kg**	1.41	1.47	1.14	1.07	0.79	0.54	0.71	0.82
V	170		1.51	1.41	1.18	1.06	0.69	0.48	0.59	0.71
VI	180		1.53	1.42	1.12	0.99	0.74	0.52	0.61	0.75
Mean →			1.46	1.47	1.12	1.03	0.78	0.59	0.70	0.81
Std Error of Mean			0.02247	0.01764	0.01874	0.01563	0.02172	0.03556	0.03180	0.02836
Standard Deviation			0.05505	0.04320	0.04590	0.03830	0.05320	0.08710	0.07789	0.06947
% Inhibition			**6.13**	**17.60**	**31.07**	**49.92**	**53.87**	**70.52**	**53.24**	**41.49**

volume of the Carrageenan injected into the foot was measured at 0, 30, 60, 90, 120, and180, 240.300 minutes 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 = $1- V_t / V_c \times 100$

Where V_t and V_c are the volumes of edema in control and drug treated rats.

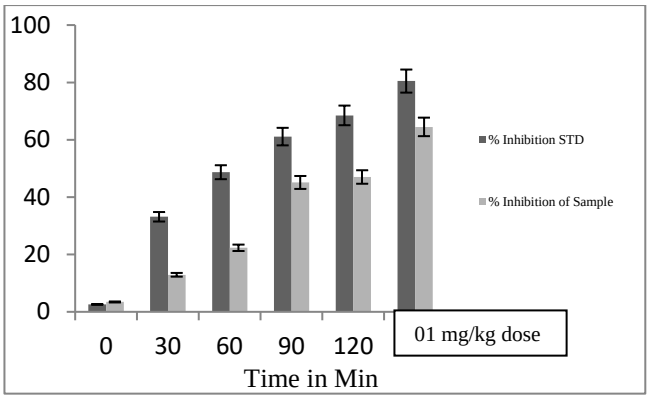
Statistical analysis

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.

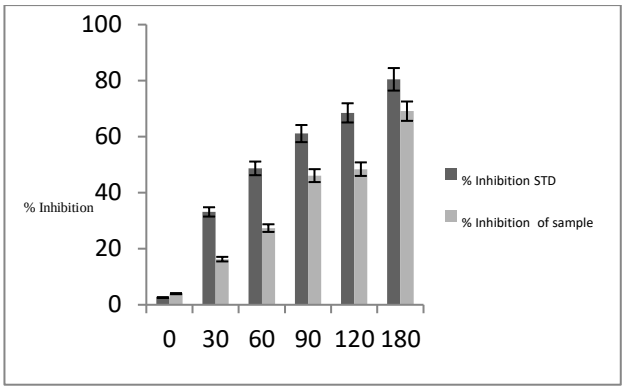
RESULTS

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

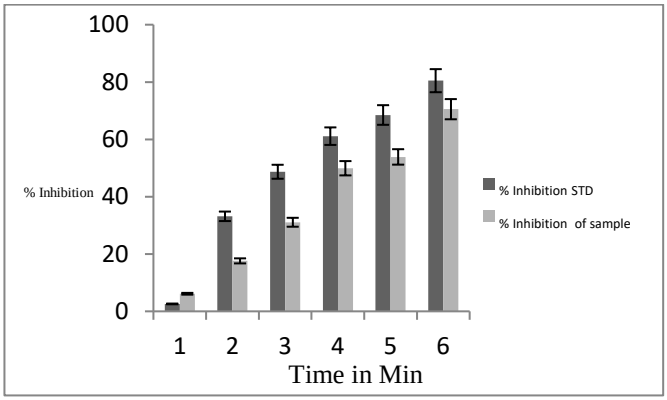
Graphical comparison of % inhibition and time is as follows.



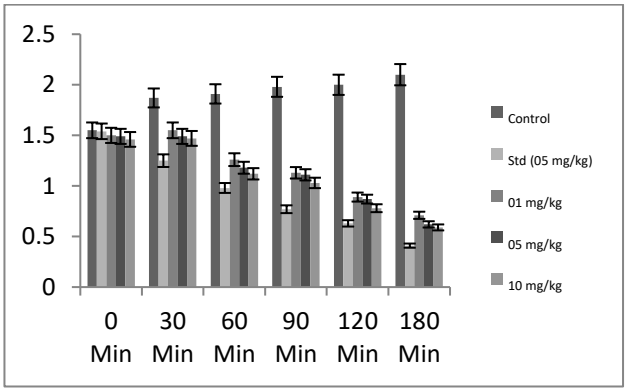
Graph 1: dose:01 mg/kg.



Graph 2 :dose 05 mg/kg.



Graph 3: dose 10 mg/kg.



Graph 4: graph of std, Control and sample.

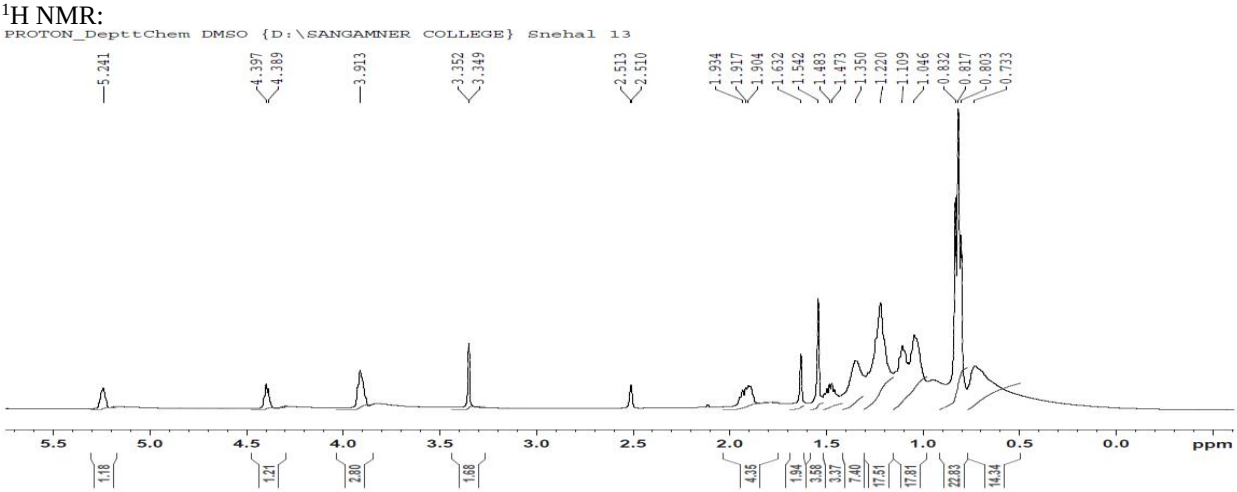
Edema in Control

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).

^{1}H NMR:



^{13}C NMR:

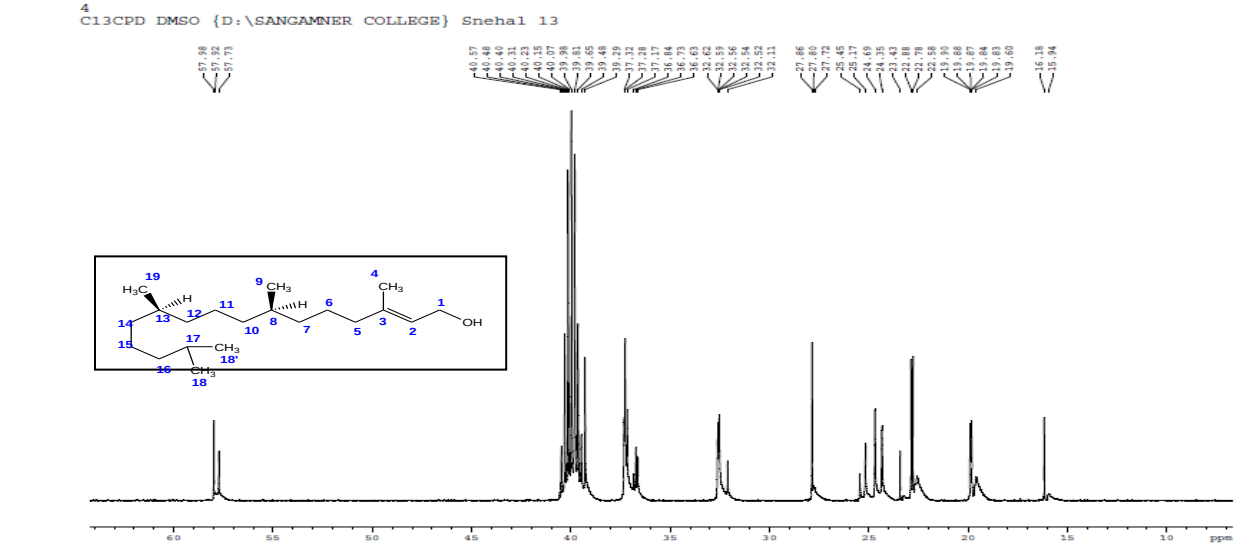


Figure 5: (A) ^{1}H NMR (B) ^{13}C NMR.

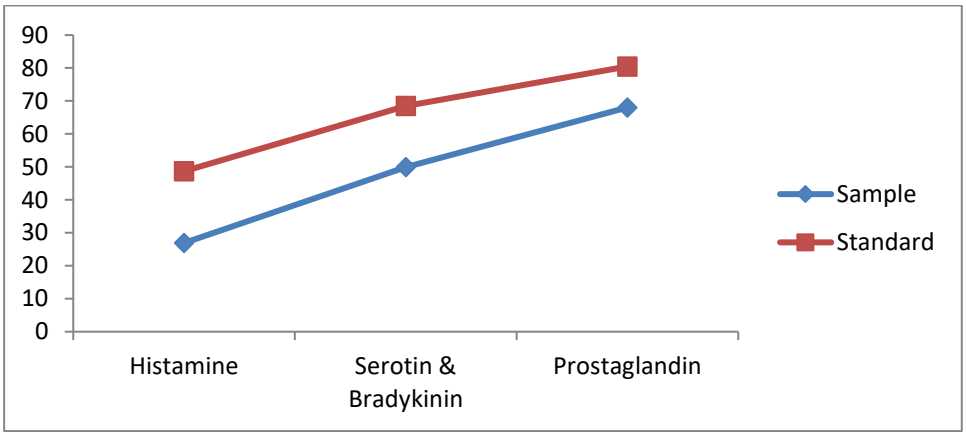
Table 5: Paw edema of Standard (Diclofenac 5mg/kg).

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

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

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	1.5 ± 0.01	1.55 ± 0.02***	1.26 ± 0.02	1.13 ± 0.02	0.89 ± 0.02***	0.71 ± 0.03***
05 mg/kg	1.49 ± 0.016	1.49 ± 0.046**	1.18 ± 0.020	1.11 ± 0.026	0.87 ± 0.024***	2.10 ± 0.013***
10 mg/kg	1.46 ± 0.02	1.47 ± 0.02**	1.12 ± 0.02	1.03 ± 0.02	0.78 ± 0.02***	0.41 ± 0.011**



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

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 by Mass spectra

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 Phytol three important major peaks shows in Fig.02.

IR spectra of Phytol

CONCLUSION

Fragmentation Pattern: Fragmentation pattern is as follows Phytol shows significant Anti-inflammatory activity (68.03%) with respect to standard (Diclofenac 5mg/kg). Histamine release (26.92%) after 1 hr, serotonin and bradykinin release (49.9%) and prostaglandin release (68.03%) indicate remarkable percentage inhibition.

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