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Phorbol is an active anti-inflammatory compound of *Woodfordia floribunda* Salisb

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Abstract

Inflammation is a complex disease which is associated with various symptoms. These symptoms are manged with non-steroidal and steroidal anti-inflammatory drugs, which lead to severe side effects on long-term. But natural product can be used as an alternative source with the discovery of new entities to be used as anti-inflammatory compounds to overcome this problem. The compound isolated by using the soxhlet apparatus and column chromatography. The objective of the present study is to evaluate the anti-inflammatory activity of isolated compound Phorbol from methanolic extract of W. *floribunda* Salisb for scientific validation of the folklore claim of the plant. The plant flowers is rich in antioxidants and has been suggested as a potential source of anti-inflammatory compounds. Thus, this study has elevated the anti-inflammatory activities of the methanolic extract of flowers injecting vitro with widespread use

in folk medicine. In addition to this focus is on the preliminary phytochemical analysis, inorganic quantification, isolation of pure compound a, and characterization. The formulations are pharmacologically evaluated for anti-inflammatory activity using the Carrageenan-induced paw edema model in Wistar rats. The histamine release (45.30% with Serotonin bradykinin release (58.25%) and prostaglandin (69.44%) are compared with standard diclofenac (5 mg/kg). All the formulations are passed the microbial contamination test. None of the heavy metals is detected with higher than permissible limits in any formulations. The results have revealed that in vitro methanolic extract possess significant antinflammtory activities of flowers and thus can be explored as an important source of novel anti-inflammatory agents. The Phorbol is evaluated at three different concentrations of 01, 05 and 10 mg/kg, p.o. Significant dose-dependent anti-inflammatory activity is observed.

Keywords: Carrageenan, Paw edema model, Antinflammtory activity, diclofenac.

1. INTRODUCTION

Woodfordia floribunda Salisb is a shrub of Lythraceae family. Mostly found in India and other countries such as South East and East Asia [1]. The Ayurvedic medicine report is commonly used in traditional medicine for curing various diseases including skin diseases, allergies, intestinal parasites, ulcers, worms etc. Most of the markets drug includes the leaves, fruits, flowers an addition to buds mixed with the pedicles and twigs of the plant. The leaves of the plant are used in traditional medicine in India [2]. This plant contains many bioactive compounds polyphenols, tannins, anthraquinone glycosides, and flavonoids reported in *W. floribunda* Salisb and different tannins in the form of *Woodfordin* A, B, and C and in addition to this hydrolyzable tannin, endothelin B is reported in the plant [3]. Hence, inflammation is a protective mechanism that is essential to health. However, when its use persists for a long time, it results in a numerical pathological condition rheumatoid arthritis and septic shock [4].

The immune system is invulnerable to harmful stimuli such as pathogenic bacteria, damaged cells, harmful materials, or irradiation is inflammation, which functions by removing those stimuli and restoring the immune system [5]. The steroidal and non-steroidal Antinflammtory drugs immunosuppressant are suggested by the researcher to manage the inflammation condition and get free from inflammatory diseases [6]. A person needs long-term treatment because of these synthetic medications. These are mainly linked to adverse responses since they inhibit the cyclooxygenase isozymes COX1 and COX-2, which catalyse the conversion of arachidonic acid into prostaglandins and thromboxane [7]. For performing the important function of vasodilation and platelet adhesion prostaglandins and thromboxane have become an important role [8]. The inhibition of cyclooxygenase influence cardiovascular, hepatic gastric mucosa, renal and hematologic system. Besides the resistance of infection-causing microorganism against antibiotic has a serious problem and create a problem for a human healthy life. These synthetic antibiotics are available in well-developed countries but they are expensive and not efficient for curing diseases caused by the microorganism. When humans consume this antibiotic but it has adverse side effects [9]. The symptoms are found in diarrhea, abdominal pain, edema, coughing and loss of appetite. Due to the adverse effect of the synthetic drug used for the treatment of inflammation against the pathogenic microorganism, researchers are more focused on the bioactive constituents isolated from the natural product nowadays [10].

There are several unexplored plant species that the locals in the northern Himalayan region utilize for traditional medicinal purposes. Ethnopharmacological common names for *Woodfordia floribunda* members

of the Lythraceae family are called Dhatki, Dawi, Dhai and Dhavdi. It is used to treat a variety of diseases, including urinary problems, wounds, diarrhea, bleeding wounds, and headaches, as well as immunomodulatory, antitumor, hepatoprotective, and antiulcer activity. The dried flowers are used in the treatment of the disorder of mucus membranes and flowers are also used for curing asthma and cough [11].

The previous study focuses on the egg protein denaturation method for the *vitro* assessment of the anti-inflammatory activity of leaves of *woodfordia fruticosa*. The main reason of inflammation is due to the denaturation of protein. So the present researcher has confirmed that in vitro study of different plant extracts of *Woodfordia fruticosa* leaves shows the presence of significant anti-inflammatory activity [12]. The researcher also focuses on the *W. fruticosa* plant and finding the Anti-inflammatory activity of leaves ethanoic extract. For that purpose, the previous researcher found took rats 200mg/kg dose was given orally to them and inflammation was studied by using carrageenan-induced paw edema, they got satisfactory results [13]. The present researcher has found the Anti-inflammatory activity of the ethanolic extract of *W. fruticosa* by taking phytochemical analysis and Gas Chromatography Spectroscopy study and utilized it as a magnificent source of phenols [14]. Besides different examinations using distinctive extraction procedures for the isolation of bioactive compounds appreciative of biological activities have been performed. These methods, however, are not very compelling [15-16]. The previous researcher has only focused on biological and pharmaceutical studies. But the present research article overcomes this difficulty of isolation of extraction by correlating previous work, having the following objectives: extraction of methanolic extract using a modified technique such as soxhlet extraction was used and its characterization is done using GC-MS, ^{1}H-NMR, ^{13}C-NMR and COSY. The bioactivity of isolated compound phorbol is carried out with in vitro anti-inflammatory activity by using a carrageenan-induced paw edema model. The isolated compound phorbol has significant Anti-inflammatory activity.

1. MATERIAL AND METHODS

2.1 Collection of Plant material and Authentication

The flowers of *W. floribunda* Salisb are collected from the Kalasubai mountain region at Rajur, Ahmednagar Maharashtra, India. Plant authetification was done at the Botanical Survey of India (BSI) Pune and the herbarium (No.BSI/WRC/Iden.Cer./2021/1905210003955)

Fig. 1: *W. floribunda* flowers

Extraction outline

Plant material is dried in the shade and ground to a coarse powder using a commercial grinder. The dried powder was defatted with petroleum ether and further extracted with methanol at 40°C in Soxhlet apparatus [17].

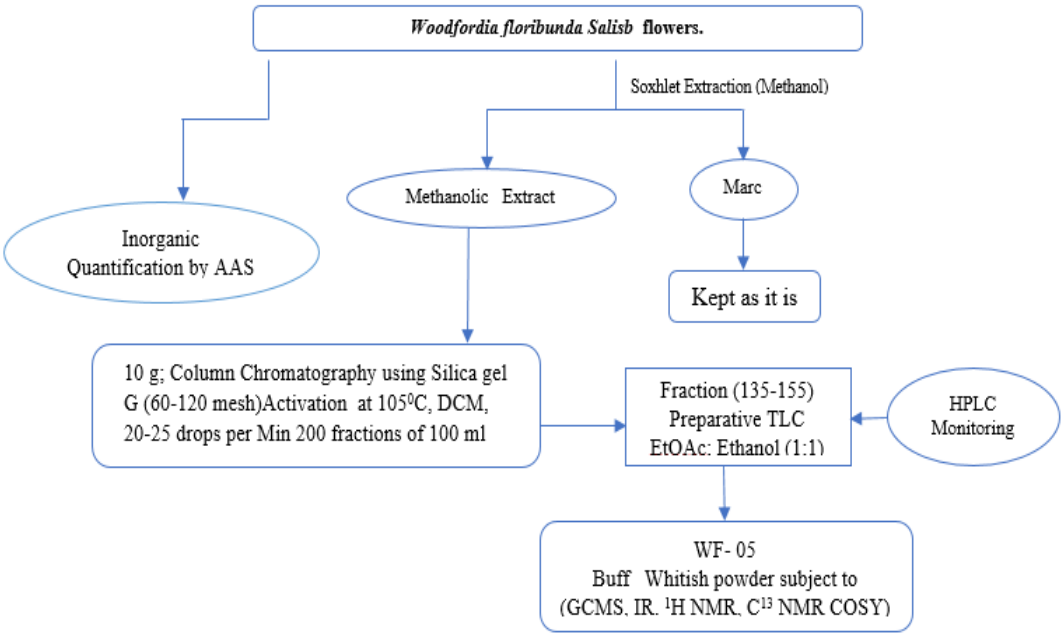

Fig. 2: Experimental work outline of isolation of Phorbol (WF-05) from the extract of *W. floribunda* flowers.

It requires 5-7 days to complete its cycle and get the Crude methanolic extract. It was sent to SAIF Bombay for GC-MS analysis and elemental detection. The subsequent part is the column chromatography by using DCM solvent and taking the TLC of each fraction having mobile phase ethyl acetate and ethanol (1:1) ratio. The isolated compound was sent to spectroscopic analysis such ^{1}H-NMR, ^{13C}-NMR and COSY. After the structure, confirmation checks the purity of the compound by using the HPLC technique. Lastly, examine the Anti-inflammatory activity of the isolated compound [18].

GCMS

The concentrated extract was passed to the GC-MS for analysis of bioactive constituents. The GC-MS analysis was performed using the instruments Agilent 7890, FID detector, Headspace injector, combipal autosampler (SAIF, IIT, Bombay), and Jeol, Model Accu TOF GCV, Time of flight analyzer mass range 10-200 amu, mass resolution 6000. After analysis get bioactive constituents.

2.4 INORGANIC QUANTIFICATION

The dried flower ash was analysed by atomic absorption spectroscopy for inorganic elemental detection quantitatively [19].

2.5 RPHPLC

RPHPLC specification details (model no. HPLC 3000 series, Analytical Technologies Ltd.) With detector (UV-3000-M) and column (Cosmosil C18 (250 mm x4.6ID, Particle size: 5 microns) carried out at RAP analytical research institute center, Nashik, India.

METHODS: -

The UV spectrum of phorbol is found between 200-400 nm at 238 nm wavelength. The wavelength is 238 nm and the mobile phase methanol: water (90:10), and the pH of the mobile phase: is 3 (pH is adjusted with o-phosphoric acid). The sample volume is about 20µl . While the flow rate

1.00ml/min and pressure 9-10MPa and the run time 8.21 min and 10.21 min [20].

Animal

All animal experiments are performed with prior permission from the Institutional Animal Ethics Committee (IAEC) of the Amruthwahini Pharmacy College, Sangamner. It confirmed the "Guidelines for care and use of animals in scientific research" (INSA 1998, Revised 2000) (AVCOP/IAEC/2021-22/1153/26/01) using the carrageenan-induced rat paw edema model. Wistar rats of either sex weighing 200 ± 20 g were used in the present investigation [21]. Animals were randomly selected from the animal house of APCS and Housed in a group of six in separate cages under the controlled condition of temperature (22 ± 2°C).

Carrageenan Induced Paw Edema

Animals were divided into three groups. Animals of groups 1, 2 and 3 were given distilled water (10 ml/kg), Phorbol (01mg/kg, 05mg/kg, 10mg/kg) and Diclofenac (10mg/kg) respectively. After the interval of 1hr, 2hr and 3hr paw edema was induced in experimental animals [23]. Intra peritoneal carrageenan (0.1%) was injected into the hind paw using a 26 gauge needle. Paw thickness was measured with a plethysmometer at 1,2,3,4 and 5h following carrageenan administration. Percentage inhibition of edema was measured using the flowing formula:

% Inhibition = [(A control - A test)/ A control] x100

2.9 STATISTICAL CALCULATION

All data were analysed by One Way ANOVA followed by Bonferroni's test. $P<0.05$ was considered significant.

1. Result and Discussion

3.1 GC-MS Study of Methanolic Extract

The bioactive compound is present in the methanolic extract flower of *W. floribunda* as tabulated in the following manner. The results were obtained and identified nine bioactive compounds by measuring their retention time and peak area. The obtained compounds are finger pointed into the table and w.r.t. data with reported biological activity by NIST [24].

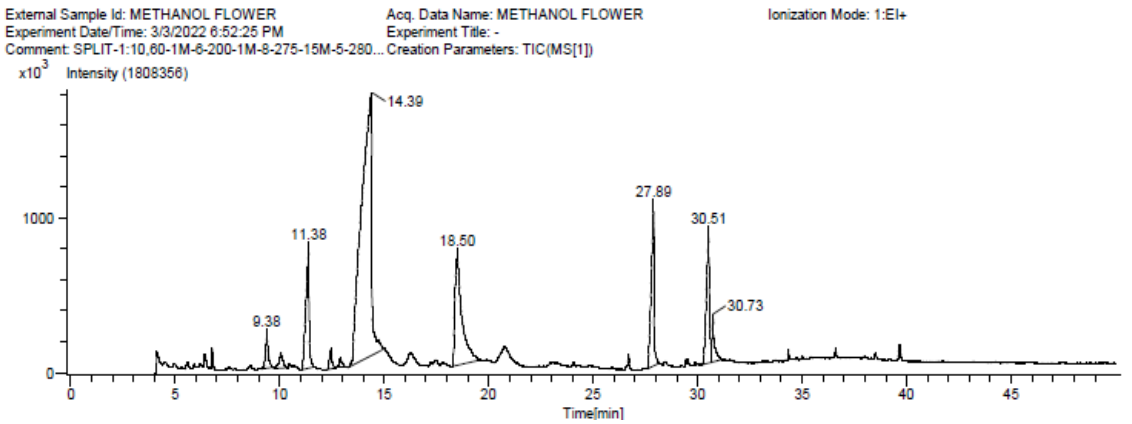

Fig. 3: Chromatogram of methanolic extract flower of *W. floribunda*

Table 1: List of bioactive compound present in methanolic extract of flower of *Woodfordia floribunda* Salisb

Sr.no.	Compound present in the extract	Formula for molecules	Molecular Weight	Retention Time	Biological Activity
1	**Phorbol**	**$C_{20}H_{28}O_6$**	**364**	**9.38**	**Antinflammtory Activity**
2	4HCyclopropa[5',6']benz[1',2',7',8']azuleno[5,6-b]oxiren-4-one,8-8a-bis(acetyloxy)-2a{(acetyloxy)methyl}-1,1a,1b,1c,2a,3,3a,6a,6b,7,8,8a ,dodecahydro	$C_{26}H_{34}O11$	522	11.38	Antioxidant
3	4H-Pyrane-4-one,2,3-dihydro-3,5dihydroxo-6-methyl	$C_6H_8O_4$	144	14.39	Antimicrobial, Antinflammtory
4	Cholestanol(3,2-c)isoquinolin-1'(2'H)-one,3',4'-dihydro-6',7'-dimethoxy	$C_{36}H_{55}NO_3$	549	18.50	Anticancer, Antinflammtory
5	Cholestano[3,2-c]isoquinolin-1'[2'H]-one,3',4'-dihydro-6'-7'-dimethoxy	$C_{36}H_{55}NO_3$	549	27.89	Anticancer, Antinflammtory
6	2-Furancarboxyaldehyde,5-(hydroxymethyl)	$C_6H_6O_3$	126	30.51	Antibacterial Activity
7	1,2,3-Benzenetriol	$C_6H_6O_3$	126	30.73	Antibacterial activity

INORGANIC QUANTIFICATION

Major micronutrients like Fe, Zn, Cu while minor micronutrients such as Mg, B estimated in *W. floribunda* Salisb by AAS. The results from our research unveiled key aspects on interrelation among some mineral and metal due to higher concentration of Fe and Zn [25].

Table 2: Elemental analysis of *Woodfordia floribunda* Salisb flower in the ppm unit

Sr.no.	Name of the Element	Standard Range of Element in unit ppm	Observed Range of Element in unit ppm
1	Iron	71.1-214	196.5
2	Mg	29.1-88	35.6
3	Zinc	14.1-72	68.5
4	Copper	29.1-72	55.2
5	Boron	20.1-30	25.6

RPHPLC Analysis

The analysis of these compounds has been successfully carried out by HPLC with UV and ToF-MS detection. Quantification could be achieved via standard addition and use of phorbol as reference standard. Statistical evaluation of the method shows adequate precision with low variances in results. The purity of isolated compound phorbol 61.72% obtained [26].

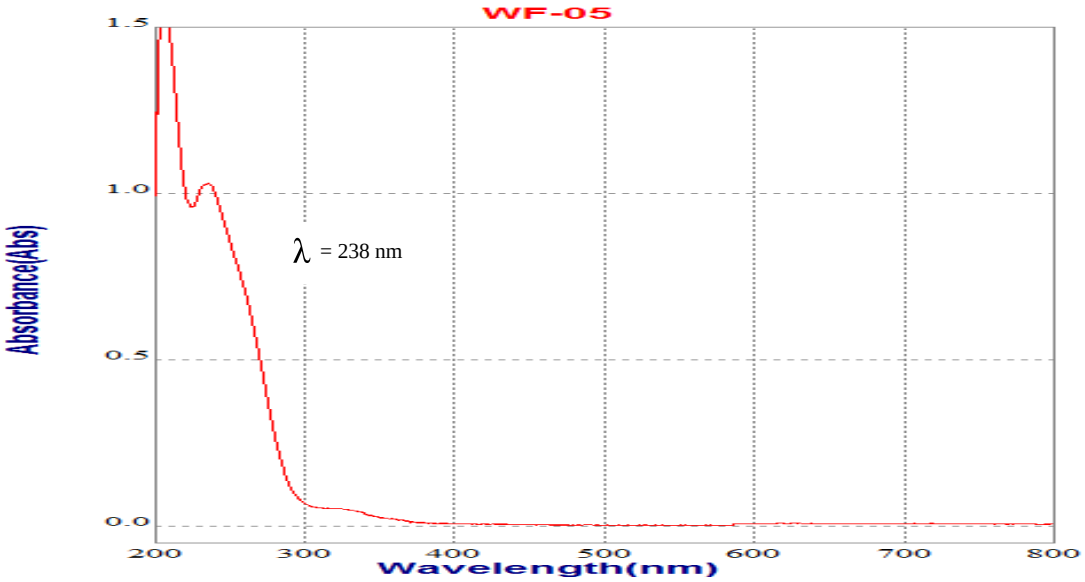

Fig. 4: UV spectrum of phorbol (WF-05)

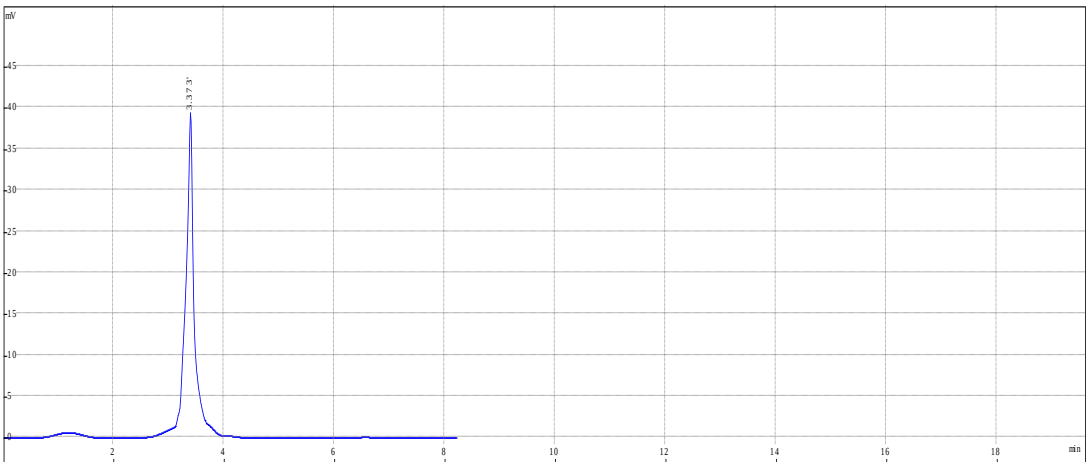

Fig. 5: Typical chromatogram of standard compound phorbol

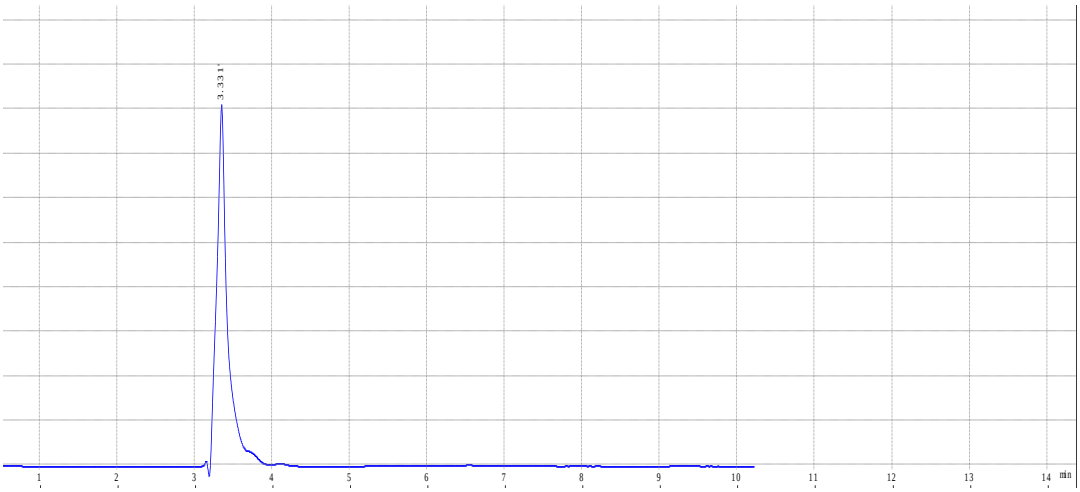

Fig. 6: Typical chromatogram of isolated compound Phorbol (WF-05)

Table: 4 The retention time and peak area of the Phorbol and its purity in percentage (WF-05)

Particulars	Retention Time	Area	% Purity of Compound
Standard	3.37	453164	
Sample (WF-05)	3.33	279693	61.72%

Characterization of Phorbol

3.5.1 Mass Spectrometry

Mass fragment (m/z):- 296(M^+),149,123,95,81,71,57,43,41,31,29

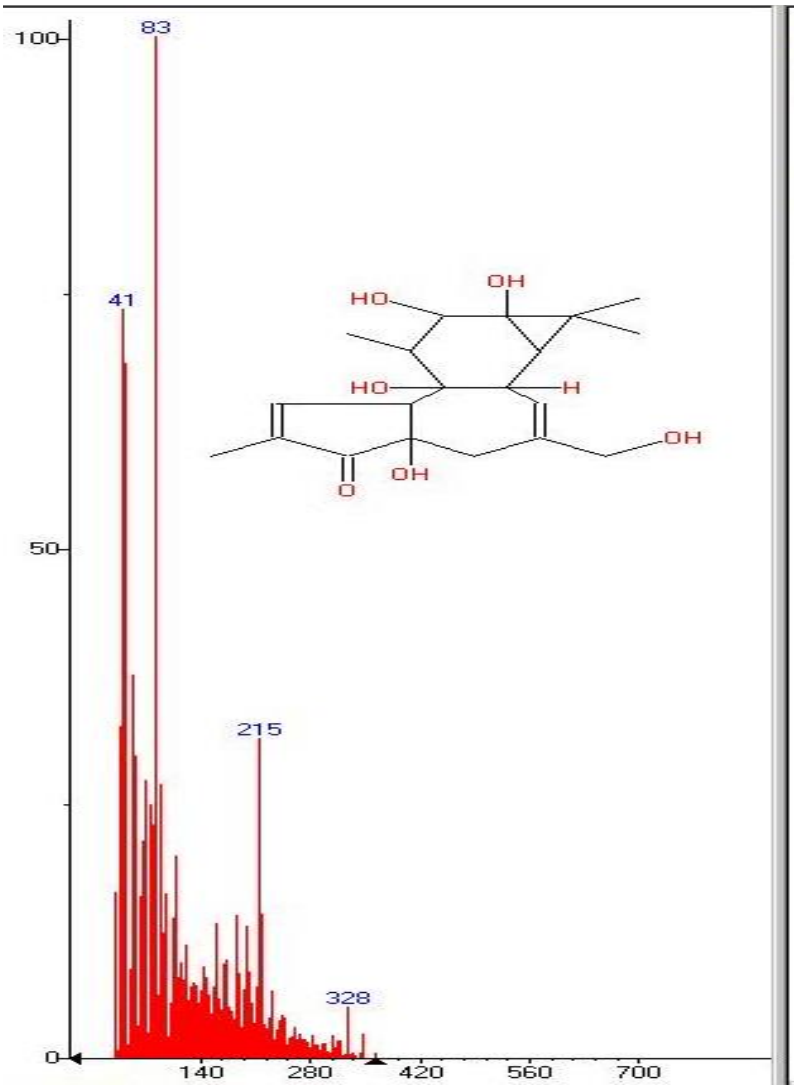

Fig. 7: MS spectrum of Phorbol (WF-05)

IR Spectroscopy

From the IR spectrum, we observed that OH stretching was observed at 3250 cm^{-1} to 3500 cm^{-1}, whereas alkyl C-H stretching frequency at 2900 cm^{-1} and the C-O stretching frequency was observed that 1005 cm^{1}.The double bond stretching frequency is at C=C bond due to α-OH.

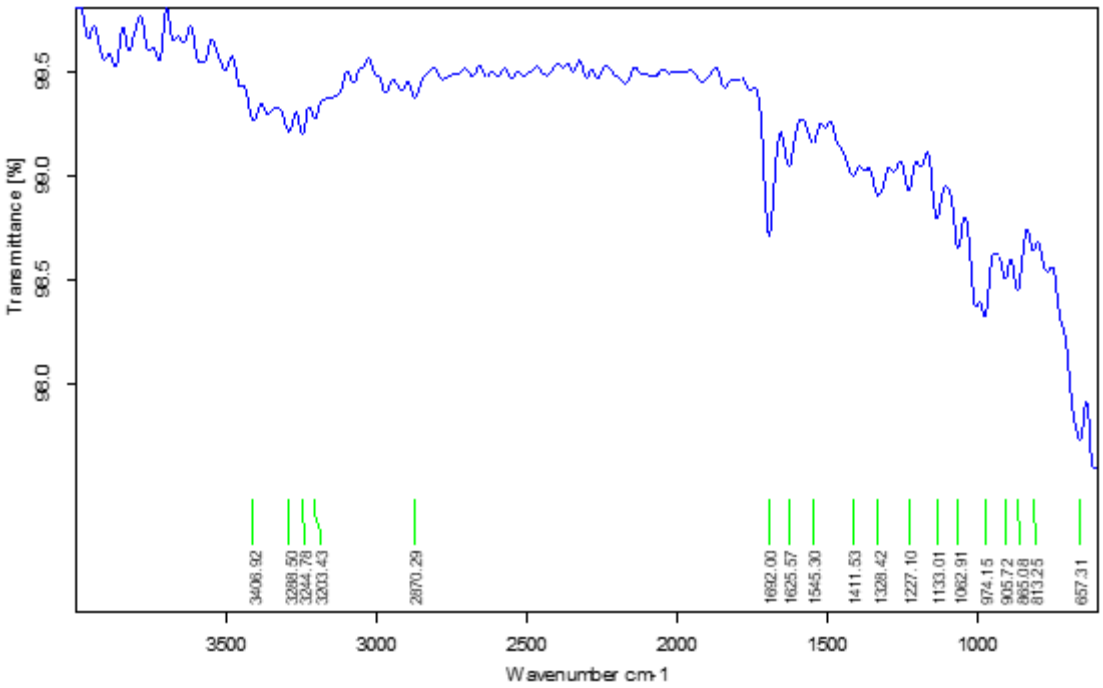

Fig. 8: IR spectrum of Phorbol (WF-05)

^{1}H NMR (500 MHz, DMSO):-

7.54 (s, 1H), 5.54 (s, 1H), 5.45 (d, J = 4.5 Hz, 1H), 4.81 (s, 1H), 4.70 (s, 1H), 4.32 (d, J 3.0 Hz, 1H), 3.91 (d, J=8.2 Hz, 1H), 3.82 (d, J = 8.9 Hz, 1H), 3.80-3.72 (m, 2H), 2.95 -2.89 (m, 2H), 2.34 (d, J = 18.7 Hz, 1H), 2.26 (d, J = 18.8 Hz, 1H), 1.70 (td, J= 13.0, 6.6 Hz, 1H), 1.65 (dd, J=2.8, 1.2 Hz, 3H), 1.14 (s, 3H), 1.02 (s, 3H), 0.92 (d, J 6.4 Hz, 3H), 0.52 (d, J = 5.3 Hz**,** 1H).

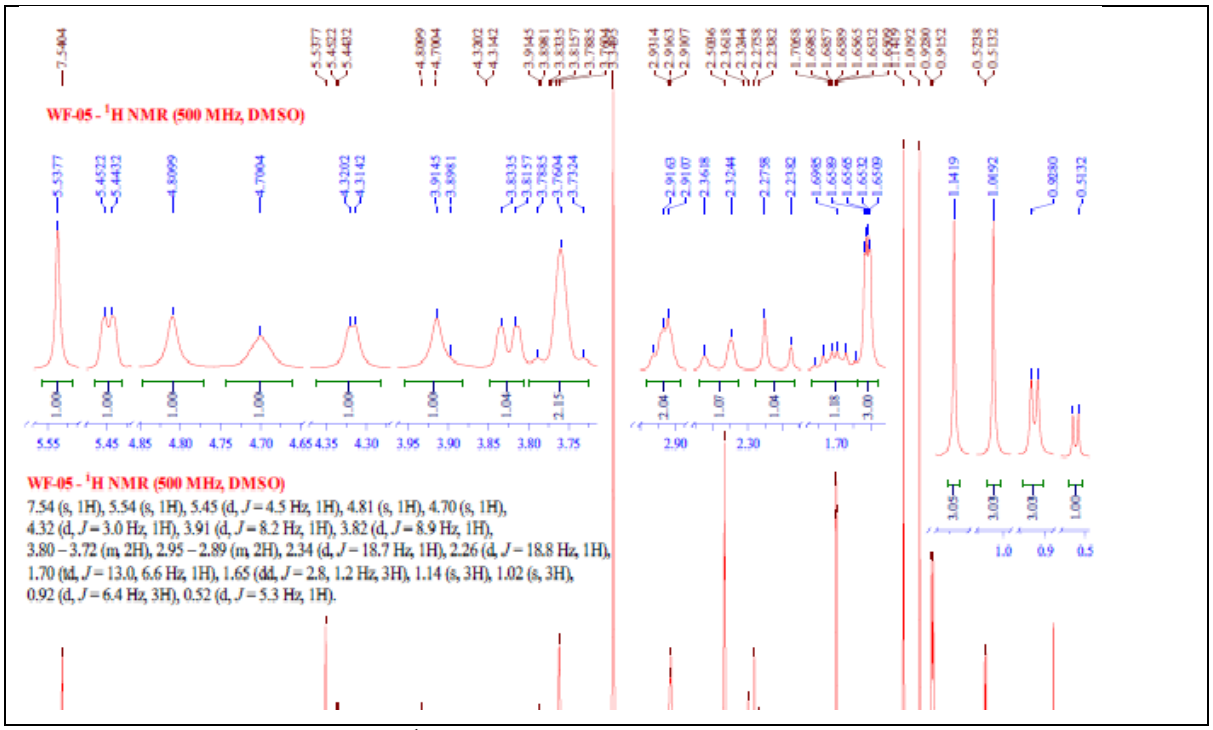

Fig. 9: ^{1}H-NMR spectrum of Phorbol (WF-05)

3.5.3 ^{13}C NMR (126 MHz, DMSO)

207.88, 159.35, 139.88, 130.97, 128.37, 78.74, 76.75, 72.50, 65.53, 60.48, 56.23, 43.94, 37.92, 36.66, 35.30, 23.89, 23.37, 16.83, 14.45, 9.42.

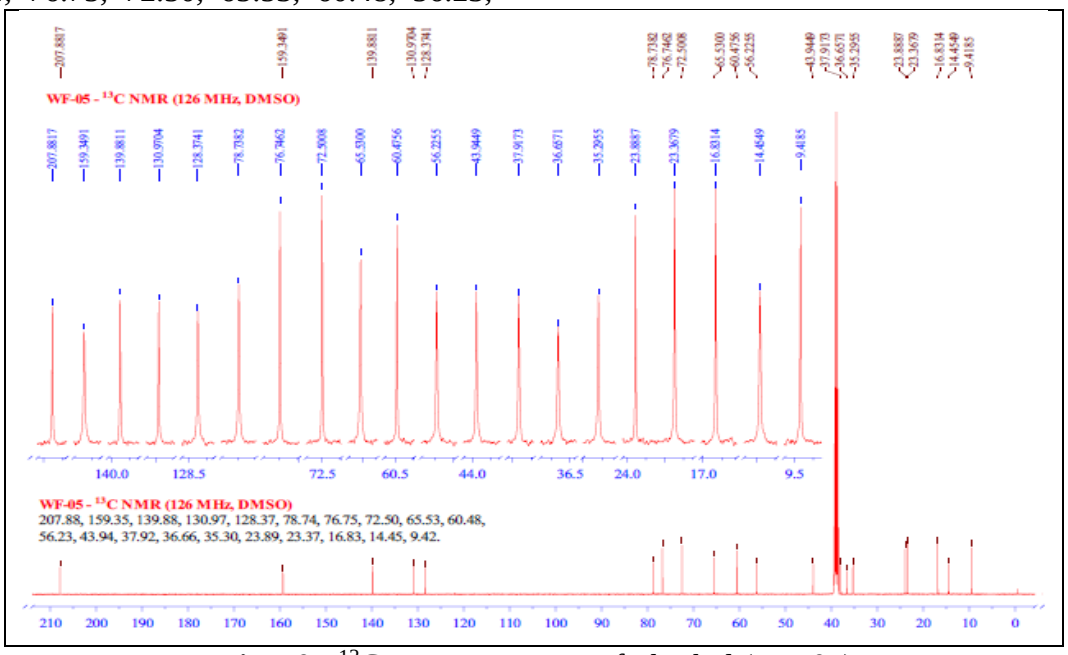

Fig. 10: ^{13}C-NMR spectrum of Phorbol (WF-05)

3.5.4 COSY

The proton having chemical shift value 2.34 (d) is only coupled with 5.45(d).The 2.95-2.89 (m) coupled with 0.52, 1.65, 7.54 and 2.95-2.89 protons. The 3.82 proton shows doublet with 4.32(d) proton. Also proton 5.45 is coupled with 2.34, 2.95-2.89 and 3.80-3.72 protons.

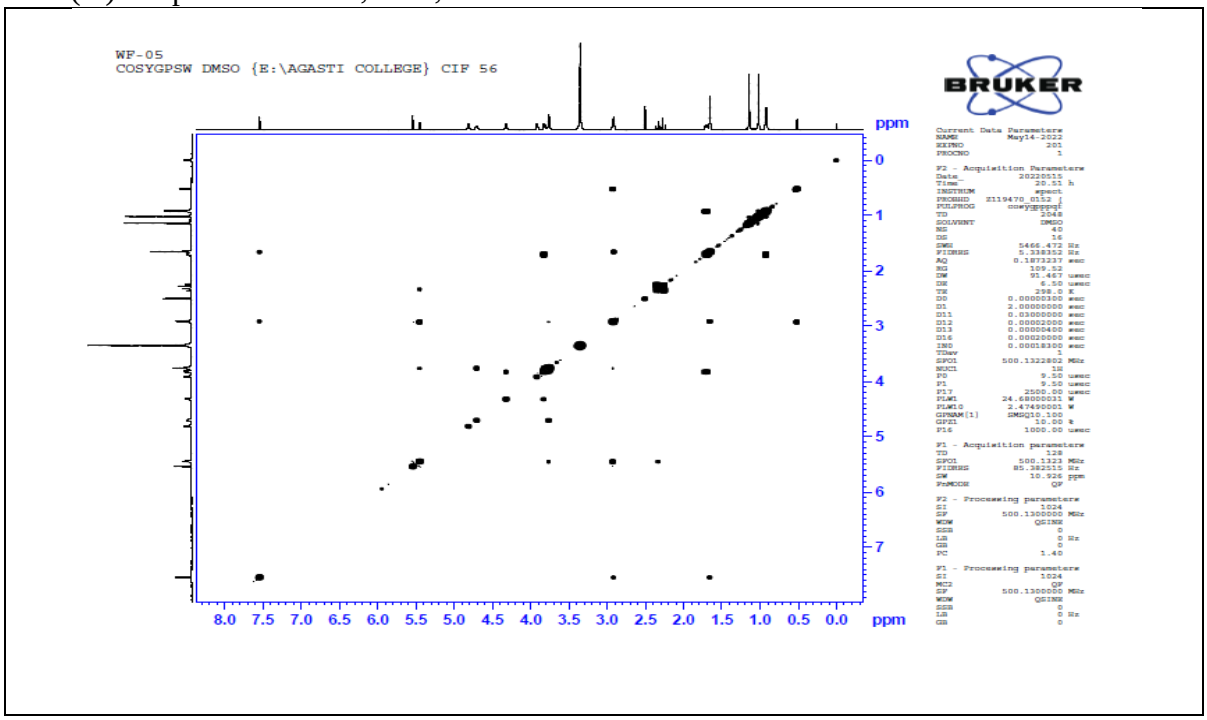

Fig. 11: COSY spectrum of Phorbol (WF-05)

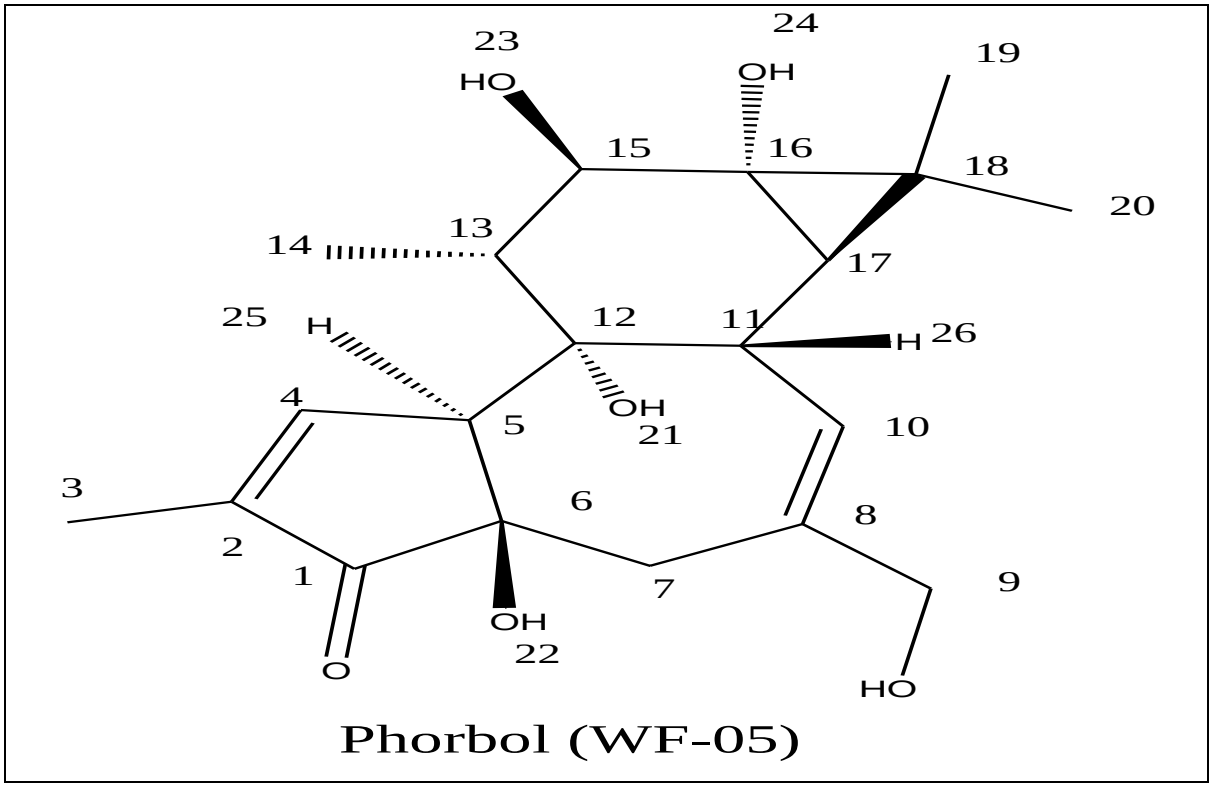

Fig. 12: Correct structure of isolated compound Phorbol (WF-05)

2. Anti-inflammatory activity

Table 3: Inhibition of paw edema in percentage of Phorbol (n = 06) compared with standard (Diclofenac)

Dose	0 Min	30Min	60 Min	90 Min	120 Min	180 Min	240 Min	300 Min
01 mg/kg	3.23	19.89	35.79	40.10	59.30	49.73	45.53	34.93
05 mg/kg	3.87	21.51	35.26	42.64	59.80	56.22	53.69	41.61
10 mg/kg	5.16	22.04	44.74	47.72	58.29	63.81	52.91	42.69
STD 05mg/kg	0.65	32.80	48.42	60.91	68.34	80.48	76.14	70.27

Table 4: 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	240 Min	300 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	1.97 ± 0.02	1.85 ± 0.057
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	0.47 ± 0.012	0.55 ± 0.011
01 mg/kg	1.50 ± 0.014	1.49 ± 0.017	1.2 ± 0.013**	1.17 ± 0.011*	0.81 ± 0.018***	0.75 ± 0.007**	0.81 ± 0.0142**	0.90 ± 0.0126**
05 mg/kg	1.49 ± 0.018	1.46 ± 0.013	1.22 ± 0.016**	1.13 ± 0.015**	0.80 ±0.023***	0.68 ± 0.049**	0.69 ± 0.0242**	0.82 ± 0.0147**
10 mg/kg	1.47 ± 0.016	1.44 ± 0.011	1.01 ± 0.014**	1.036 ±0.011***	0.82 ± 0.025***	0.56 ±0.026***	0.70 ± 0.0324***	0.80 ±0.0316***

The present result of anti-inflammatory activity was used as the irritant to induce paw edema due to induction of inflammation. The phorbol was administered at 1 mg/kg, 5mg/kg and 10 mg/kg and it was found to be significantly effective. Paw thickness was found to significantly less (P<0.05) in treated animal. With progress in time on half an hour. Test sample significantly decreased the thickness with percentage of 56.22 and 63.81 on the 3rd hour at 5 mg/kg and 10mg/kg respectively. The detail result are shown in Table 1.

The graphical representation of % inhibition concerning the time in min is plotted and it is compared with the standard and gives significant anti-inflammatory activity of isolated compound Phorbol.

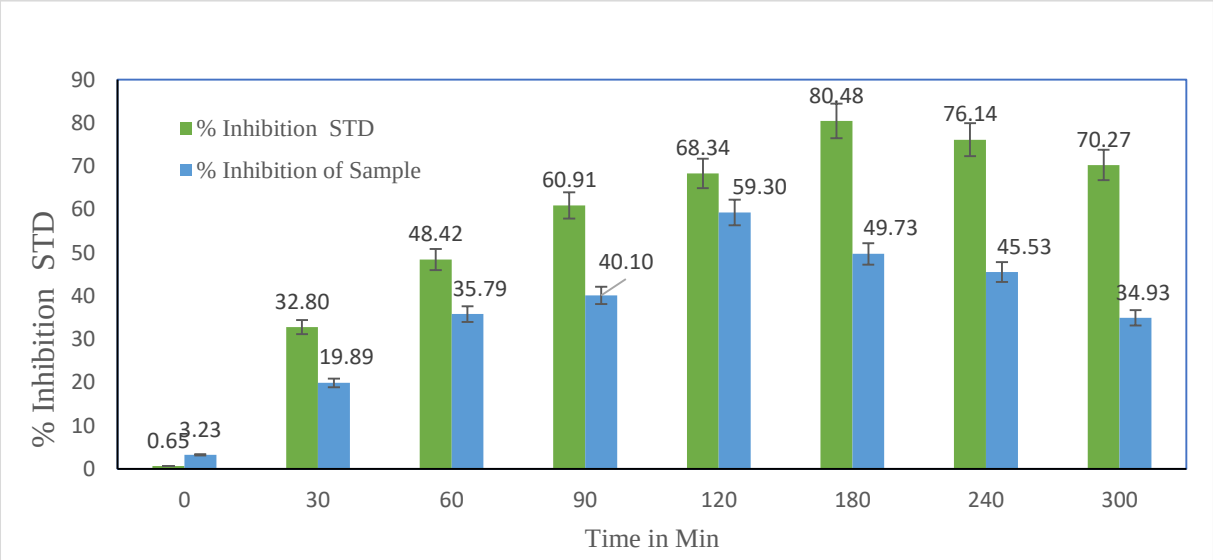

Fig. 13: The % inhibition of sample dose 1mg/kg

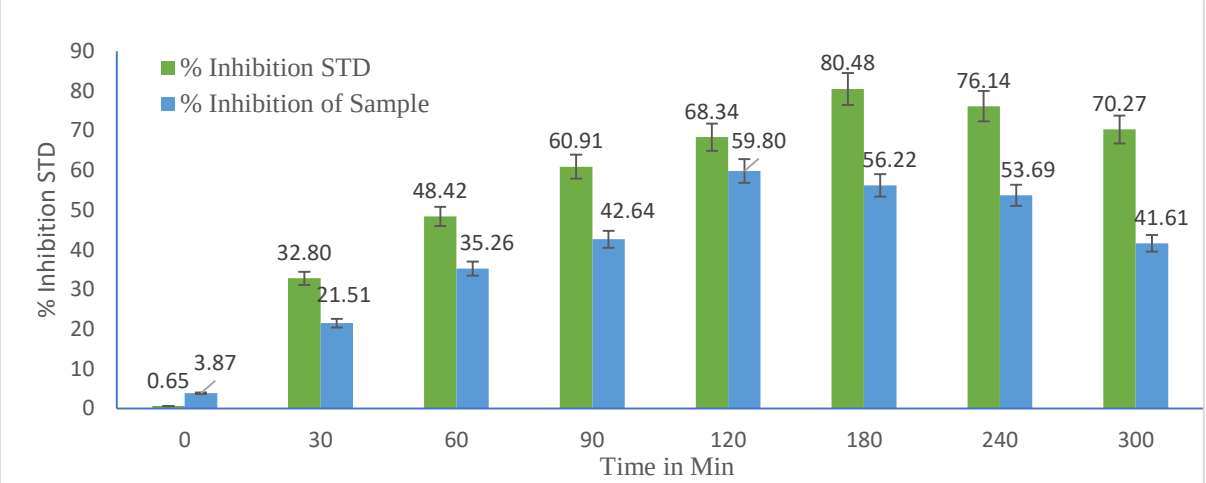

Fig. 14: The % inhibition of sample dose 5mg/kg

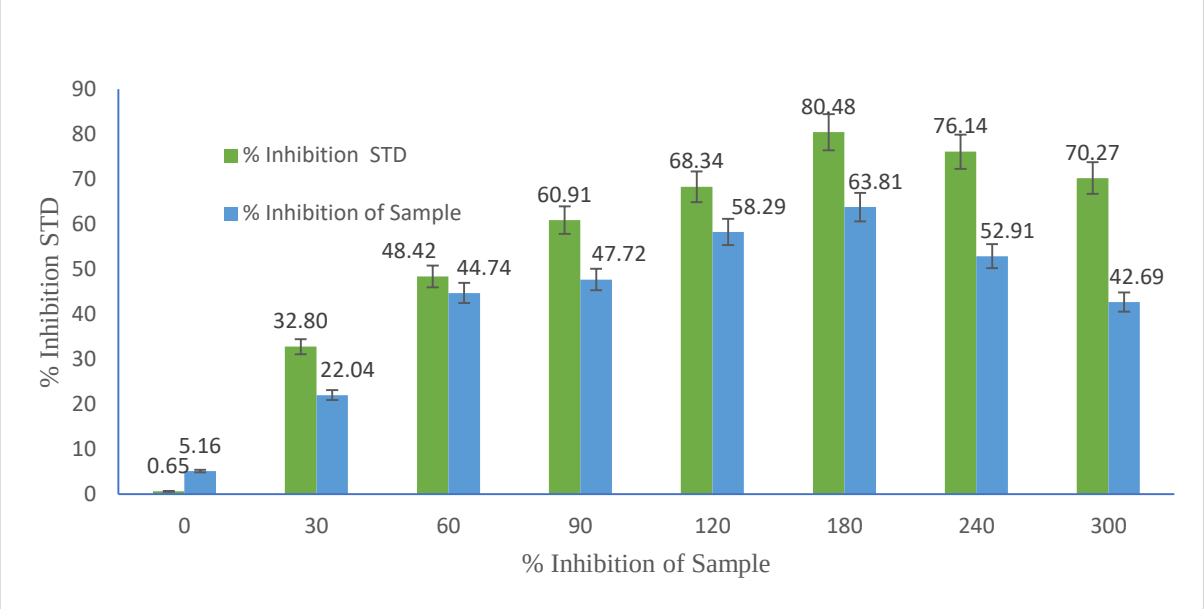

Fig. 15: The % inhibition of sample dose 10mg/ml

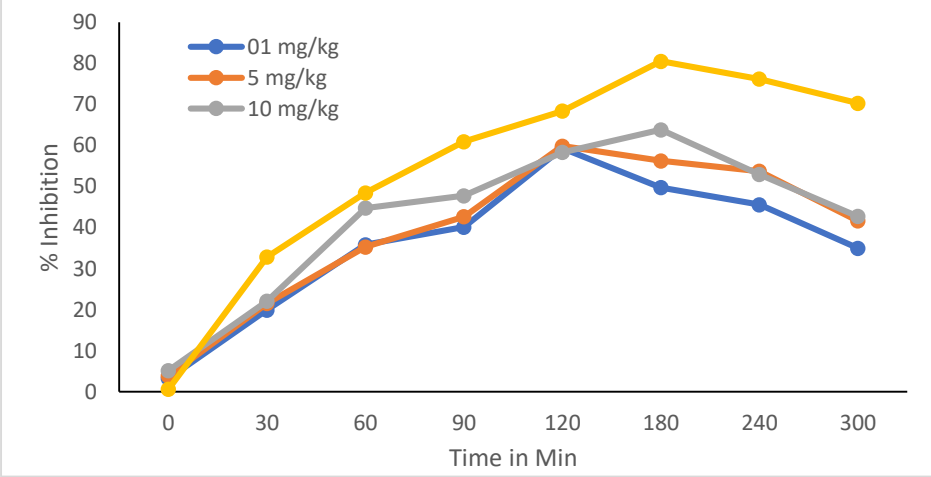

Fig. 16: comparative graph of time vs % inhibition of sample dose

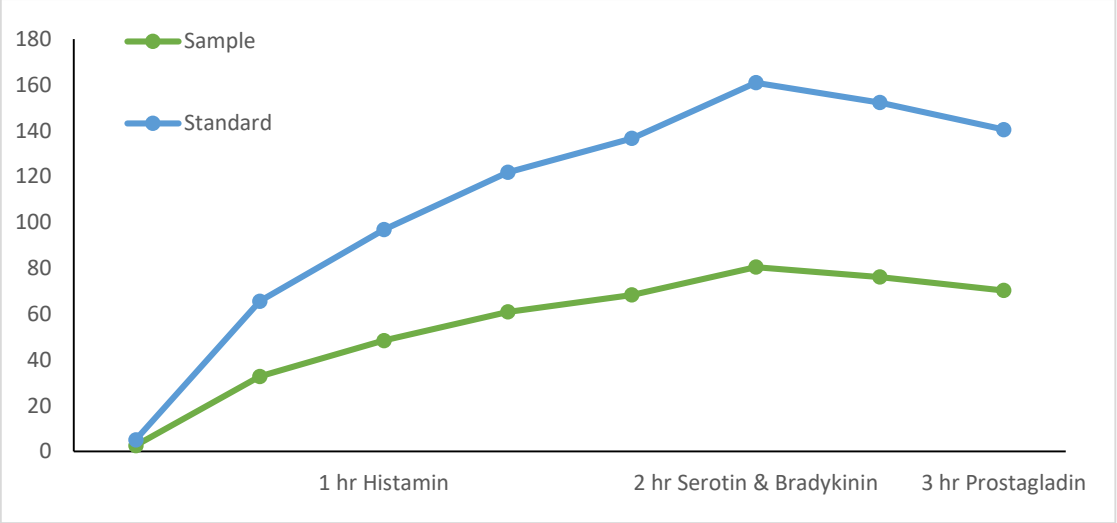

Fig. 17: Release in Histamine (1 hr), Serotonin and bradykinin (2hr) and prostandinin (3 hr.)

Herbs are considered to be an important source of bioactive components useful in treatment of various diseases .The present investigation revealed that WFM possesses significant anti-inflammatory activity. *W. floribunda* consist of flavonoids, anthraquinones [27].

The anti-inflammatory activity of this plant has been reported by taking ethanolic extract of *W. floribunda* flowers. Here the researcher used different models to induce inflammation such as carrageenan, autacoid induced hind paw edema, formaldehyde and cotton pellet granuloma in mice. Similarly the anti-inflammatory activity of ethanolic extract of the plant was evaluated on the asthma induced by the combination of Histamine [28].

In carrageenan-induced paw edema is one of the widely accepted models for screening of acute phase of inflammation [29]. Mechanism of inflammation in this model can be divided into two phases, in which the first phase is associated with the release of histamine and serotonin (COX-1). On the other hand, the second phase is associated with the release of prostaglandins like mediators. Most of the steroidal and nonsteroidal anti-inflammatory agents were found to be effective in the second phase and they are related to COX-2 inhibition [30].

Among the various pro-inflammatory mediators tumor necrosis factor α (TNF-α) plays an important role in inflammation associated with arthritis [31]. Using anti-TNF-α therapy in patients with long standing rheumatoid arthritis is bases on the involvement of TNF-α in its pathophysiology. It is also suggested that other pro-inflammatory cytokines also get inhibited if TNF-α is decreased [32]. In the previous study, it was observed that WFE not only decreased edema associated with the administration of adjuvant but it also decreased

Elevated TNF-α in serum. Further anti-inflammatory potential of WFE was also ascertained by

Using one another model of inflammation associated with arthritis, MIA-induced arthritis. After MIA injection, pain occurs during the initial stage of synovial inflammation [33].

Conclusion

The research scholars focus on the different extract of *W. floribunda* Salisb extract and reported that ethanolic, methanolic extract has abundant source of alkaloids, tannins and steroids. Also proves the role and their biological activity by applying different model and their significance. In the present scenario isolation of the bioactive

compound and evaluate their anti-inflammatory efficacy of isolated compound. Using this modified techniques of soxhlet extraction and column chromatography to isolate the bioactive compound Phorbol. Lastly it is found that the anti-inflammatory efficacy of phorbol and significant anti-inflammatory activity by using carrageenan induced paw edema model. The present study is the first one to conduct to standardize these marketed synthetic drug. So this study can be used as a standardizing tool for future prospects and by the researchers willing to work on these herbal formulations. This study also forms the basis for niche researchers to extend the work on these herbal formulations and work to explore the pharmacological potential of these formulations.

The formulations are pharmacologically evaluated for anti-inflammatory activity using the Carrageenan-induced paw edema model in Wistar rats. The histamine release (45.30% with Serotonin bradykinin release (58.25%) and prostaglandin (69.44%) are compared with standard diclofenac (5 mg/kg). All the formulations are passed the microbial contamination test. None of the heavy metals is detected with higher than permissible limits in any formulations. The results have revealed that in vitro methanolic extract possess significant antinflammtory activities of flowers and thus can be explored as an important source of novel anti-inflammatory agents. The Phorbol is evaluated at three different concentrations of 01, 05 and 10 mg/kg, p.o. Significant dose-dependent anti-inflammatory activity is observed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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