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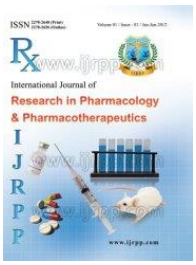

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Role of β-sitosterol enriched fraction from the methanolic extract of leaves of *Mallotus philippensis* (Lam.) Muell. Arg. on inflammatory cytokines

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ABSTRACT

The potent pro-inflammatory cytokines (IL-1β & TNF-α) and anti-inflammatory cytokine (IL-10) were investigated for its effect on β-sitosterol enriched fraction from the methanolic extract of leaves of *Mallotus philippensis* (Lam.) Muell. Arg. The *n*-butanol part of the methanolic extract was eluted using mixtures of solvents of increasing polarity. From the combined eluted fractions, three fractions such as F1, F2 and F3 were collected by column chromatography using *n*-hexane: Chloroform (1:1) as a solvent and the compounds present in the fractions were identified by GC-MS. Among the collected fractions, the F1 was found to be the β-sitosterol enriched fraction (β-SEF). The maximum non-lethal dose of β-SEF was found to be 1000 mg/kg body weight using OECD 423 guidelines. The effect of β-SEF (100 mg/kg), almost 10 times less than the standard drug Diclofenac sodium (10 mg/kg), and it was determined against inflammation by carrageenan induced hind paw edema method. β-SEF produced a dose dependent decrease in pro-inflammatory cytokines like IL-1β and TNF-α and increase anti-inflammatory cytokine IL-10 level in the inflamed paw. Treatment with Diclofenac sodium (10 mg/kg) and β-SEF (30 and 100 mg/kg) significantly decreased the NF-κB expression in inflamed rats which concludes the reduction of inflammation was probably by decreasing the expression of NF-κB.

Keywords: *Mallotus philippensis*, β-sitosterol enriched fraction, Inflammatory cytokines, Western blot analysis

INTRODUCTION

Inflammation is the tissue reaction that leads to injury, infection, or irritation. This is characterized by pain, swelling, redness and heat. Inflammation accomplishes its protective mission by diluting, destroying, or otherwise neutralizing harmful agents. The inflammatory response is mediated by prostaglandin type of mediators, locally released lesser active chemicals and cytokines [1]. Some cytokines act to make the disease worse (pro-

inflammatory), whereas others serve to reduce inflammation and promote healing (anti-inflammatory). The pro-inflammatory mediators like TNF-α and IL-1β act as chief components in inflammatory response. It was reported that blocking TNF-α or IL-1β has been highly successful in patients with rheumatoid arthritis, inflammatory bowel disease, or graft-vs-host disease. Various forms of these inflammations are treated by non-steroidal anti-inflammatory agents, disease-modifying anti-rheumatic drugs, corticosteroids, antibodies against chemokines and cytokines, but, continuous use of these synthetic medicines produces various adverse effects in human beings. Therefore, when compared to synthetic drugs, herbals are considered to be the best source of treatment for inflammation [2].

The fruit powder of *Mallotus phillipensis* is used as an anthelmintic and anti fertility drug for a long period of time [3-5]. The glands on fruits are bitter pungent, heating, purgative, cathartic, styptic, anthelmintic, detergent, carminative, heal ulcers and wounds, tumours, stone in the bladder, useful in bronchitis, enlargement of spleen. The leaves are bitter, cooling and acts as appetizing agent. The decoction of bark is used for abdominal pain. Among the tribe of Chota Nagpur, the well ground root is rubbed on the painful parts in articular rheumatism. In katha, burma, the seeds are ground to a paste and applied to wounds and cuts. The powdered seeds are mixed with sulphur and sandalwood oil and the mixture is very effective when applied externally in rheumatic joints and also in dermatitis [6]. According to the literatures, all the parts of the plant, except leaf possessed anti-inflammatory activity. To confirm the folklore use of the drug against inflammation and also to determine the effect of inflammatory cytokines, the leaf was taken for the present study.

MATERIALS AND METHODS

Collection and authentication of the plant material

The leaves of *Mallotus philippensis* (Lam.) Muell. Arg. were collected from Topsengattupattu of Pachaimalai hills during February 2008 and authenticated by Prof.P.Jeyaraman, Director of Plant Anatomy Research Centre, Pharmacognosy Institute, West Tambaram, Chennai–45. The voucher specimen was deposited in the Museum of the Department of Pharmacognosy, Adhiparasakthi College of Pharmacy, Melmaruvathur, Tamil Nadu, India (PARC/2008/M-123).

Pharmacognostical evaluation

The pharmacognostical evaluations of the leaf of *Mallotus philippensis* (Lam.) Muell. Arg. were carried out as per the pharmacopoeial standards [7].

Extraction

About 850 gm of coarsely powdered (10/44) dried leaves of *Mallotus philippensis* were extracted with 1.5 L of 90% methanol at 60 ºC using soxhlet apparatus for 48 h by continuous hot extraction method [8]. The same procedure was repeated with remaining 3.4 kg of drug material to obtain the methanolic extract. All these extracts were combined together. The solvent was evaporated to dryness under reduced pressure in the Buchi Rotary Evaporator (Switzerland) at 40-45 ºC. The extract obtained was a dark green powder and the yield was found to be 15% w/w.

Fractionation

The concentrated extract (115 g) was partitioned between *n*-butanol and water. The *n*-butanol part was evaporated under reduced pressure. The residue (75 gm) was then purified on silica gel (60 - 120 mesh, Fischer) using column chromatography with solvent systems of gradually increasing polarity. It was eluted using *n*-hexane (5.1 g), *n*-hexane: Chloroform (7:3, 35 g) and Chloroform: methanol (1:1, 4.5 g) and methanol (3 g). The eluted fractions were monitored by TLC using pre-coated aluminium plates (E. Merck, Germany) using different solvent system to find out the presence of phytoconstituents.

The collected fractions were pooled together and further fractioned by column chromatography using *n*-hexane: chloroform (1:1) solvent system. Three different fractions were collected as F1 (dull white powder, 32.7 g), F2 (dull white powder, 0.21 g) and F3 (dull white powder, 0.34 g). Based upon the results of TLC, fraction F1 was selected for further studies [9].

GC-MS analysis of fraction, F1

The GC-MS analysis was carried out for F1 [10]. The Shimadzu GC-MS model no. QP 2010 was used and the chromatographic conditions were as follows: The VF-5ms Column (Agilant) was operated in electron impact mode at 70 eV; helium (99.999%)

was used as carrier gas at a constant flow of 1.51 mL/ min and an injection volume as per requirement (split ratio of 10:1). The injector temperature was 240 °C and ion-source temperature was taken as 200 °C. The oven temperature was programmed from 70 °C (isothermal for 3 min), with an increase of 10 °C/min, to 300 °C. The MS results were obtained from the libraries, NIST08s, WILEY8 and FAME.

PHARMACOLOGICAL EVALUATION

Animals

Male Sprague Dawley rats weighing between 150-180 g bred in Centre Animal House Facility of Manipal University were separated and acclimated in the experimental area for 1 week. They were maintained at housing conditions of 25 ± 2 °C with relative humidity of 45 to 55 % of 12:12 h light: dark cycle with free access to food (Lipton India Ltd., Mumbai, India) and watered till one day before the experimental day. Rats were fasted overnight, a day before the experiment. The experimental protocol was approved by (Approval No. IAEC/KMC/94/2012 dated 19.10.2012) Institutional Animal Ethics Committee, Central animal house, Manipal University, Manipal (Reg. No. CPCSEA/94/1999) and the study was conducted according to the CPCSEA guidelines for the use and care of experimental animals.

Chemicals

Carrageenan was procured from Sigma, St. Louis, Mosourri, USA. Diclofenac sodium was obtained as gift sample from Cadila Pharmaceuticals Ltd., Ahmedabad, India. Rat Cytokines assay kits were purchased from Thermo scientific & Invitrogen BioServices India Pvt. Ltd, Bangalore, India. BCA™ protein assay kit was purchased from Thermo Fisher Scientific Inc., Waltham, MA, USA.

Acute oral toxicity study

Prior to the dosing, the animals were fasted overnight for 24 h. Following the period of fasting, the fasted body weight of each animal was determined and the dose was calculated according to the body weight. The starting dose of 2000 mg/kg body weight of an animal was used and prepared at 200 mg/mL using 1 % Tween 80 as the solvent. $1/10^{th}$ of the LD_{50} dose was selected for the pharmacological activity. After the extract was orally administered, animals were observed for about 48 h and that was extended for 14 days [11].

Anti-inflammatory activity

The rats were divided into five groups of 6 rats each and the grouping was as follows:
Group I - 1% Tween 80 (control)
Group II -Diclofenac sodium 10 mg/kg (standard)
Group III, IV and V- 10, 30 and 100 mg/kg of β-SEF

After 1h of treatment, 0.1 mL of 1% carrageenan solution was injected into sub-planter region of the left paw to all the treated groups. Immediately after carrageenan administration, left paw volume was measured plethysmometrically (UGO-Basile, Italy). Followed by this measurement, the paw volumes of the each animal were measured at 1, 3 and 5 h of post carrageenan administration [12-14].

Determination of inflammatory mediators [15-18]

Rats were sacrificed with over dose of isoflurane at the end of 5 h. The soft tissue from each inflamed paws was recovered by scalpel, immediately and separately processed to obtain cytosolic and nuclear fractions using nuclear extraction kit (Cayman Chemical Company, Michigan, USA). The fractions were stored in different aliquots at -80 °C.

Cytosolic and nuclear extracts

Soft tissue sample of inflamed paws was weighed and cut into very small pieces. The pieces were collected in a pre-chilled, clean Dounce homogenizer. The sample was kept on ice. To this, 3 mL of ice-cold 1X Hypotonic Buffer supplemented with DTT and Nonidet P-40 (3 µL of 1M DTT and 3 µL of 10% Nonidet P-40) per g of tissue was added. The sample was homogenized with a Dounce homogenizer and incubated on ice for 15 min. This was transferred to prechilled microcentrifuge tubes and centrifuged at 300X g for 10 min at 4 ºC. Then, the supernatant liquid was transferred into a pre-chilled microcentrifuge tube (tube 1). Although the tissue was homogenized, most of the pelleted cells were not yet lysed. The cells were resuspended in 500 µL of 1X Hypotonic Buffer by pipetting up and down several times and transferred to a pre-chilled microcentrifuge tube.

The cells were incubated on ice for an additional 15 min. 50 µL of 10 % Nonidet P-40 was added and mixed gently pipetting up and down. This was

centrifuged and the supernatant was transferred which contained the cytosolic fraction to a new tube and combined with Tube 1. The supernatant was the cytoplasmic fraction, aliquoted and stored at -80 °C. The pellet was resuspended in 50 µL ice-cold 1X Extraction Buffer (with protease and phosphatase inhibitors). This was vortexed for 15 s at the highest setting and the tube was gently rocked on ice for 15 min using a shaking platform. The sample was vortexed for 30 s at the highest setting and gently rocked for an additional 15 min. The procedure was repeated for four more times for a total of six cycles. Then, this was centrifuged at 14,000 X g for 10 min at 4 °C. The supernatant contained the nuclear fraction was used for western blot analysis. The aliquot was transferred to clean chilled tubes, flashed freeze and stored at -80 °C. A small aliquot of the cytosolic extract was kept for the quantitative determination of the protein concentration using BCA kit was used with the working range 1:8 by micro plate method as per manufacture's instruction.

Measurement of serum TNF-α concentration

The cytosolic fraction was kept for 30 min, centrifuged at 3000 rpm for 10 min and stored at -20 ºC prior to the analysis. The TNF-α concentration was quantified using ELISA kit of 120 µL of sample.

Estimation of interleukin-1β

50 µL per well of supernatant obtained from cytosolic fraction was used to determine the concentration of IL-1β using ELISA kit. The samples to be assayed were stored within 24 h at 2-8 °C. For long-term storage, aliquot and freeze samples at -70 °C.

Estimation of interleukin-10

ELISA kit was used to estimate IL-10 concentration. 20 µL of the sample per well was preferred.

Western blot analysis

Nuclear fraction of the homogenized rat paw was used to estimate the C-reactive protein content using commercially available BCA™ protein assay kit.

Nuclear fraction were adjusted to 25 – 30 µg/mL of protein concentration and was resolved in 10 % SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, then the membrane was blocked for 1 h at room temperature with 5% non-fat milk protein in TBS containing 0.1% Tween 20. Then blot was incubated with 1:1000 dilutions of primary antibodies of interest (NF-κB, Cell signaling, USA) and the GAPDH antibody (housekeeping standard) over-night at 4 °C. The blot was then washed with washing buffer and subsequently incubated with a 1:1000 dilution of IgG–horseradish peroxides conjugate. Amplified Opti-4CN™ substrate kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to visualize the proteins.

The experiments were performed in triplicate. The blot was scanned and image was processed using the software ImageJ (ImajeJ 1.4 Waynerasband, NIH, USA). The band intensities of the protein of interest and GAPDH were estimated. The values were expressed as Mean ± SEM of relative band intensity of protein of interest and the means of groups were compared with mean of negative control by one way ANOVA followed by Dunnett's post hoc analysis using Prism Version 6.01 Demo version (GraphPad Inc., LaJolla, CA, USA).

Statistical analysis

The results were expressed as the Mean ± SEM for the parametric data sets and as the median (minimum, maximum) for the nonparametric data sets. The significant difference between the means (parametric) was evaluated by one-way ANOVA followed by Dunnett's post hoc multiple comparison test for normal data.

RESULTS AND DISCUSSION

Pharmacognostical evaluation

The leaves of *Mallotus philippensis* (Lam.) Muell. Arg. was collected in February 2008, identified and authenticated.

Extraction

The methanolic extract obtained from the leaf powder by continuous hot percolation was a dark green powder and the yield was found to be 15% w/w. The *n*-butanol part of methanolic extract, on further more fractionation, gave three different fractions which were collected and named as F1 (dull white powder, 32.7 g), F2 (dull white powder, 0.21 g) and F3 (dull white powder, 0.34 g). The F1 was analysed by GC-MS.

GC-MS analysis of F1

The F1 showed the presence of 16 numbers of compounds, in which β-sitosterol found with 49.99% and the fraction was named as β-sitosterol enriched fraction (β-SEF). The result was shown in Figure 1 and Table 1. All the compounds identified by GC-MS of F1 were possessed the super impossibility more than 85%, so, it was selected for further studies.

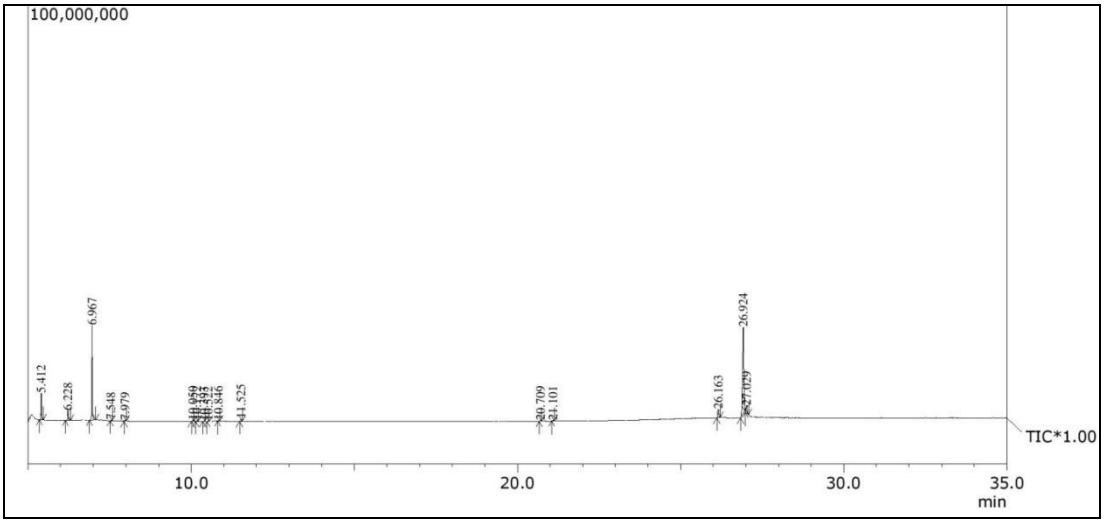

Figure 1 GC-MS chromatogram of F1

Table 1 R_f values and peak areas of F1

S. No.	Peak No.	Retention Time	Area %	Name of the Compound	SI (Super Impossibility Factor)
1.	15	26.924	49.44	β-Sitosterol	88
2.	3	6.967	28.35	Benzene, 1-methoxy-4-(2-propenyl)-	98
3.	1	5.412	8.4	1,6-Octadien-3-ol, 3,7-dimethyl-	99
4.	14	26.163	4.25	Ergost-5-en-3-ol, (3.beta.)-	85
5.	16	27.029	3.79	Stigmastanol	90
6.	2	6.228	3.01	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1R)-	98
7.	11	11.525	0.83	.α. 1,4,8-cycloundecadiene, 2,6,6,9-tetramethyl-, (E,E,E)- caryophylli	92
8.	6	10.050	0.35	Caryophyllene	94
9.	7	10.172	0.31	Bicyclo [3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	92
10.	5	7.979	0.23	trans-3,7-dimethyl-2,6-octadienal	92
11.	13	21.101	0.23	2,6,1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester 6,9-tetramethyl-1,4,8	93
12.	10	10.846	0.22	1,6-cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-,[S-(E,E)]-	93
13.	12	20.709	0.20	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	86
14.	4	7.548	0.18	2,6-Octadienal, 3,7-dimethyl-,(z)-	91
15.	8	10.393	0.10	1,6,10-dodecatriene, 7,11-dimethyl-3-methylene-, (z)	87
16.	9	10.522	0.09	1,4,8-cyclodecadiene, 2,6,6,9-tetramethyl-, (E,E,E)-	89

PHARMACOLOGICAL EVALUATION

Acute oral toxicity studies

The maximum non lethal dose was found to be 1000 mg/kg body weight for β-SEF following the OECD 423 guidelines. The 1/10th dose was taken as the effective dose.

Anti-inflammatory activity of β-SEF

Administration of phlogestic agent like carrageenan (1% w/v) produced inflammation of paw up to 5 h. Pre-treatment with β-SEF (10, 30 and 100 mg/kg) produced dose dependent decrease in edema volume at 1, 3 and 5 h. Significant reduction ($p<0.001$) in edema volume was observed at 100 mg/kg dose level at all the time points of measurement. The effect of β-SEF was almost 10 times less than the standard drug Diclofenac sodium which was shown in Table 2.

Table 2 Anti-inflammatory activity of β-SEF

S. No.	Treatment	Edema volume (ml)		
		1 h	3 h	5 h
1.	Control (1% Tween 80)	0.27 ± 0.03	0.87 ± 0.04	0.95 ± 0.06
2.	Diclofenac (10 mg/kg)	0.10 ± 0.02**	0.49 ± 0.05***	0.59 ± 0.06**
3.	β-SEF (10 mg/kg)	0.13 ± 0.02	0.83 ± 0.08	0.89 ± 0.08
4.	β-SEF (30 mg/kg)	0.17 ± 0.38*	0.83 ± 0.05	0.79 ± 0.05*
5.	β-SEF (100 mg/kg)	0.05 ± 0.01***	0.51 ± 0.12**	0.57± 0.13***

Values are expressed as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001 vs control using one way ANOVA followed by Dunnett's test.

Effect of β-SEF on inflammatory mediators

Carrageenan injection increased the levels of IL-1β and TNF-α. Treatment with β-SEF produced a dose dependent decrease in pro-inflammatory cytokines like IL-1β and TNF-α and increased anti-inflammatory cytokine IL-10 in the inflamed paw. The extent of cytokine modulation by Diclofenac (10 mg/kg) and β-SEF (100 mg/kg) were almost similar (Table 3).

Table 3 Effect of β-SEF on cytokine levels in carrageenan induced rat paw edema

Treatment	Cytokine levels (pg/ml)		
	IL-1β	TNF-α	IL-10
Control (1% Tween 80)	4959 ± 307.89	68.8 ± 2.90	13.8 ± 1.28
Diclofenac (10 mg/kg)	2686.83 ± 291.85***	89.67 ± 2.89**	36 ± 1.93***
β-SEF (10 mg/kg)	4281.67 ± 280.48	69.17 ± 2.56	17.67 ± 1.48
β-SEF (30 mg/kg)	4097.17 ± 290.92	75.83 ± 3.91	24.5 ± 1.78**
β-SEF (100 mg/kg)	3183.17 ± 301.31**	92.50 ± 2.73**	34.67 ± 2.08***

Values are expressed as mean ± SEM. **p<0.01; ***p<0.001 compared to control group using One way ANOVA followed by Dunnett's post hoc test.

Western blot analysis

Effect of NF-κB expression in carrageenan induced inflamed paw was determined. The subcutaneous injection of carrageenan significantly increased NF-κB expression as compared to normal rats. Treatment with Diclofenac and β-SEF known as F1 (30 and 100 mg/kg) significantly decreased the NF-κB expression in inflamed rats (Figure 2 & Figure 3). These results suggested that the β-SEF (F1) and Diclofenac reduce the inflammation probably by reducing the expression of NF-κB.

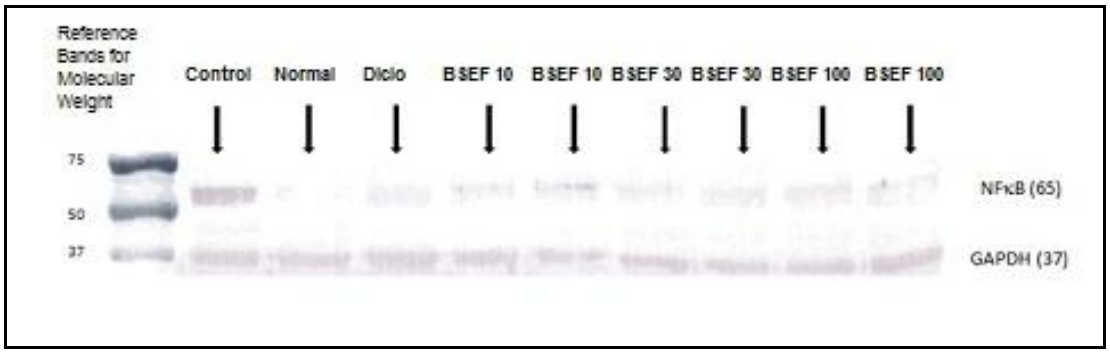

Figure 2 Effect of β-SEF on NF-κB expression in carrageenan induced rat paw edema

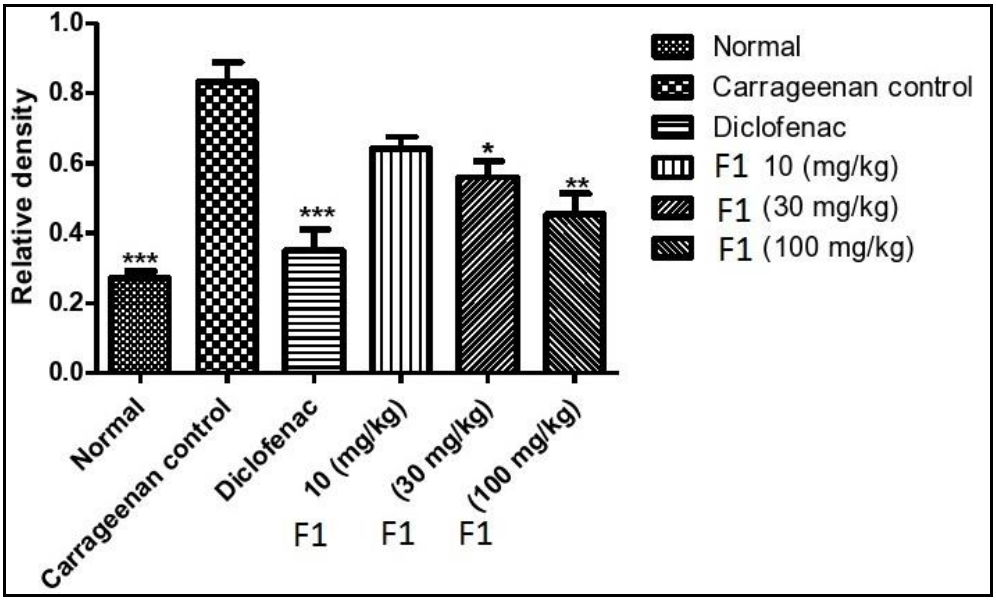

Figure 3 Relative densities of protein expression of western blot analysis

CONCLUSION

The β-SEF was isolated from the methanolic extract of leaves of *Mallotus phillipensis* (Lam.) Muell. Arg. which belongs to the family Euphorbiaceae. The different doses of β-SEF produced dose dependent decrease in edema volume and also observed that the effect β-SEF (100mg/kg) was almost 10 times less than the standard drug, Diclofenac sodium. Administration of Carrageenan increased the levels of IL-1β and TNF-α. Treatment with β-SEF produced a dose dependent decrease in pro-inflammatory cytokines like IL-1β and TNF-α and increased anti-inflammatory cytokine IL-10 in the inflamed paw. Treatment with β-SEF (30 and 100 mg/kg) significantly decreased the NF-κB expression in inflamed rats. The involvement of cytokines such as IL-1β, TNF-α and NF-κB were seemed to be of particular importance in bone physiology. The NF-κB represents a central factor in all types of inflammation. It can be activated by a great variety of stimuli and a complex network of signaling pathways, which can also influence each other. Furthermore, it regulates cytokines, growth factors, adhesion molecules, intracellular signaling molecules, transcription factors etc.

This study concluded that the NF-κB expression is being controlled by reducing the pro-inflammatory cytokines (IL-1β & TNF-α) and increasing the anti-inflammatory cytokine (IL-10).

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