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Research Article

SUPPRESSION OF PRO-INFLAMMATORY CYTOKINES AND MEDIATORS VIA THE INHIBITION OF THE NF-κB IN LPS-INDUCED RAW 264.7 MACROPHAGE CELLS BY PURIFIED TERPENOID EXTRACT FROM *HYPNEA MUSCIFORMIS* (WULFEN) J V LAMOUROUX.

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ABSTRACT

Sea weeds utilization originates from Chinese *Materia medica* and are traditional local medicine used to treat multiple disorders including acute and chronic inflammation. Knowledge on sea weeds biological activity underlying its mode of anti-inflammation action is not yet traced to prove its value for pharmacological and clinical usage. Prostaglandin (PGs) play crucial role in the induction of the inflammatory reactions and is over expressed in the inflamed or injured tissues. This study presents evidence to justify the antinociceptive and anti-inflammatory usage of terpenoid extract from *Hypnea musciformis*. The methanolic extract of the red algae was purified by silica gel column and was fractionated by TLC and GC-MS. The analysis of the purified fraction of *H. musciformis* revealed the presence of 8 major peaks of terpenoids compatible with their fragmentation patterns. The major terpenoids noticed were eicosane, heneicosane, 2-pentadecanone, hexadecanoic acid, methyl ester, n-hexadedcanoic acid, hexadecanoic acid, ethyl ester, heptadecanoic acid,methyl ester , 11- octadecanoic acid, metyl ester. Purified terpenoid extract of *H. musciformis* obviously decreased the expression levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, nuclear factor (NF)-κB and inducible nitric oxide synthase (iNOS). In addition, cyclooxygenase (COX)-2 activity and nitric oxide (NO) content were inhibited substantially. The molecular mode underlying these effects is through the down regulation of NF-κB and iNOS expressions coupled with other inflammatory mediators like TNF-α, IL-1β, and IL-6. Thus, the present data provided biochemical evidences that purified terpenoid extract mitigates inflammatory related pain disorders by inhibiting the expression of inflammatory mediators.

Keywords: Anti-inflammation, cyclooxygenase-2, inducible nitric oxide synthase, nuclear factor- kappa B, purified terpenoid extract

1. INTRODUCTION

Normally, inflammation related disorders are resolved in a timely manner; however, deregulated inflammatory responses can cause excessive tissue damages leading to acute or chronic inflammatory disorders. NF-κB is a central mediator of pro-inflammatory induction among innate and adaptive immune cells. Many works have been documented in the physiopathology of inflammation and the role of free radicals in pathogenesis. Inflammation is characterized by redness, swelling, pain, heat and dysfunction of the tissue and organs [1]. The transcription factor nuclear factor κ B (NF κ B) is the major regulator of immune and inflammatory responses. The humoral and cellular mechanisms of inflammation involve the nuclear factor-kappa B (NF-κB) and signaling substances such as cytokines and prostaglandins 2. During the inflammatory event the host activates cellular immune responses that increase production of pro-inflammatory mediators, including the tumour necrosis factor alpha (TNF-a). TNF- α has been responsible for multiple illness in humans, including immune and inflammatory diseases, cancer, psychiatric disorders. IL-1α, another cytokine which exerts a pro-inflammatory activity [2].

There are many natural products that have anti-inflammatory and analgesic properties with relatively low side effects. Among these natural products, the compounds isolated from marine macroalgae were unique in terms of analgesic and antiulcerogenic ways.

Steroid drugs, nonsteroidal anti-inflammatory drugs and immune suppressants, are commonly used for the treatment of inflammatory diseases. Most of them were associated with serious side effects such as gastrointestinal bleeding and peptic ulcers. The discovery and development of new bioactive natural products with anti-inflammatory and antinociceptive properties are need of the hour. In this juncture, secondary metabolites from seaweeds have been showed to be effective in the treatment of inflammation and pain by suppressing the production of inflammatory mediators [3]. Thus, this study aimed at evaluating the role of terpenoids from the red algae *H. musciformis* against pro-inflammatory cytokines and mediators in LPS-induced RAW 264.7 macrophage cells and their probable mode of action.

2. MATERIAL AND METHODS

The red algae *H. musciformis* was collected during March 2018, from the Mandapam coast (latitude 9° 17' N, longitude79° 22' E) of Gulf of Mannar. The thallus was washed thoroughly until all the debris were removed. Initially, 50 g of the dried algal powder was subjected to soxhlet extraction with 250 ml methanol. The extraction was repeated thrice. The extract was then filtered and kept at room temperature for evaporation. Fractionation of the crude methanolic extract was done by silica gel column chromatography using different ratios of petroleum ether: ethyl acetate solvent combinations. The 95:5 eluted fractions showed optimal terpenoid content and were then subjected to TLC and further analysed by GC-MS. For GC-MS analysis, the sample was injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 ìM film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. A chromatogram was obtained and the mass spectrums of the unknown components were compared with the spectrum of the known components available in the NIST terpenoid library.

2.1. Cell culture

RAW264.7 macrophages obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in DMEM containing 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37°C in a 5% CO_2 humidified air environment.

2.2. ELISA analysis of TNF-α, IL-1β, and IL-6 level

TNF-α, IL-1β and IL-6 cytokines in the culture supernatant and the cell lysate were measured by sandwich ELISA, using specific monoclonal antibodies according to the manufacturer's instructions. To determine the level of cell-associated cytokines, cells were washed with phosphate-buffered saline (PBS) and then lysed in buffer containing 0.5% NP-40 [4]. Standard recombinant proteins were also diluted in PBS at the same concentrations of NP-40 as the cell lysate. The amounts of cytokines in the culture supernatant and the cell lysate are calculated as those produced from 1×10^6 cells. The optical density (OD) value was detected at 450nm by an ELISA Reader (Thermo Fisher Scientific, Inc.)

2.3. Western Blot analysis

The Raw 264.7 cells (3.2×10^5 cells /60 mm culture dish plate) were washed three times with 50 µL PBS and lysed with lysis buffer (Mammalian Cell-PE LB, GBio sciences, St. Louis, MO, USA). The proteins were separated on polyacrylamide mini-gels and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Chalfont, UK). Following incubation overnight with the appropriate primary antibody, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase. The immune reactive bands were visualized using an ECL detection system (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.4. Estimation of nitric oxide concentration

Treated sample solutions from murine cells were taken in test tubes and subjected to Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2.5% hydrochloric acid). The colorimetric reaction was allowed to proceed for 10 min at room temperature, and OD was measured at 550 nm. The concentration of nitrite was calculated from a standard curve established with serial dilutions of sodium nitrite [5].

Nitrite concentration = (Absorbance (net O.D) of test x Concentration of Standard)/ Absorbance of standard

2.5. Assay for cyclooxygenase-2 (COX-2) inhibition

The COX -2 inhibiton assay measures PGF2α by stannous chloride reduction of COX derived PGH2 so produced. The reaction system consists of reaction buffer, haem, enzyme and plant extract pre-incubated at 37 °C for twenty minutes with background and enzyme controls. The absorbance was read at 420 nm. The data was plotted as % B/B_0 (Standard Bound / Maximum

Bound) versus log concentration using a 4-parameter determined from a standard curve with appropriate dilutions and used to calculate the percent inhibition as per the formula given below:

Percent Inhibition (%) = (Activity of Control – Activity of Test/Activity of Control) x 100

The percent inhibition was plotted against the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition).

2.6. Statistical analysis

Results of all biological studies were expressed as means±SD. One-way analysis of variance (ANOVA) was used to determine significance when compared to the control group. p values less than 0.05 and 0.01were considered significant (*p <0.05, **p <0.01).

3. RESULTS AND DISCUSSION

Initially, the methanolic extract of the red algae *H. musciformis* was purified by silica gel column chromatography. By using different solvent combinations of petroleum ether and ethyl acetate the fractions were eluted. Elution was started with 100% PE and gradually increased the polarity up to 100% EA.The column eluted fractions were analyzed for the presence of terpenoids and subjected to TLC using 6 ml toluene: 1ml ethyl actate as solvent combination and further to GC-MS analysis. The solvent combination of 95:5 PE:EA showed significant amount of terpenoids. This fraction was then subjected to thin layer chromatography for confirming the presence of terpenoids. By using the retention time and the relative abundance of each compound was analysed. The 95:5 PE: EA eluted fraction showed a single band on TLC with Rf value 0.89. This fraction revealed the presence of 8 major peaks of terpenoids compatible with their fragmentation patterns (Fig.1). The major terpenoids found in *H. musciformis* were eicosane, heneicosane, 2- pentadecanone, hexadecanoic acid, methyl ester, n-hexadedcanoic acid, hexadecanoic acid, ethyl ester, heptadecanoic acid, methyl ester , 11-octadecanoic acid, metyl ester.

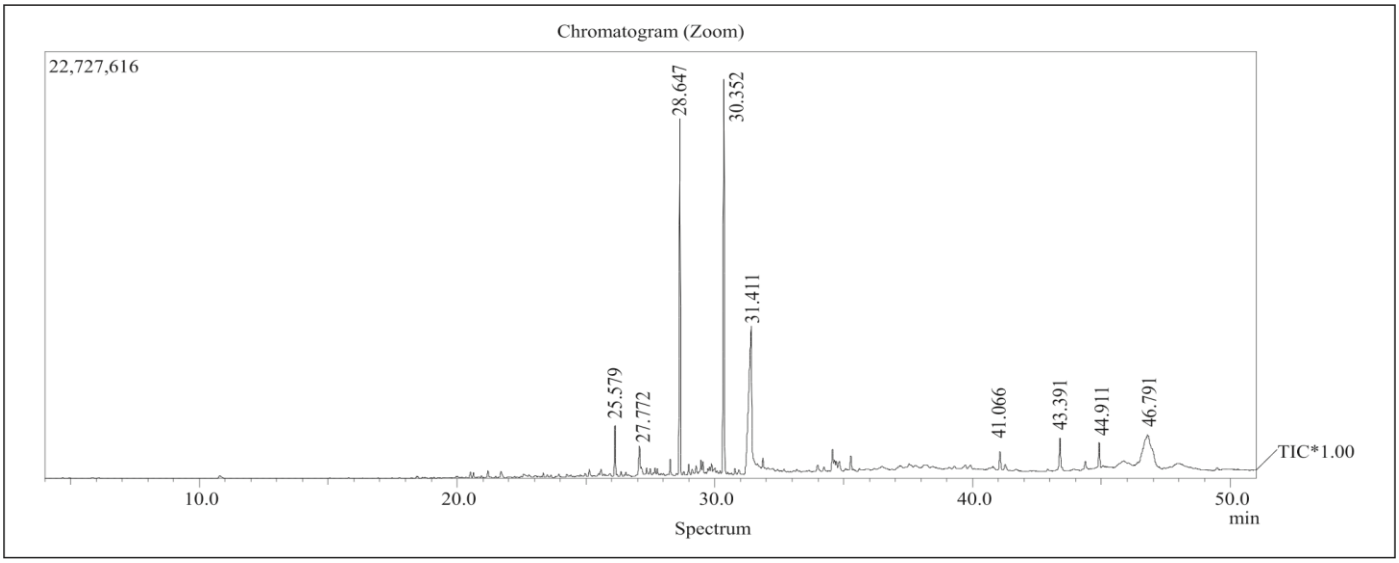

Fig. 1: GC- MS spectrum showing the terpenes composition from *H. musciformis*

The terpenoid extract of *H. musciformis* treated murine cell lines showed significant reduction in the nitric oxide content (Table 1). Nitric oxide is usually associated with many life style disorders. NO is a short-lived molecule that has an important role in various physiologic processes. However, NO overproduction can activate nuclear factor-κB to induce the expression of pro-inflammatory mediators. NO is a chemical mediator having microbicide activity which is produced during inflammation by activated phagocytes. Similarly, pathogen infections causes overproduction of NO that leads to many neurodegenerative disorders. Inducible nitric oxide synthase (iNOS) triggers NO synthesis that results in vasodilation and hypotension. Pro-inflammatory cytokines such as TNF-α and IL-6 evoke iNOS activity and their by a significant increase in the production of NO [6]. Therefore, NO inhibitors are novel molecules for the treatment of inflammatory

issues. Gutierrez and Hoyo-Vadillo [7] confirmed that the crude extracts of *Petiveria alliacea* regulates NO synthesis and thereby regulates anti-inflammatory disorders. The infectious diseases like tuberculosis showed increased expression of iNOS in alveolar macrophages, together with excessive production of TNF-α that triggers NO synthesis leads to fever and necrosis of pulmonary tissue [8]. Chronic generation of NO radical is associated with various cancers and inflammatory conditions, including diabetes mellitus and arthritis. The red algae *Laurencia okamurae* was reported to be a potent inhibitor of the production of pro-inflammatory mediators, such as prostaglandin E2, interleukin-6 (IL-6), NO, and TNF-α [9]. The anti-inflammatory activities of extracts from the micro alga *Tetraselmis suecica* with respect to nitric oxide (NO) production, tumor necrosis factor (TNF)-α and interlukin (IL)-6 release in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells was evaluated by Jo et al. [10].

Table 1: Production (µM) and % of NO inhibition by terpenoid extracts

H. musciformis		
Concentration (µg/ml)	**NO (µM)**	**NO inhibition (%)**
25	39.6	10.2
50	28.3	18.6
75	21	24
100	14	50
200	8.5	64.7

NO: Nitirc oxide

H. musciformis extracts showed a potent significant inhibition of the COX-2 enzyme and was comparable with the positive control, Ibuprofen. The results were expressed as percent of inhibition of activity. The IC_{50} values were 74.2 µg/ml. The pro-inflammatory cytokine TNF-α induces COX-2 expression and consequently, the release of prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α) [11]. Hence, an inhibition of the secretion of TNF-α results a decrease in the COX-2 expression. The anti-inflammatory activity (reduced COX-2 activity) observed with the terpenoids may be attributed possibly by decreasing the production of the pro-inflammatory cytokines, especially TNF-α. Similar result was observed in methanolic extracts of brown algae *Undaria pinnatifida* that inhibited iNOS and COX-2 expression in a dose dependent manner [12]. C-phycocyanin, a biliprotein isolated from *Spirulina platensis*, suppressed inflammation by inhibiting the production of pro-inflammatory cytokines and suppressed expressions of inducible nitric oxide synthase and cyclooxygeanase-2 level. Subedi *et al.*, [13] proved that γ-tocopherol is potential to inhibit COX activity and there by PGE2 production in macrophages and epithelial cells. The present results also showed that terpenoids inhibited COX enzyme activity effectively in a concentration dependent manner. A new morpholine alkaloid was isolated from the thalli of red seaweed *Gracilaria opuntia* showed profound COX-2 inhibitory activity in conjunction with *in vitro* 5-lipoxidase inhibitory activity as compared to non-steroidal anti-inflammatory drugs [14].

3.1. Pro-inflammatory cytokines

The experiment was further aimed to explore whether the anti-acute inflammatory effects of terpenoid extract of *H. musciformis* was associated with regulation of the major pro-inflammatory cytokines that induces the inflammatory reactions. The effects of terpenoid extract of *H. musciformis* on TNF-α, IL-1β and IL-6 levels were showed in the Figure 2 a, b, & c. Compared with the data for the control group, a significant reduction in the TNF-α level was noticed in RAW264.7 macrophages treated with different concentrations viz. 50, 100, 200 and 400 µg/ml of purified terpenoid extract from *H. musciformis* (9.82 to 2.65 ng/ml, $P < 0.01$). The terpenoid extracts of *H. musciformis* showed a dose-dependent response and was comparable with that of dexamethasone (synthetic drug). Similarly, as compared to the control group, treatment with 50, 100, 200 and 400 µg/ml terpenoid extract of *H. musciformis* significantly reduced IL-1β levels. The values were statistically significant. Although the inhibitory effect of terpenoid extract of *H. musciformis* on IL-1 was slightly attenuated with increasing dose, the effect was still within the effective range.

A significant reduction in IL-6 was also observed following treatment with 50, 100, 200 and 400 µg/ml terpenoid extract of *H. musciformis*. The reduction observed following administration of terpenoid extract of *H. musciformis* at the tested concentrations was similar to that of standard drug.

Similarly, the terpenoid extract of *H. musciformis* attenuated NF-κB protein expression in RAW264.7 macrophages treated with LPS (Figs 3 a & b.). At 1 h, 50, 100, 200 and 400 µg/ml terpenoid extract of *H. musciformis* showed inhibitory effects on NF-κB protein expression soundly as compared to untreated control.

Dexamethasone also down regulated NF-κB expression effectively.

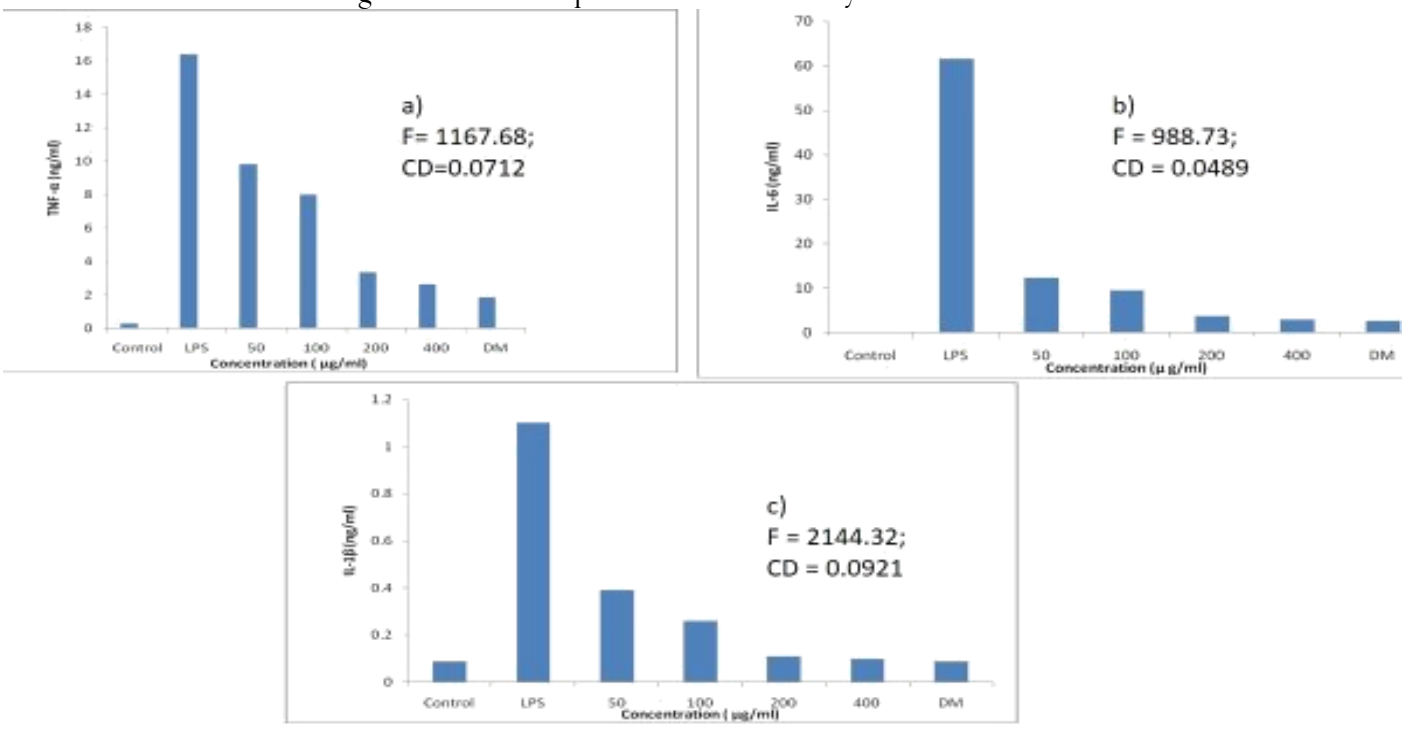

Fig. 2 a, b, c: Effect of terpenoid extract on pro-inflammatory cytokine production in LPS induced RAW264.7macrophages.

RAW264.7 cells were pretreated with terpenoid (50–400 µg/mL) for1h and stimulated with LPS (100 ng/mL) for 24h. Levels of TNF-α (a), IL-6 (b), and IL-1β (c) that were present in the supernatants were quantified using ELISA.

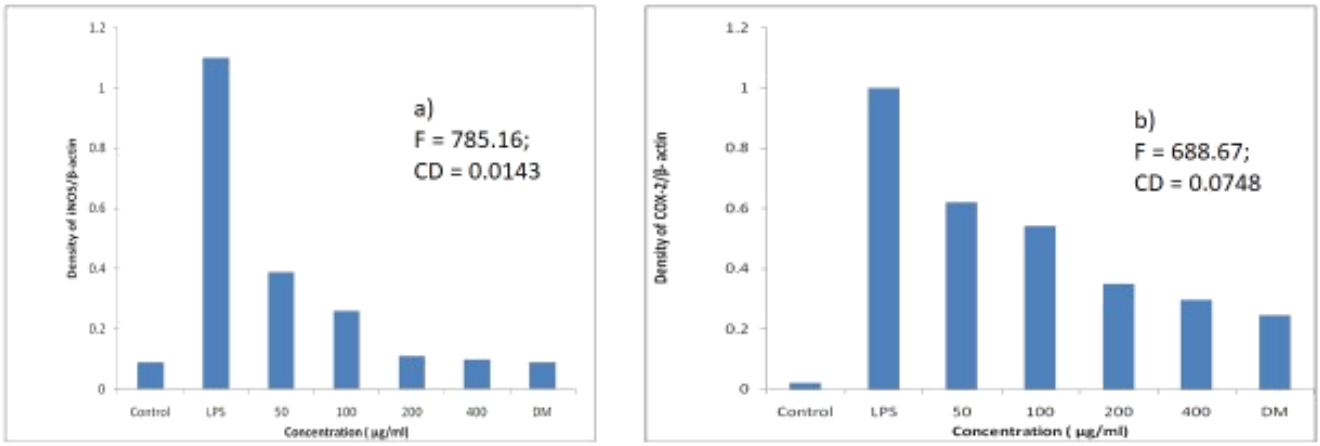

Fig. 3 a and b: Effect of terpenoid extract on pro-inflammatory cytokine production in LPS induced RAW264.7macrophages.

RAW264.7 cells were pretreated with terpenoid (50-400 µg/mL) for1h and stimulated with LPS (100 ng/mL) for 24h. iNOS ((a), for 24h) and COX-2((b), for7 h) protein levels were analyzed in whole cell lysate using western blotting. β-actin expression was used as an internal control.

Terpenoid extract of *H. musciformis* and dexamethasone inhibited the expression of iNOS in the macrophage cells (Figs 3 a & b). The results showed that administration of terpenoid extract of *H. musciformis* at 50, 100, 200 and 400 µg/ml inhibited iNOS protein expression profoundly as compared to control level ($P < 0.05$) at 1 h. Dexamethasone significantly inhibited iNOS protein expression ($P < 0.05$). Similarly, the COX-2 expression

was also reduced in a dose dependent manner by terpenoid extract and dexamethasone.

Anti-inflammatory studies on macrophages have documented that LPS induces the release of specific pro-inflammatory cytokines, namely TNF-α, IL-1β, and IL-6. These cytokines induce pathological reactions such as edema, allodynia, neutrophil leaching, hypersensitivity and pain. However, Ovuakporie-Uvo *et al.,* [15] and Mi-Jin *et al.,* [16] have addressed the functions of TNF-α, IL-1β, and IL-6 cytokines in LPS induced inflammation. In the present work, a remarkable reduction in TNF –α, IL-1β, and IL-6 expression levels were seen following treatment with purified terpenoid extract of *H. musciformis* similar to that of synthetic drug dexamethasone. iNOS and COX-2 protein expression also displayed similar results (Fig. 4).

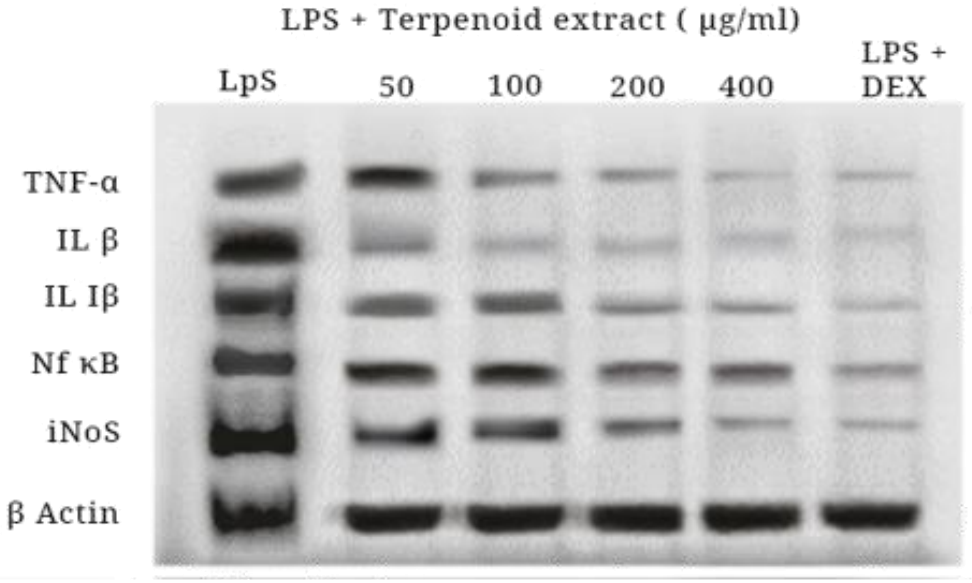

Fig. 4: Expression of proinflammatory cytokines and NF-kB and iNOS by Western blot analysis where β-actin was used as internal standard

Cytokines induces the expression of the α1A-adrenoceptor subtype on immune tissues and also on other cells of the injured tissue. Generally, nor-adrenaline critically up-regulates α1A-adrenoceptor to enhance the synthesis of the pro-inflammatory cytokine IL-6. Further, the discharge of inflammatory mediators and nerve growth factor (NGF) from keratinocytes and others may instigate the expression of α1-adrenoceptors on peripheral nerve fibers. Subsequently, nociceptive afferents develops an abnormal excitability to adrenergic factors, and inflammatory processes developed [17]. Additionally, Qomaladewi *et al.,* [18] and Vargas and Petricevich [19] have reported that TNF-α and IL-1β are the major factors involved in neutrophil leaching and the over expression of mRNA levels of IL-1β, IL-6 and TNF-α in RAW264.7 macrophages treated with LPS. Meanwhile, IL-6 instigates the synthesis of the TNF-α and IL-1β pro-inflammatory cytokines and indirectly fosters the protein expression of iNOS and COX-2 activity, and ultimately the peroxynitrite production and tissue damage. The results of this work revealed that different doses of terpenoid extract of *H. musciformis* indeed down regulated the activities of TNF-α, IL-1β and IL-6, although the efficacies were different.

After the administration of terpenoid extract of *H. musciformis*, the levels of TNF-α, IL-1β and IL-6 were reduced, and the expression of NF-κB and iNOS protein was inhibited (Fig 4). At 1 h, terpenoid extract of *H. musciformis* showed significant inhibitory effects on NF-κB and iNOS protein expressions that were similar to the effects of dexamethasone. NF-κB, a crucial transcription element, can control the COX- 2 and iNOS expression [20]. In succession, COX-2 can enhance PG levels to activate inflammatory injuries [21]. iNOS are primarily involved in NO synthesis in response to inflammatory and nociceptive reactions. Therefore, it is justifiable that the antinociceptive role of terpenoid extract of *H. musciformis* may be in accordance to that of peripherally

acting synthetic drugs, and was due to its anti-inflammatory potential. The molecular modes underlying the anti-inflammatory impact of terpenoid extract of *H. musciformis* may involve blocking of the cytokine activities of TNF-α. Tumour necrosis factor (TNF) is the established most effective physiological inducers of NF-κB, and both are mutually influence each other. In addition, terpenoid extract of *H. musciformis* inhibited LPS induced changes in RAW264.7 at 1 h as well as NF-κB and iNOS protein expressions. It also indirectly regulated the activities of NO and COX-2. iNOS is regulated by TNF-α and NF-κB. Subsequently, another putative mode for the antiinflammatory effects of terpenoid extract of *H. musciformis* was via the inhibition of NF-κB. NF-κB play a crucial role in the anti-inflammatory and analgesic potentialities of terpenoid extract of *H. musciformis*. Killeen *et al.* [22] also reported that the regulation and control of NF-κB activation was an effective therapeutic strategy for the control and treatment of NF-KB-related human inflammatory disorders. Previous studies reported that the transcription factors NF-κB and AP-1 regulate the production of many inflammatory mediators. Ovuakporie-Uvo *et al.,* [23] correlated analgesic, pro and anti-inflammatory activities of *Desplatsia dewevrei* with gene expression of cytokines of Wistar rats and mice.

NF-κB plays a crucial role in inducing inflammatory reactions. It comprises two subunits, p65 and p50 that guild to form homo-and hetero dimmers with multiple roles includes alliance with IκB, nuclear translocation, and DNA binding [24] .Therefore, the present results tempt to suggest that terpenoid extract may reduce both p65 and p50 expressions in the nucleus. p65 is more critical for NF-κB induction in the inflammatory events. There were similar reports on anti-inflammatory potential of *Carpomitra costata* ethanolic extracts *via* inhibition of NF-κB and AP-1 activation in LPS-stimulated RAW264.7 macrophages. NF-κB has been implicated in the pathogenesis of a number of inflammatory diseases, such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), multiple sclerosis, atherosclerosis, systemic lupus erythematosus, type I diabetes, chronic obstructive pulmonary disease and asthma. NF-κB mediates the induction of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, in monocytes/macrophages. Many of these cytokines are capable of activating NF-κB in innate immune cells and fibroblasts, thereby inducing the expression of additional inflammatory cytokines and chemokines, leading to further recruitment of inflammatory immune cells and dissemination of inflammation. Recently, it was reported that the ethanolic extract of *Sargassum serratifolium* attenuates interleukin-1β-induced oxidative stress and inflammatory response in chondrocytes by suppressing the activation of NF-κB, p38 MAPK, and PI3K/Akt [25]. Zang *et al.*, [26] reported the anti-inflammatory effects of *Polygonum hydropiper* stalks extracts on 2,4,6-trinitrobenzenesulphonic acid-induced intestinal inflammation in rats and its anti-inflammatory effects was through the inhibition of the NF-κB signal pathways. The cytokines such as IL-1, IL-6, and TNF-α are produced mainly by activated monocytes or macrophages. They stimulate the cell proliferation in various types of cells. TNF -α affect various biological processes including the regulation and the production of other cytokines [27] .TNF-α activates macrophages and promotes inflammation and expression of cell adhesion molecules to inflammatory tissue thereby plays a key role in the induction and perpetuation of inflammation. In this study also, the terpenoid fraction of *H. musciformis* significantly inhibit proinflammatory cytokines production in a dose-dependent manner in LPS stimulated RAW 264.7 cells. The anti-inflammatory effects from the microalgal strain *Aurantiochytrium limacinum* 4W-1ba and its mechanism of action in LPS-stimulated murine macrophage cells was reported by Takahashi *et al.,* [28]. Many studies have reported the anti-inflammatory effects of algal extracts on mammalian cells. The green algae, *Chlorella vulgaris* was found to potently inhibit the pro-inflammatory mediators in a dose dependent manner and also the methanol extracts significantly decreased the LPS induced TNF-α and IL-6 [29]. Thus, the obtained result in the red algae in terms of anti-inflammatory mode of action was justifiable.

4. CONCLUSION

The present study interprets the anti-inflammatory molecular mode of action of terpenoids from of *H. musciformis* in LPS-treated macrophage cells through NF-κB regulation. The inhibitory effects of terpenoid fractions on NO and COX 2 production in the LPS-induced RAW264.7 macrophages was remarkable. Furthermore, the terpenoids markedly attenuated the expression of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α. These effects were associated with the inhibition of LPS-induced NF-κB expression inactivation. This may further inhibit c-Jun N-terminal kinase/stress-

activated protein kinase (JNK) and phosphatidylinositol 3′-kinase/Akt (PI3K/Akt) by phosphorylation. Thus, it is possible to state that the terpenoid fraction from *H. musciformis* possesses potential anti-inflammatory activities and a good alternate against conventional steroidal and nonsteroidal anti-inflammatory drugs.

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