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RESEARCH ARTICLE

Preliminary screening of two marine algae and sea grass harvested from Sri Lankan waters against the LPS-induced inflammatory responses in RAW 264.7 macrophages and *in vivo* zebrafish embryo model

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Abstract: The present study was designed to investigate the secondary metabolites from under-explored marine flora inhabiting the coastal areas of Sri Lanka for evaluating their anti-inflammatory properties. The hexane, chloroform, ethyl acetate and aqueous solvent fractions obtained from 80 % methanol extracts of two marine algae; *Chaetomorpha antennina*, *Gracilaria edulis* and the sea grass; *Halophila ovalis* harvested from the Kalpitiya area of Sri Lanka were screened for anti-inflammatory properties using LPS stimulated RAW 264.7 macrophages and by *in vivo* zebrafish embryo model. The chloroform fraction of *G. edulis* (GEMC), and hexane fractions of *G. edulis* (GEMH) and *C. antennina* (CAMH) demonstrated the best anti-inflammatory activity against LPS induced NO production in RAW 264.7 macrophages. The anti-inflammatory effects in RAW 264.7 macrophages were mediated through the inhibition of iNOS, COX-2 and PGE_2 expression. Moreover, GEMH, GEMC and CAMH solvent fractions also exerted pronounced anti-inflammatory activity against LPS induced NO production, ROS production and cell death in the zebrafish model. The active components responsible for the anti-inflammatory activity could be identified with further purification of the solvent fractions.

Keywords: Anti-inflammatory, *Chaetomorpha antennina*, *Gracilaria edulis*, RAW 264.7 macrophages, Sri Lankan marine algae, zebrafish.

INTRODUCTION

Screening biofunctional properties of natural products has laid the foundation for the isolation of functional molecules for further systematic studies to develop pharmaceuticals. In this regard, marine organisms are a promising source of functional molecules due to its great diversity represented by 34 of the 36 phyla inhabiting the massive marine ecosystem (Gul & Hamann, 2005). These organisms have been found to possess a number of intriguing metabolites bearing unusual structures accounting for a range of biological functionalities (Wijesekara *et al*., 2011; Zhou & Guo, 2012). They are proven to be effective as anti-cancer, anti-oxidant, anti-inflammatory, anti-microbial, immunomodulatory, anti-coagulant, anti-diabetic, anti-obesity and anti-herpetic compounds (Newman & Cragg, 2004; Holdt & Kraan, 2011). Although marine natural products lack a renowned ethnomedical history, modern research has revealed fascinating biological properties of these metabolites (Dias *et al*., 2012).

Marine flora includes algae, seagrass, fungi, bacteria, and cyanobacteria, the primary producers of the marine

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ecosystem (Boopathy & Kathiresan, 2010). Marine flora that inhabits the coastal areas of Sri Lanka represents an under-explored natural resource. The bioactive potentials and nutraceutical values of these organisms have not been explored widely except for a few biochemical and taxonomical studies. Ratnasooriya *et al.* (1994), and Premakumara *et al.* (1995;1996) have reported about the post-coital contraceptive activity of *Gracilaria corticata* and *Gelidiella acerosa* crude extracts, and isolation of a sphingosine derivative from *G. acerosa*. Lakmal *et al.* (2014) described the antioxidant and anti-cancer activity of the 80 % methanol extract of six marine algae species. The locals are unaware of the potential use of these natural resources as food or their possible utilisation in food, pharmaceutical, cosmetic and other industries. Therefore, the under-explored marine algae inhabiting Sri Lankan coastal waters could be further investigated to promote their conservation, aquaculture and imperishable utilisation in producing functional ingredients. The objective of the present study was to explore the potential anti-inflammatory properties of algae extracts obtained from several selected algae species abundant in the coastal areas of Sri Lanka.

METHODOLOGY

RAW 264.7 macrophages were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO Inc. (NY, USA). 2′,7′-2′ 7′-dichlorodihydrofluorescein diacetate (DCFH2-DA), acridine orange, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma, Aldrich, USA. All the organic solvents used in the extraction and fractionation were of analytical grade.

Sample collection and preparation

The two algae *Chaetomorpha antennina* and *Gracilaria edulis* and the seagrass *Halophila ovalis* samples used in this study were collected off Kalpitiya, Sri Lanka (N 6º4'54.19"/ E 80º8'51.78"). The samples were identified based on the morphological characteristics. After thorough washing, all the samples were freeze-dried and ground into powder.

Extraction and fractionation

The extraction was done using 80 % methanol in water with continuous sonication for 3 h under 37 ºC. The crude extracts were obtained by removing the solvent under vacuum (37 ºC) and fractionated into hexane, chloroform and ethyl acetate. Finally, the 4 fractions including the water fraction were dried using a vacuum. Hereafter, the fractions will be named following their scientific name, extraction and fractionated solvent conditions; GEMH- *Gracilaria edulis* 80 % methanol extract hexane fraction, GEMC- *Gracilaria edulis* 80 % methanol extract chloroform fraction, etc.

The proximate composition of marine floral material and their extracts

The analysis was carried out according to the official methods of AOAC (AOAC International, 2005). Accordingly, the protein and lipid contents were determined by standard Kjeldahl and Soxhlet methods, respectively. The ash content was analysed by dry ashing in a furnace at 550 °C for 6 h. The total phenolic and sterol content of each crude extract was analysed using the phenol-sulfuric acid method and modified Liebermann Burchard method described by Chandler and Dodds (1983) and Xiong *et al.* (2007), respectively.

Cell culture

RAW 264.7 macrophages were maintained in DMEM supplemented with 10 % FBS and 1 % antibiotics (penicillin and streptomycin). The cells were maintained in incubators at 37 ºC in a humidified atmosphere with 5 % CO_2. Cells under exponential growth were seeded for the experiments with a concentration of 1.0×10^5 cellsmL^{-1}. Cell viability as a measurement of cytotoxicity of the samples was evaluated using MTT assay according to the method described by Samarakoon *et al.* (2013).

Determination of LPS induced NO production

Lipopolysaccharides (LPS) induced nitric oxide (NO) production of the samples was evaluated using RAW 264.7 macrophages (Ko & Jeon, 2015). LPS, at 1 µgmL^{-1} was introduced into each well pretreated with samples at varying concentrations. After 24 h, 100 µL of the cell culture medium withdrawn from each well was mixed with 100 µL of Griess reagent. The absorbance was recorded at 540 nm using a synergy HT multi-detection microplate reader.

Western blot analysis for evaluation of the expression of inflammatory mediators

RAW 264.7 macrophages were cultured in 6 well plates and incubated for 24 h. Then different sample concentrations were treated to the wells following LPS stimulation and incubated for another 24 h period. The cells were harvested using PBS and cell lysates were prepared using lysis buffer under ice cold conditions. The

cell lysates were centrifuged at 16000 g for 20 min at 4 ºC and the supernatants were collected. The protein contents of the supernatants were standardised to 50 μgmL^{-1} after analysing using Thermo Scientific Pierce BCA protein assay kit. Lysates were subjected to electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gel. The protein bands were then transferred onto polyvinylidene fluoride membranes. First, the membranes were incubated with primary antibodies (1:1000) in 5 % nonfat dry milk in TBST at 4 ºC for 12 h, and then incubated with the secondary antibodies at 1:3000 concentration for nearly 3 h. Westar EtaC enhanced chemiluminescent substrate (Cyanagen Srl, Bologna, Italy) was used to develop the western blot signals and image analysis was done using a fluorescence molecular imaging system (Vilber Lourmat, Paris, France) (Wijesinghe *et al*., 2014).

Evaluation of PGE_2 and pro-inflammatory cytokine, TNF-α, IL-β and IL-6 production

RAW 264.7 macrophages were cultured in 24 well plates with 10^5 $cellsmL^{-1}$ concentration. After 24 h incubation, GEMH, GEMC and CAMH samples were introduced into each well achieving final concentrations of 25, 50 and 100 μgmL^{-1}. After 1 h the stimulant LPS was added to each well except for the control, achieving a final concentration of 1.0 μgmL^{-1}. Following 24 h cell culture, supernatants were withdrawn from each well and evaluated for the levels of PGE_2. Experiments were performed using a commercial enzyme immunoassay kit according to manufacturer's instructions.

In vivo evaluation of anti-inflammatory effects of the samples in zebrafish embryo model

Adult zebrafish were obtained from a commercial fish dealer. After acclimating the fish to laboratory environment under carefully monitored controlled conditions, embryos were obtained by inducing spawning in the morning by turning on the light. The collected embryos were sterilised by keeping immersed in a solution of methylene blue for 1 h. Embryos were then mounted in 12 well plates with 15 embryos per well in embryo media containing 0.002 % of 1-phenyl 2-thiourea. After 1 h, GEMH, GEMC and CAMH samples were added to each well in triplicate except for the controls, achieving final concentrations of 25, 50 and 100 μgmL^{-1}. After 1 h, LPS was introduced into each well except for the control. Embryo media were replaced after each 24 h until the 3rd day of postfertilisation (dpf). The hatched larvae, 4 from each group, were allocated into separate wells of 3 different 24 well plates. The well plates were separately stained with different fluorescence dyes, DAF-DM-DA, DCF-DA and acridine orange to analyse the inhibition of NO production, ROS production and cell death, respectively (Kang *et al*., 2013). Images were taken using a CoolSNAP-Pro colour digital camera (Olympus, Japan) connected to a fluorescence microscope. The fluorescence intensities were quantified by image J programme.

Statistical analysis

All data values are expressed as mean ± standard deviation based on at least 3 independent experiments. Statistical analysis for comparing the data was performed using IBM SPSS Statistics 20 software using one-way ANOVA by Duncan's multiple range test. P values less than 0.05 ($p < 0.05$) '*' and less than 0.001 ($p < 0.001$) '**' were considered as significant.

RESULTS AND DISCUSSION

Proximate composition

Proximate composition of the samples according to official methods of AOAC International (2005) are indicated in Table 1. The highest ash content was recorded in the sea grass *H. ovalis*, whereas the highest protein and carbohydrate contents were observed in the green alga *C. antennina*. The highest lipid content was observed in the red alga *G. edulis*.

Table 1: Proximate chemical composition of the algae

	Sample name	Moisture content (%)	Ash content (%)	Protein content (%)	Lipid content (%)	Carbohydrate content (%)
GE	*Gracilaria edulis*	2.61 ± 0.02	38.17 ± 0.49	7.56 ± 0.29	0.45 ± 0.07	49.15 ± 0.28
CA	*Chaetomorpha antennina*	3.56 ± 0.04	14.54 ± 0.02	12.3 ± 0.20	0.25 ± 0.05	67.71 ± 0.36
HO	*Halophila ovalis*	0.51 ± 0.04	43.2 ± 0.16	5.2 ± 0.31	0.37 ± 0.07	22.82 ± 0.66

Results represent means ± standard deviation of triplicate determinations

Table 2: Analysis of polyphenolic and sterol contents of the solvent fractions.

Sample	Polyphenolic content (%)	Sterol content (%)
GEMH	4.14 ± 0.51	40.79 ± 2.40
GEMC	6.46 ± 0.01	15.15 ± 3.10
GEME	7.86 ± 0.15	1.16 ± 0.80
GEMW	2.43 ± 0.11	0.20 ± 0.02
CAMH	4.84 ± 0.02	60.60 ± 3.66
CAMC	4.92 ± 0.16	40.79 ± 4.20
CAME	10.14 ± 0.02	4.66 ± 3.66
CAMW	4.31 ± 0.05	2.49 ± 0.50
HOMH	6.46 ± 0.24	75.07 ± 2.20
HOMC	10.8 ± 0.02	61.77 ± 1.50
HOME	14.05 ± 0.05	22.14 ± 1.60
HOMW	2.14 ± 0.02	2.33 ± 2.20

Results represent means ± standard deviation of triplicate determinations

Chemical analysis of the solvent fractions

Natural products such as polyphenols and sterols have been identified as possessing a broad range of beneficial biological functionalities (Fernando *et al*., 2016a). As shown in Table 2 the highest level of polyphenols (14.05 ± 0.05 %) was reported from the HOME solvent fraction. The highest level of sterols (75.07 ± 2.2 %) was reported from the HOMH fraction. From all solvent fractions, the hexane fraction of each sample had the highest level of sterols.

Anti-inflammatory activity inhibition of LPS induced NO production in RAW cells

A significant reduction of LPS induced NO production could be observed in GEMH, GEMC, CAMH, CAMC, HOMC and HOME samples (Figure 1). However, CAMC, CAME and HOMC appear to be cytotoxic to RAW 264.7 macrophages, and this could have affected

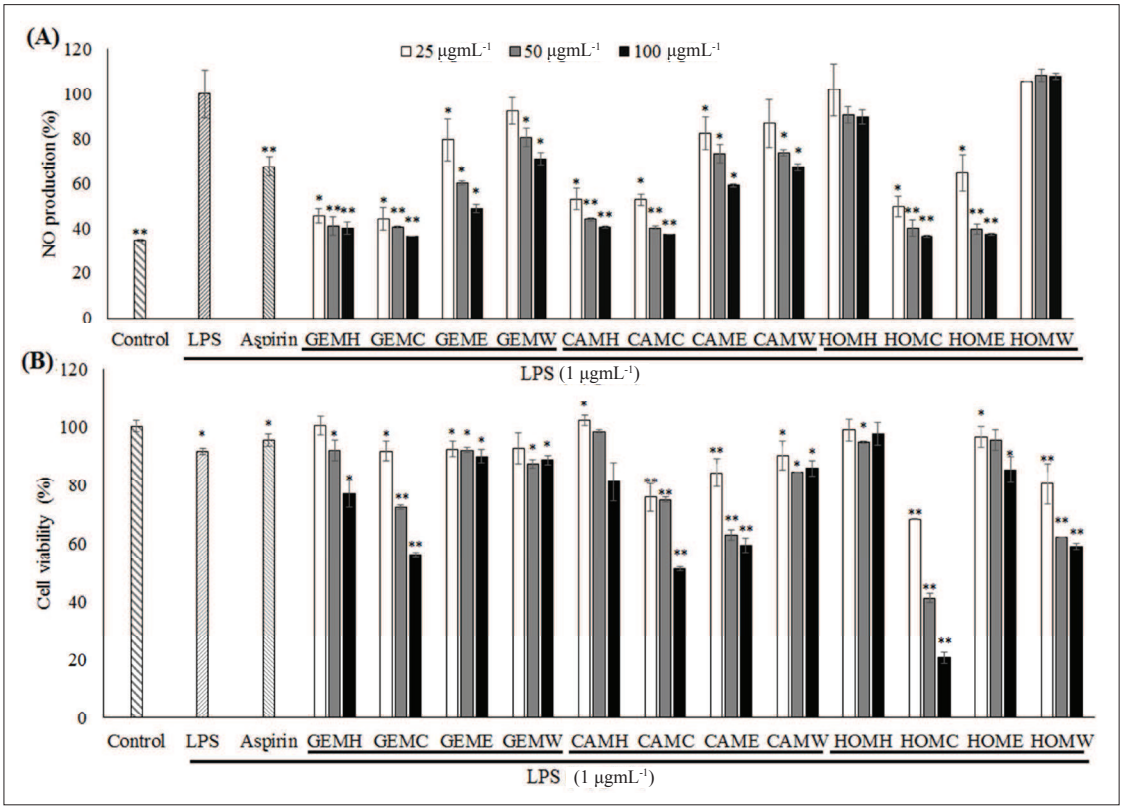

Figure 1: Inhibitory effects of the solvent fractions on LPS induced NO production in RAW 264.7 macrophages. Results represent the percentage (%) of NO production and viable cells 24 hours after the treatment of the solvent fractions. Results were obtained from three independent experiments and represented as means ± SD. With regard to NO production, * $p < 0.05$ and ** $p < 0.001$ were considered as significant compared to the positive control (LPS) and with regard to cell viability, * $p < 0.05$ and ** $p < 0.001$ were considered as significant compared to the control.

the observed reduction in NO production. Hence the fractions, which exhibited anti-inflammatory activity (GEMH, GEMC and CAMH) could be further studied for their anti-inflammatory potential. When considering the chemical composition of polyphenols and sterols the observed anti-inflammatory activity in GEMH and CAMH could most probably be due to sterols. However, such a claim cannot be made regarding GEMC.

Mediation of pro-inflammatory cytokines iNOS, COX-2 and PGE_2

Inflammatory responses are mediated through a complex system of signalling pathways. Pro-inflammatory cytokines such as iNOS, COX-2 and PGE_2 are three of the most important signalling molecules that regulate the production of NO and the formation of prostaglandins, which result in inflammation (Fernando *et al*., 2016b). Expression of these pro-inflammatory cytokines is associated with detrimental health issues, which cause inflammatory disease conditions that include tissue destruction, inflammatory bowel disease, rheumatoid arthritis, or graft-*vs*-host disease (Dinarello, 2000). After evaluating the inhibitory effects of GEMH, GEMC and CAMH on iNOS, COX-2 and PGE_2 protein expression in LPS induced RAW 264.7 macrophages, they were subjected to western blot analysis and ELISA analysis for detecting the levels of inflammatory mediators (iNOS, COX-2 and PGE_2). The expression of all studied cytokines was markedly increased in response to LPS stimulation (Figure 2). However, with increasing sample concentrations, all GEMH, GEMC and CAMH fractions effectively inhibited the production of cytokines in a dose-dependent manner. Densitometric analysis of the western blot protein bands revealed a dose-dependent decrease of iNOS and COX-2 inhibition (Figure 2B).

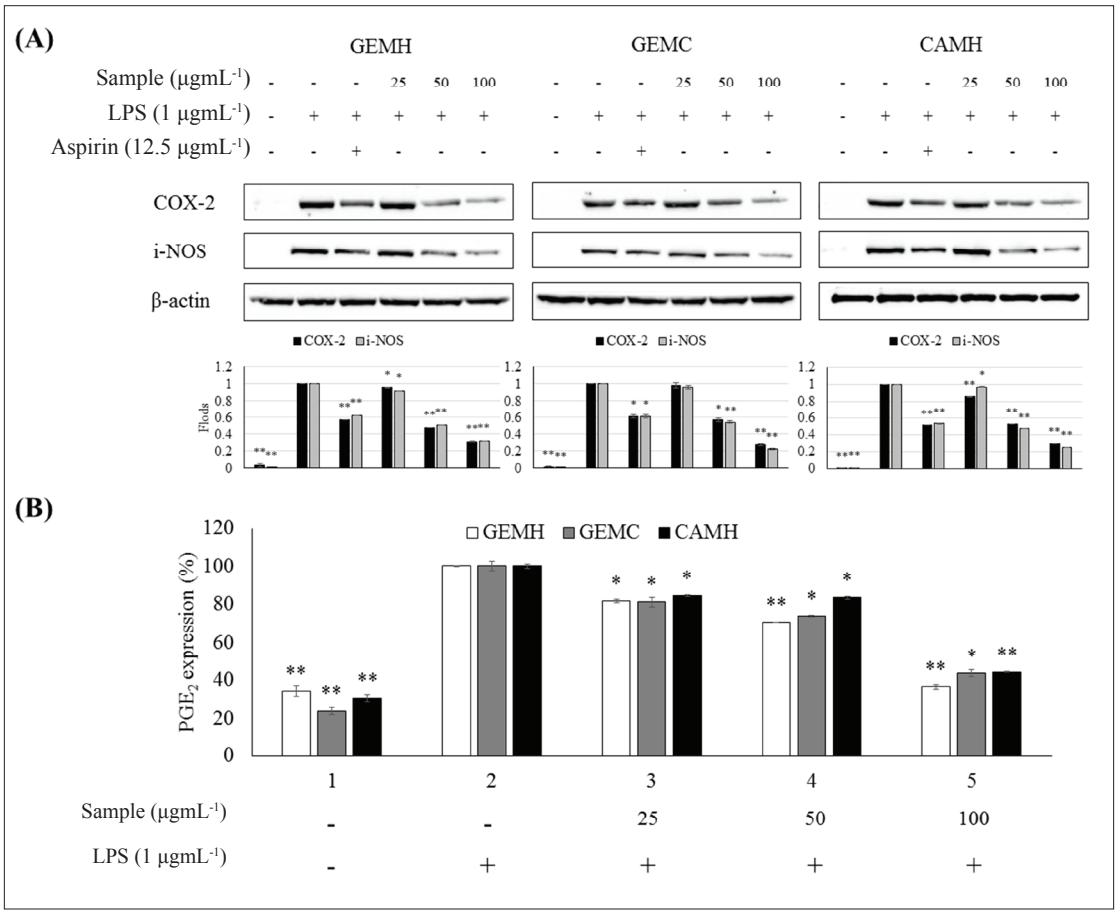

Figure 2: Inhibitory effects of the GEMH, GEMC and CAMH solvent fractions upon the expression of pro-inflammatory cytokines in LPS induced RAW 264.7 macrophages. (A) Western blot analysis of the expression of iNOS and COX-2; (B) percentage of PGE_2 production. Results were obtained by three independent experiments and represented as means ± SD. * $p < 0.05$ and ** $p < 0.001$ were considered as significant compared to the positive control (LPS).

Expression of COX-2 is in turn mediated by PGE_2 since the downregulation of PGE_2 has resulted in reduced expression of COX-2. These observations suggest that the reduction of NO production in RAW 264.7 macrophages could be due to the inhibition of iNOS expression. Further, they could inhibit the expression levels of PGE_2, in turn downregulating COX-2 and related inflammatory responses. Hence, all GEMH, GEMC and CAMH solvent fractions have the potential to mediate anti-inflammatory activity in LPS induced RAW 264.7 macrophages.

Inhibition of LPS induced NO production, ROS production and cell death in zebrafish embryo model

In vitro cellular anti-inflammatory responses on exposure to LPS could be insignificant when considering their effect on the whole organism. Therefore, evaluation of anti-inflammatory responses under *in vivo* conditions could provide a better understanding of the functionality of the studied samples. According to recent findings, mimicking human disease conditions in zebrafish models is gaining popularity over mouse model due to a number of desirable reasons, including the degree of similarity with human genome and organ system, short life span, handling simplicity and the ability for nonintrusive visualisation (Kim *et al*., 2016). As shown in Figure 3 relative fluorescence intensity was highest in LPS treated positive control group of the zebrafish model for all the NO production, ROS production and cell death. However, sample treatment with increasing concentrations reduced the relative fluorescence intensity levels similar to that of the control indicating profound anti-inflammatory responses of the samples. These results suggest that GEMH, GEMC and CAMH could contain biofunctional secondary metabolites with profound anti-inflammatory activity.

CONCLUSION

According to the findings of the present study ethyl acetate fraction of *Halophila ovalis* (HOME) demonstrated the highest anti-inflammatory activity although it appeared

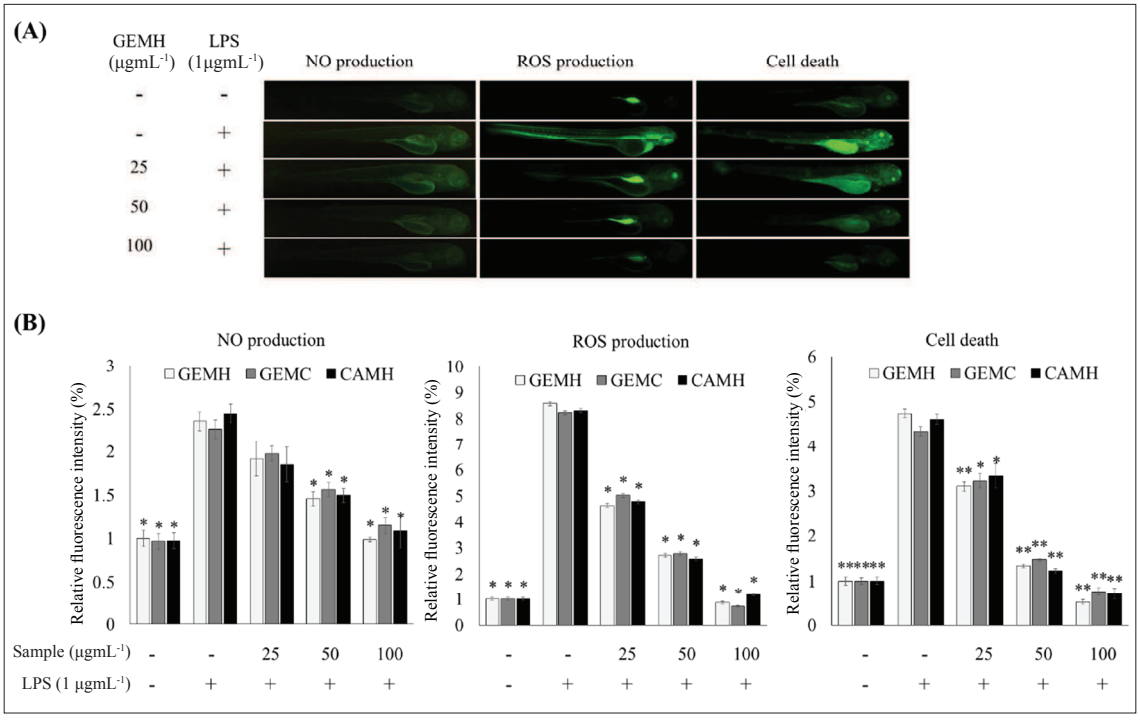

Figure 3: *In vivo* analysis of the inhibition of LPS induced NO production, ROS production and cell death in zebrafish embryo model. (A) The fluorescence microscopic images of the NO production, ROS production and cell death in LPS induced zebrafish after treatment with different concentrations of GEMH; (B) densitometry analysis of the fluorescence intensities corresponding to NO production, ROS production and cell death in LPS induced zebrafish after treatment of GEMH, GEMC and CAMH. Results were obtained by three independent experiments and represented as means ± SD. * $p < 0.05$ and ** $p < 0.001$ were considered as significant compared to the positive control (LPS).

to be cytotoxic to RAW 264.7 macrophages. In contrast, GEMH and GEMC exerted higher anti-inflammatory effects without significant toxicity, indicating an effective reduction of LPS induced NO production in RAW 264.7 macrophages without a considerable toxic effect. The anti-inflammatory effects of all these fractions appear to be mediated through the inhibition of iNOS and COX-2 expression.

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REFERENCES

1. AOAC International (2005). *Official Methods of Analysis of AOAC International*, 18th edition. AOAC International, Rockville, Maryland, USA.
2. Boopathy N.S. & Kathiresan K. (2010). Anticancer drugs from marine flora: an overview. *Journal of Oncology* **2010**: article ID 214186.
3. Chandler S.F. & Dodds J.H. (1983). The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasodine in callus cultures of *Solanum laciniatum*. *Plant Cell Reports* **2**: 205 − 208. DOI: https://doi.org/10.1007/BF00270105
4. Dias D.A., Urban S. & Roessner U. (2012). A historical overview of natural products in drug discovery. *Metabolites* **2**: 303 − 336. DOI: https://doi.org/10.3390/metabo2020303
5. Dinarello C.A. (2000). Proinflammatory cytokines. *Chest* **118**: 503 − 508. DOI: https://doi.org/10.1378/chest.118.2.503
6. Fernando I.P.S., Kim M., Son K.-T., Jeong Y. & Jeon Y.-J. (2016a). Antioxidant activity of marine algal polyphenolic compounds: a mechanistic approach. *Journal of Medicinal Food* **19**: 1 − 14. DOI: https://doi.org/10.1089/jmf.2016.3706
7. Fernando I.P.S., Nah J.-W. & Jeon Y.-J. (2016b). Potential anti-inflammatory natural products from marine algae. *Environmental Toxicology and Pharmacology* **48**: 22 − 30. DOI: https://doi.org/10.1016/j.etap.2016.09.023
8. Gul W. & Hamann M.T. (2005). Indole alkaloid marine natural products: an established source of cancer drug leads with considerable promise for the control of parasitic, neurological and other diseases. *Life Sciences* **78**: 442 − 453. DOI: https://doi.org/10.1016/j.lfs.2005.09.007
9. Holdt S. & Kraan S. (2011). Bioactive compounds in seaweed: functional food applications and legislation. *Journal of Applied Phycology* **23**: 543 − 597. DOI: https://doi.org/10.1007/s10811-010-9632-5
10. Kang M.-C., Cha S.H., Wijesinghe W.A.J.P., Kang S.-M., Lee S.-H., Kim E.-A., Song C.B. & Jeon Y.-J. (2013). Protective effect of marine algae phlorotannins against AAPH-induced oxidative stress in zebrafish embryo. *Food Chemistry* **138**: 950 − 955. DOI: https://doi.org/10.1016/j.foodchem.2012.11.005
11. Kim E.-A. *et al*. (13 authors) (2016). A marine algal polyphenol, dieckol, attenuates blood glucose levels by Akt pathway in alloxan induced hyperglycemia zebrafish model. *RSC Advances* **6**: 78570 − 78575. DOI: https://doi.org/10.1039/C6RA12724J
12. Ko S.-C. & Jeon Y.-J. (2015). Anti-inflammatory effect of enzymatic hydrolysates from *Styela clava* flesh tissue in lipopolysaccharide-stimulated RAW 264.7 macrophages and *in vivo* zebrafish model. *Nutrition Research and Practice* **9**: 219 − 226. DOI: https://doi.org/10.4162/nrp.2015.9.3.219
13. Lakmal C., Chaminda H., Samarakoon K.W., Lee W., Lee J.-H., Abeytunga D., Lee H.-S. & Jeon Y.-J. (2014). Anticancer and antioxidant effects of selected Sri Lankan marine algae. *Journal of the National Science Foundation of Sri Lanka* **42**: 315 − 323. DOI: https://doi.org/10.4038/jnsfsr.v42i4.7730
14. Newman D.J. & Cragg G.M. (2004). Marine natural products and related compounds in clinical and advanced preclinical trials. *Journal of Natural Products* **67**: 1216 − 1238. DOI: https://doi.org/10.1021/np040031y
15. Premakumara G.A.S., Ratnasooriya W.D. & Tillekeratne L.M.V. (1995). Studies on the post-coital contraceptive mechanisms of crude extract of Sri Lankan marine red algae, *Gelidiella acerosa*. *Contraception* **52**: 203 − 207.
16. Premakumara G.A.S., Ratnasooriya W.D. & Tillekeratne L.M.V. (1996). Isolation of a non-steroidal contragestative agent from Sri Lankan marine red alge, *Gelidiella acerosa*. *Contraception* **54**: 379 − 383.
17. Ratnasooriya W., Premakumara G. & Tillerkaratne L. (1994). Postcoital contraceptive activity of crude extracts of Srilankan marine red algae. *Contraception* **50**: 291 − 299.
18. Samarakoon K.W., Lee J.-H., De Silva E.D., Kim E.-A., Wijesundara R.L., Lakmal H.C. & Jeon Y.-J. (2013). Bioactivity evaluation of organic solvent extractions of *Ganoderma lucidum*: a Sri Lankan basidiomycete. *Journal of the National Science Foundation of Sri Lanka* **41**(3): 249 − 257. DOI: https://doi.org/10.4038/jnsfsr.v41i3.6049
19. Wijesekara I., Pangestuti R. & Kim S.-K. (2011). Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. *Carbohydrate Polymers* **84**: 14 − 21. DOI: https://doi.org/10.1016/j.carbpol.2010.10.062
20. Wijesinghe W., Kang M.-C., Lee W.-W., Lee H.-S., Kamada T., Vairappan C.S. & Jeon Y.-J. (2014). 5 [Beta]-Hydroxypalisadin B isolated from red alga *Laurencia snackeyi* attenuates inflammatory response in

lipopolysaccharide-stimulated RAW 264.7 macrophages. *Algae* **29**: 333.
21. Xiong Q., Wilson W. & Pang J. (2007). The Liebermann–Burchard reaction: sulfonation, desaturation, and rearrangment of cholesterol in acid. *Lipids* **42**: 87 − 96. DOI: https://doi.org/10.1007/s11745-006-3013-5
22. Zhou Z.-f. & Guo Y.-w. (2012). Bioactive natural products from Chinese marine flora and fauna. *Acta Pharmacologica Sinica* **33**: 1159 − 1169. DOI: https://doi.org/10.1038/aps.2012.110