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Research article

Anti-inflammatory and antioxidant properties of *Agelas sp.*, *Ircinia sp.*, and *Aaptos suberitoides*

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ABSTRACT

Reactive oxygen species (ROS) and cyclooxygenase-2 (COX-2) can be therapeutic targets for inflammatory diseases. COX-2 expression which can be triggered by ROS might consequently promote inflammation. Inhibition of ROS by the antioxidant, however, may prevent or decrease the expression of COX-2. In this study, the antioxidant and anti-inflammatory properties of the hexane (H), dichloromethane (DCM), and methanol-water (MW) extracts of marine sponges *Agelas* sp. (Asp), *Ircinia* sp. (Isp), and *Aaptos suberitoides* (As) were determined. The correlation between their antioxidant properties and ability to inhibit COX enzymes was also established. The MW extracts of the marine sponges and the AsDCM contain a significant ($p<0.05$) amount of phenolics. The same extracts exhibited potential antioxidant activity via reduction (79.65-85.76%). About 53.63–56.68% of the DPPH radicals were significantly ($p<0.05$) scavenged by the AspMW, IspMW and IspDCM. These extracts possess potential radical scavenging activity. The AspMW and the DCM and MW extracts of *Ircinia* sp. and *A. suberitoides* were COX-2 active and COX-2 selective. They inhibited >50% of the COX-2 and gave a selectivity index (COX-2/COX-1) >1.0. This suggests that the extracts can be sources of compounds with promising and selective inhibitory properties against COX-2. Correlation analysis further showed a positive linear correlation between the extracts' inhibitory activity against the COX enzymes and their TPC, reducing power, and DPPH radical scavenging activity. Thus, the antioxidant activity of the extracts may have influenced their anti-inflammatory property via COX inhibition.

Keywords: DPPH inhibition, Reducing power, Cyclooxygenase, COX selectivity index, Correlation.

INTRODUCTION

Inflammation is part of the body's protective mechanism against infection and injury. It is the process through which the immune system detects, recognizes, and eliminates damaging and foreign stimuli, thereby initiating the healing process. The biosynthesis of prostaglandins, which are the critical mediators of inflammation, is catalyzed by the cyclooxygenase (COX) enzymes. The COX enzyme comes in two isoforms: COX-1 and COX-2. COX-1, which is constitutively expressed, is believed to mediate housekeeping functions while COX-2, whose expression is induced, acts as a key player in initiating inflammatory responses.

The progression of inflammatory diseases is associated with the production of reactive oxygen species (ROS) [1]. ROS are partially reduced oxygen metabolites with significant oxidizing capabilities. They notably harm the cells at high concentrations. ROS exerts injurious effects on cells by oxidizing protein and lipid cellular constituents resulting in damage to the DNA [2]. Prolonged production of ROS is believed to be fundamental to the progression of inflammatory disease that may lead to chronic diseases.

The relationship between ROS and COX-2 expression in different cell types has been reported. ROS triggers COX-2 expression

via activation of the NF-κB and ERK1/2 during monocyte differentiation[3]. Similarly, ROS was observed to contribute to the inflammatory effect on the PDL (periodontal ligament) cells by inducing COX-2 expression through activation of the NF- κB signaling [4]. ROS may act as a signaling factor that triggers gene expression by activating oxidative-stress-responsive transcription factor NF- κB [5]. Inhibition of ROS by the antioxidant, however, may prevent or decrease the expression of COX-2 [6]. Thereby, ROS and COX-2 can be therapeutic targets for inflammatory diseases. Elucidation of their relationship can be undeniably helpful in developing effective treatments.

Over time, nature has proven to be an essential source of novel compounds with unique pharmacological properties that are safer. Marine sponges have increasingly attracted attention as potential sources of bioactive natural compounds with therapeutic value. Structurally diverse molecules have been isolated from marine sponges such as alkaloids, terpenoids, macrolides, peptides, and steroids with remarkable bioactivities including anti-inflammatory, antioxidant, antimicrobial, antifungal, antiviral, and anticancer, among others [7]. This study determined the antioxidant and anti-inflammatory properties of *Agelas* sp., *Ircinia* sp., and *Aaptos suberitoides*. Their inhibitory activity against COX enzymes and their COX-2 selectivity were assessed *in vitro*. Lastly, the correlation between their antioxidant properties and the ability to inhibit COX enzymes was also established.

MATERIALS AND METHODS

The marine sponges *Agelas* sp. (Asp), *Ircinia* sp. (Isp), and *Aaptos suberitoides* (As) were collected by scuba diving at a depth of 6-7 m in Iligan Bay, Kalangahan, Lugait, Misamis Oriental, Philippines. The samples were authenticated and identified by Prof. Angelo Responte and Prof. Sharon Rose M. Tabugo, marine biologists from the Department of Biological Sciences, College of Science and Mathematics, MSU-IIT, Iligan City, Philippines. Voucher specimens (KL23, KL26, and KL27) are kept at the Natural Products and Drug Discovery Laboratory, Premier Research Institute of Science and Mathematics (PRISM), MSU-IIT, Iligan City, Philippines.

The COX-2 and COX-1 enzymes were purchased from Sigma-Aldrich. The standards used, i.e. Indomethacin, gallic acid, and ascorbic acid, were obtained from Merck (Germany).

Sample Preparation and Extraction

The samples were washed, freeze-dried, powdered, and sequentially soaked in hexane, dichloromethane, and methanol: water (1:1 v/v) for 72 h in each extraction solvent. After sequential soaking, the mixture was filtered, and the filtrate was concentrated *in vacuo* and weighed to obtain the crude hexane (H), dichloromethane (DCM), and methanol: water (MW) extracts. The concentrated extracts were then stored at -20°C until further analysis.

Total Phenolic Content (TPC)

A modified 96-well microplate Folin-Ciocalteu method[8] was employed to determine TPC. In an Eppendorf tube, 300 uL of 1000 ug/mL test solution in methanol was added with 300 uL of 10% Folin-Ciocalteu reagent and 1.2 mL of 10% Na_2CO_3. The reaction mixture was, then, incubated in the dark for 90 min at room temperature (RT) and centrifuged at 11000 rpm for 3 min. A 200 uL of the mixture was placed into the wells and its absorbance was measured at 750 nm with a microplate reader (Thermo Scientific SkyHigh). The same procedure was performed for the blank (methanol). A calibration curve was prepared using gallic acid (0-40 ug/mL). The TPC of the samples, expressed as mg gallic acid equivalents per g extract (mg GAE/g), was then calculated using Equation 1

$$\text{TPC, mg GAE/g extract} = \frac{A}{B} \quad (1)$$

where: A = GAE of the test solution based on the equation of the calibration curve, mg GAE/mL

B = concentration of the test solution, g/mL

Antioxidant Assays

Reducing Power

A 200 uL of 0.2 M phosphate buffer (pH 6.6) and a 200 µL of 1% (w/v) potassium ferricyanide were added to 1 mL of test solution (1000 µg/mL). The mixture was incubated at 50°C for 30 min. After cooling to RT, 200 uL of 10% (w/v) trichloroacetic acid was added, and the mixture was centrifuged for 3 min at 11000 rpm. An aliquot (200 uL) was pipetted to the 96-well microplate and added with 20 uL of 0.1% (w/v) ferric chloride. The absorbance was then measured at 620 nm using a microplate reader (Thermo Scientific Multiskan Sky High). The ascorbic acid and methanol were used as positive and negative controls, respectively. The percent reducing power was calculated using Equation 2 [9].

$$\text{Reducing power,\%} = \frac{Absorbance_{blank} - Absorbance_{sample\ or standard}}{Absorbance_{blank}} \text{ x } 100 \quad (2)$$

DPPH Radical Scavenging Activity

A 1000 ug/mL test solution (in methanol) was prepared. A 150 uL of the test solution was dispensed into a 96-well microplate. The addition of 50 uL of 0.1 mM DPPH and incubation of the mixture in the dark at room temperature for 30 min followed. The absorbance of the mixture at 517 nm was recorded using a microplate reader (Thermo Scientific Multiskan SkyHigh). The ascorbic acid and methanol were used as positive and negative controls, respectively. The DPPH radical scavenging activity was calculated as percent DPPH inhibition using Equation 3 [10].

$$\text{DPPH inhibition,\%} = \frac{Absorbance_{blank} - Absorbance_{sample\ or\ standard}}{Absorbance_{blank}} \text{ x } 100 \quad (3)$$

Anti-inflammatory Assay

COX Inhibition Assay

The ability of the sponge extracts to inhibit COX-2 and COX-1 enzymes was determined based on the method of Opog et al.

(2019) [11]. Samples that gave ≥ 50% COX-2 inhibition and ≥ 1.00 COX-2/COX-1 ratio are considered COX-2 active and COX-2 selective. In a scintillation vial containing 5.184 mL of 100 mM Tris buffer (pH 8), 96 uL of 250 U/mL of COX-2 or COX-1 enzyme and 480 uL of 20 uM Hematin were added. This mixture constitutes the enzyme-cofactor solution. After dispensing 50 uL of the same buffer into each well, 120 uL of the enzyme-cofactor mixture was added. Then, 10 uL of 200 ug/mL of sponge extracts in dimethyl sulfoxide (DMSO) was added to make a final well concentration of 10 ug/mL. An 8 mM indomethacin in DMSO and 5% DMSO (final well concentration) were used as the positive and the negative control, respectively. Then, the mixture was incubated for 15 min at 25°C. A 10 uL of 200 uM Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) and 10 uL of 2000 uM arachidonic acid were added to each well. The reaction mixture was mixed and purged with N_2. The reaction was then monitored for 2 min using a microplate reader (Thermo Scientific Varioskan LUX) at an excitation wavelength of 535 nm and emission wavelength of 590 nm. The fluorescence intensity was measured at 12-sec intervals. The percent inhibition was determined based on the average slope of each replicate by using Equation 4.

$$\% \text{ Inhibitory Activity} = \frac{\text{Slope}_{\text{uninhibited}} - \text{Slope}_{\text{inhibited}}}{\text{Slope}_{\text{uninhibited}}} \times 100\% \quad (4)$$

where, $\text{Slope}_{\text{uninhibited}}$ is the slope of the line from the fluorescence vs. time plot of the negative control group and the $\text{Slope}_{\text{inhibited}}$ is the slope of the line from the fluorescence vs. time plot of the samples/positive control.

Statistical Analysis

Measurements were done in triplicate. Mean results were analyzed by One-Way Analysis of Variance (ANOVA). A post hoc Tukey's test at a 5% level of significance was used to compare the mean results. Pearson's correlation coefficient was calculated to determine the correlation between the antioxidant and anti-inflammatory properties of the extracts.

RESULTS AND DISCUSSION

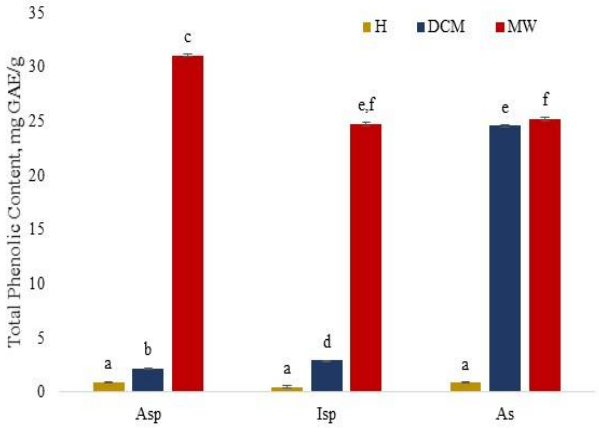
Total Phenolic Content (TPC)

The sponge extracts contain phenolic compounds (Figure 1). The phenolic compounds in marine organisms are responsible for their antimicrobial, anticancer, antiviral, antioxidant, and anti-inflammatory properties [12].

The H and DCM extracts generally gave low TPC values (0.90-2.91 mg GAE/g) while the MW extracts contained significantly high ($p<0.05$) amounts of phenolic compounds(25.21-31.08 mg GAE/g). The extractability of the phenolic compounds may account for this observation. The structural differences among the phenolic compounds affect their physical and chemical properties. Acidic phenols are soluble in more polar solvents like water. In comparison, the less acidic ones are generally soluble in less polar organic solvents such as aqueous alcohols, alcohols, or dichloromethane [13]. The results suggest that the phenolics produced by *Agelas* sp. and *Ircinia* sp. are mostly polar while those in *A. suberitoides* are both polar and less polar.

In recent years, phenolic compounds have gained interest for their pharmacological properties such as anti-inflammatory and antioxidant. Two species of Ircinia sponges, *I. spinulosa* and *I. oros*, were rich in flavonoids, tannins, and polyphenols [14]. Moreover, the detection of phenolic compounds in the methanolic extracts of *Agelas* and *Ircinia* sponges [15] and the chloroform extract of *A. suberitoides* have been reported [16].

Figure 1: Total phenolic content (mg GAE/g) of the sponge extracts. Means of the same letter superscript are statistically comparable based on Tukey's Test at a 0.05 level of significance. Error bars are SEM (n=3)



Antioxidant Activity

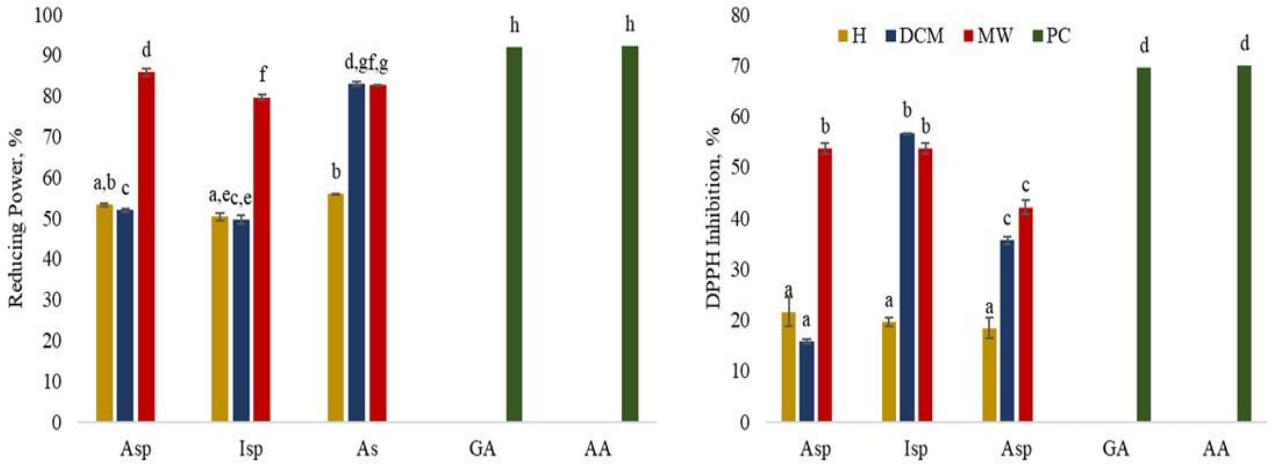
Reducing Power

The sponge extracts exhibited reducing power ranging from 49.67% to 85.76%. The AspMW and IspMW and the AsMW and AsDCM gave significantly higher ($p<0.05$) reducing power than the other extracts (Figure 2). Results imply that these extracts exhibited antioxidant activity via reduction or electron donation.

The phenolics in the sponge extracts (Figure 1) may account for their reducing power. The obtained trend in the TPC coincides with the results of the reducing power assay. The above-mentioned extracts with significantly high reducing potential also contain a high amount of total phenolics. The strong correlation between reducing power and total phenolic contents indicated that phenolic compounds contributed significantly to the antioxidant property [17]. The phenolic compounds' reducing power is found responsible for their antioxidant activity . The antioxidant activity of the phenolics may be attributed to their hydroxyl group/s which acts as electron donors [18] and has high redox potential [19].

A. *suberitoides* grown in Vietnam have shown the potential reducing power [16]. The aaptamines commonly biosynthesized by *Aaptos* species, were also known to exhibit antioxidant activity [20]. To our knowledge, however, this is the first report on the reducing power of *Agelas* sp. and *Ircinia* sp.

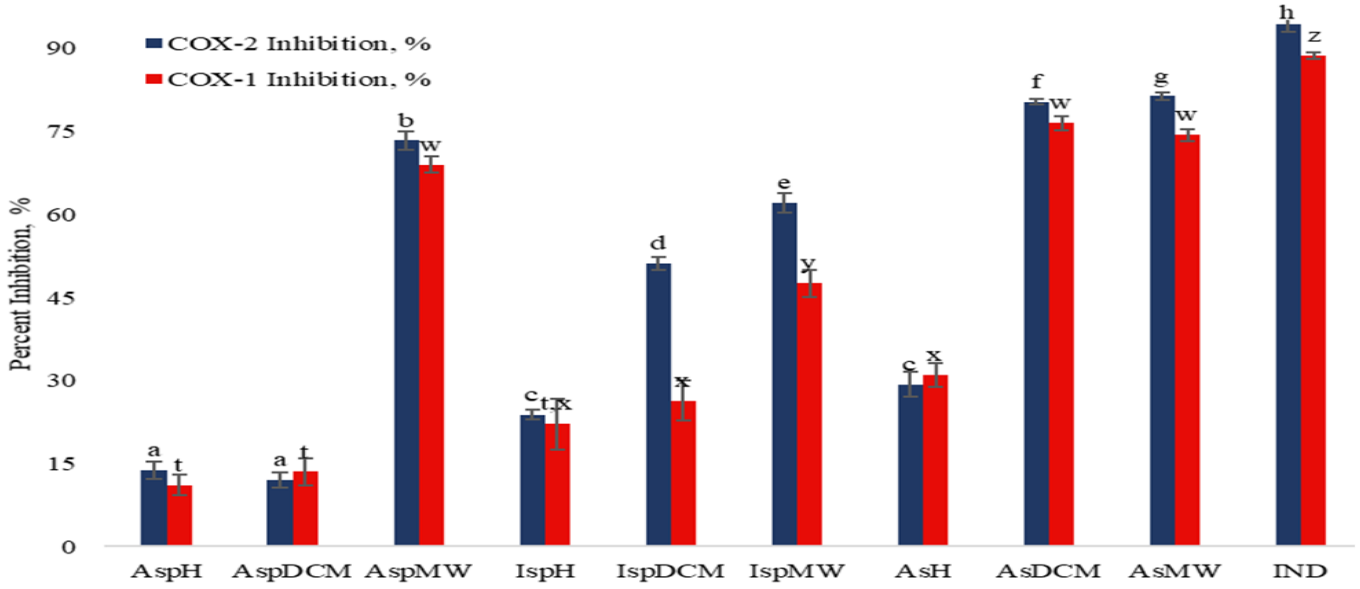
Figure 2. The antioxidant activity of sponge extracts in terms of % reducing power (left) and % DPPH inhibition (right). The positive control (PC) was gallic (GA) and ascorbic acid (AA). Means of the same letter superscript are statistically comparable based on Tukey's Test at 0.05 level of significance. Error bars are SEM (n=3).



DPPH Inhibition

The MW extracts of *Agelas* sp. and *Ircinia* sp. and the DCM extract of *Ircinia* sp. significantly scavenge 53.63% to 56.68% ($p<0.05$) of DPPH radicals (Figure 2). These extracts exhibited potential antioxidant activity via free radical scavenging. This radical scavenging activity may be attributed to the phenolic compounds in the extracts (Figure 1). By their hydrogen-donating ability, the phenolic compounds can exhibit potential free radical scavenging activity against DPPH radicals [19]. However, the high radical scavenging activity of IspDCM, despite its low TPC value, is worth noting. Antioxidants can be hydrophilic or lipophilic. Among lipophilic antioxidants, vitamin E has been found to show a potent radical-scavenging activity [21]. Thus, the exhibited activity of IspDCM may be due to the presence of lipophilic antioxidants. This finding is further supported by the result of a previous study where it was suggested that non-phenolic compounds are responsible for the observed antioxidant activity[22]. The methanolic extracts of *Agelas oroides*, *Ircinia fasciculata*, and *Ircinia spinusa* collected from the Mediterranean coast of Turkey have also shown the DPPH radical scavenging effect [23]. *Ircinia campana* gave promising results when tested for DPPH scavenging activity [24]. Lastly, *A. suberitoides* grown in Vietnam showed antioxidant activity in terms of DPPH scavenging, reducing power, and total antioxidant capacity [16].

Figure 3: The percent COX-2 and COX-1 inhibition and selectivity index (COX-2/COX-1) of sponge extracts. Means of the same letter superscript within the same target COX enzyme are statistically comparable based on Tukey's Test at a 0.05 level of significance. Error bars are SEM (n=4).



Selectivity index	1.24	0.89	1.06	1.07	1.94	1.31	0.95	1.05	1.09	1.06

Anti-inflammatory Activity: COX Inhibitory Activity

A wide array of compounds from marine sponges, like peptides, terpenoids, and bromo-indole alkaloids, were reported to exhibit anti-inflammatory properties [25].

COX enzymes play a significant role as essential mediators of inflammation. In this study, the inhibitory activity of the sponge extracts against COX-2 and COX-1 enzyme and their corresponding COX selectivity index was evaluated. Extracts that gave ≥ 50% COX-

2 inhibition and ≥1.00 COX-2/COX-1 ratio are considered COX-2 active and COX-2 selective. The extracts showed inhibitory activity against COX enzymes (Figure 3). The AspMW, IspDCM, IspMw, AsDCM and AspMW were COX-2 active. However, the AsDCM, AsMW, and AspMW showed significantly ($p<0.05$) higher inhibitory activity against the COX enzymes. Furthermore, it should be noted that MW extracts gave the highest percent COX inhibition within each sponge species than the H and DCM extracts. The result suggests that the anti-inflammatory compounds of the aqueous methanol extracts were more efficiently extracted by the methanol-water mixture than the other solvents.

The phenolic compounds in the extracts may account for their anti-inflammatory activity via COX inhibition. Although the precise mechanism of the phenolics' anti-inflammatory properties is not yet fully understood, it has been proposed that they exert anti-inflammatory activity by inhibiting the synthesis of pro-inflammatory mediators, inhibition of activated immune cells, or inhibition of nitric oxide synthase and COX-2 [26]. Other secondary metabolites produced by the studied sponges may also contribute to their exhibited bioactivity. A variabilin isomer mixture from *Ircinia felix* inhibited inflammation in a carrageenan-induced rat's paw edema [27]. On the other hand, agelasidine and several bromo-pyrrole alkaloids isolated from *Agelas* sponges have been reported to show anti-inflammatory activity [28]. For *A. suberitoides*, the ethanolic extract of *Aaptos* sp. was found to reduce the plasma interleukin (IL)-1β of the Wister rats [29]. The results of the present study showed the ability of the DCM and MW extracts of *A. suberitoides* to inhibit inflammation via COX-2 inhibition.

Using COX-2 selective drugs to target COX-2 while sparing the gastroprotective activity of COX-1 may lead to fewer toxic effects on the gastrointestinal tract [30]. From the obtained selectivity indices (COX-2/COX-1), the IspDCM, IspMW, AsDCM, and AspMW were COX-2 active and COX-2 selective (Figure 3). Compared to indomethacin, these extracts gave comparable or higher selectivity indices. This result indicates that the extracts may contain secondary metabolites with selective and potent inhibitory properties against COX-2.

Correlation of Antioxidant and Anti-inflammatory Properties

The analysis of the correlation between the antioxidant and the anti-inflammatory activity of the sponge extracts (Table 1) showed that their ability to inhibit COX-1 and COX-2 had a very strong positive correlation ($r > 0.80$) with TPC and reducing power. On the other hand, correlation analysis between COX-2 and DPPH inhibition indicated a strong positive relationship($r = 0.76$). Between COX-1 and DPPH inhibition, a moderate positive correlation ($r = 0.58$) was obtained.

Table 1. Correlation strength of the antioxidant and anti-inflammatory properties of the sponge extracts based on Pearson's correlation coefficient (r)

Variable	Pearson's Correlation Coefficient (Interpretation)*		
	TPC	Reducing Power	DPPH Inhibition Activity
COX-2 Inhibitory Activity	0.89 (positive; very strong)	0.88 (positive; very strong)	0.76 (positive; strong)
COX-1 Inhibitory Activity	0.92 (positive; very strong)	0.93 (positive; very strong)	0.58 (positive; moderate)

** descriptive interpretation of Pearson's correlation coefficient [31]*

Previous studies have found a linear, positive correlation between antioxidant and anti-inflammatory activities [32]. Our body constantly produces free radicals through physiological processes. Inflammatory response and a subsequent increase in the uptake of oxygen causes a respiratory burst resulting in the release of more free radicals like reactive oxygen species (ROS) [33]. Through the oxidative breakdown of vital cellular components, these reactive radicals and oxidants can harm cells directly and indirectly. It has become more apparent that ROS may initiate or amplify inflammation by upregulating distinct genes involved in the inflammatory response, such as COX-2 [34]. Upregulation of COX-2 and prostaglandin E2 expression has been found as a result of oxidative stress-induced phosphorylation of MAPKs and NF-kB through activation of TAK1 [34]. Nevertheless, COX-2 expression can be inhibited by antioxidant supplementation [34].

Phenolic compounds, known antioxidants, are present in the studied sponges (Figure 1). They can trap the free radicals directly or scavenge them through coupled reactions with antioxidant enzymes [35]. Phenolic compounds have potent DPPH radical scavenging and reducing power capability [36]. The phenolics in the sponge extracts may account for their anti-inflammatory activity. With a potent reducing power and free radical scavenging activity, they can reduce oxidative damage and suppress inflammation by inhibiting pro-inflammatory mediators [37]. Quantitative structure-activity relationship (QSAR) studies have also established the relationship between cyclooxygenase activity inhibition and phenolic compounds [38]. The flavonoids, a known class of phenolics, have also been reported to exhibit anti-inflammatory activity by inhibiting eicosanoid-generating enzymes like phospholipase A2, cyclooxygenases, and lipoxygenases [39]. Thus, the phenolic compounds in the extracts, their reducing power, and DPPH radical scavenging activity influenced their anti-inflammatory activity via COX inhibition.

CONCLUSION

The phenolic compounds in the AsMW, IspMW, AsDCM, IspDCM, and AspMW may account for their significant antioxidant activity by scavenging DPPH free radicals and exhibiting reducing power. The extracts also showed potential anti-inflammatory activity by inhibiting >50% of the COX-2 and giving a selectivity index (COX-2/COX-1) >1.0. Furthermore, there is a positive linear correlation

between antioxidant activities and the marine sponge extracts' anti-inflammatory activities. Thus, *A. suberitoides*, *Ircinia* sp., and *Agelas* sp. can be sources of lead compounds with promising antioxidant and COX-2 inhibitory activity and COX-2 selectivity. However, further studies are recommended to identify the compounds and mechanisms responsible for their antioxidant and anti-inflammatory activities.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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