Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Document heading doi: 10.12980/JCLM.3.201514B343 © 2015 by the Journal of Coastal Life Medicine. All rights reserved.

Bioactive profile of *Plakortis nigra*, a sea sponge from Mauritius Islands

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PEER REVIEW

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Comments

This is an interesting study in which the authors provided novel insight into the potentially important metabolites of a Mauritian sea sponge. Data in this paper appear novel and it is worth publishing.
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ABSTRACT

Objective: To investigate the *in vitro* antibacterial and antioxidant activities of crude and fractionated extracts of the *Plakortis nigra* (*P. nigra*) sea sponge from Mauritius sea waters.

Methods: Preliminary qualitative chemical screening of the sponge extracts was conducted by using standard methods while the total phenolic content (TPC) was estimated through the Folin-Ciocalteu method. Antibacterial activity was evaluated against *Staphylococcus aureus*, *Escherichia coli* and *Proteus mirabilis*. The minimum inhibitory concentration was determined by the broth microdilution method. All sponge extracts were assessed for antioxidant activity via the 1,1-diphenyl-2-picrylhydrazyl free radical scavenging *in vitro* model.

Results: Alkaloids, phenols, steroids, terpenoids, tannins and saponins were detected in the sponge extracts and TPC varied from (2.280±0.072) mg to (12.790±0.236) mg gallic acid equivalents per gram extract (P<0.05). All the extracts inhibited the growth of at least two bacterial strains whilst the most potent *in vitro* antibacterial activities were observed in the most polar ethyl acetate and butanol fractions (minimum inhibitory concentration values 0.103–0.211 mg/mL) of *P. nigra*. Each extract scavenged 1,1-diphenyl-2-picrylhydrazyl free radicals while the hexane fraction displayed the highest scavenging ability at (27.50±1.85)% (P<0.05). Antioxidant activity was positively correlated with TPC (R^2=0.843). Contrary relationships were also found between antibacterial activity and TPC.

Conclusions: The present study validates the antioxidant and antibacterial activity of marine sponge *(P. nigra)* extracts and depicts the sea sponge as a potential source of pharmaceutical leads against infectious and degenerative diseases.

KEYWORDS

Plakortis nigra, Antibacterial, Antioxidant, DPPH, Phenolics

1. Introduction

In the last several decades, research has expanded from land to ocean, which offers immense potential for biological and chemical diversity, in order to find new leads for drug candidates[1]. Sponges, principally demosponges, distinguish themselves by producing the greatest panoply of bioactive secondary metabolites from any animal group[2,3], which are of potential pharmaceutical importance[4,5].

Antioxidants are free radical scavengers, which can provide protection to living organisms from damage caused by uncontrolled production of reactive oxygen species (ROS), and have widespread applications in medicine, cosmetics and food industries. There is a need for identifying natural antioxidants due to emerging concerns about safety of synthetic preservatives[6]. Avarol, a well-studied

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Article history:
Received 8 Aug 2014
Received in revised form 9 Sep 2014
Accepted 23 Oct 2014
Available online 29 Oct 2014

antioxidant from sponges, ignited the growing interest in natural antioxidants of marine origin[7-15].

Microbial infections are still the major cause of mortality all over the world. As the infectious microorganisms evolve and develop resistance to existing pharmaceuticals, the marine sponge may provide novel leads against bacterial, viral, fungal and parasitic diseases. Psammaplin A, responsible for antibacterial activity, has been selected as a promising lead for preclinical assessment[16]. Recent studies have been conducted in the hunt for new antimicrobial substances of sponge origin[8,17-26].

Plakortis nigra (*P. nigra*) is a demosponge belonging to the Plakinidae family and 25 members of *Plakortis* species, occurring worldwide, are known to date[27]. The latter are known to produce a wide variety of bioactive compounds (antiviral, antibacterial, antimalarial and anticancer) and have yielded a number of biosynthetically diverse natural products[28-32]. In fact, the *Plakortis* genus is advertised as one of the most prolific products with respect to new marine natural compounds from 1900 to 2009, accounting for approximately 4% of all Porifera new marine natural products[3].

Mauritius is a small island (1 865 km^2) in the Indian Ocean possessing a total exclusive economic zone area of 2.3 million km^2. Such a vast maritime territory represents a niche for marine bioprospecting. Literature reveals few studies, describing biological activities of pharmacological, interest in Mauritian sponges[33-38], hence, highlighting the urgent need to put the Mauritian waters on the marine natural products research agenda. In this respect, the present study aimed at evaluating the *in vitro* antibacterial and antioxidant activities of extracted *P. nigra* secondary metabolites, so as to ultimately assess the marine sponge's pharmaceutical potential.

2. Materials and methods

2.1. Sponge collection

Samples (300 g wet weight) of *P. nigra* were collected from the northern coastal waters of Mauritius (Trou aux Biches, September 2011) via self-contained underwater breathing apparatus diving at a depth of 10-15 m. A fresh sample was deposited at the Mauritius Oceanography Institute while a sample of the specimen was sent to the Museum of the University of Amsterdam for taxonomic identification. A voucher specimen was deposited under the accession number ZMAPOR 18310. The freshly collected sponges were cleaned and stored at -70 ˚C prior to lyophilisation, using a laboratory freeze drier (Labconco), and extraction.

2.2. Extraction

Freeze-dried samples of *P. nigra* (100 g dry weight) were macerated with methanol/dichloromethane (1:1 v/v) for 96 h in the dark at ambient temperature, and the filtrates were concentrated *in vacuo* at a maximum temperature of 40 ˚C, using a rotary evaporator (Heidolph Laborota) to yield the crude extract. Thereafter, distilled water (100 mL) was added to the crude extract and the aqueous suspension was successively partitioned with different solvents in increasing order of polarity: hexane (6×200 mL), ethyl acetate (EtOAc) (5×200 mL) and butan-1-ol (BuOH) (4×200 mL), to afford the corresponding fractions, which were subsequently concentrated *in vacuo*. Methanol (MeOH) was added to the concentrated butanol extract, in order to remove any sea salt present, and the extract was ultimately concentrated *in vacuo*.

2.3. Chemical screening

Standard qualitative chemical screening tests[39] were performed on sponge extracts to detect the presence of major natural chemical groups, such as steroids, terpenes, alkaloids, phenols, tannins, coumarins, anthraquinones, leucoanthocyanins and flavonols.

The froth test was performed on freeze-dried sponge material to detect any presence of saponins. A total of 0.5 g of freeze-dried sponge material was treated with water for 5 min at 100 ˚C in a test tube, allowed to cool and shaken vigorously, then the formation of persistent froth (1-2 mL) was observed.

2.4. Determination of total phenolic content (TPC)

The Folin-Ciocalteu procedure[40], with some modifications, was employed to estimate the TPC of the sponge extracts. In capped test tubes, 200 µL aliquots of standard solutions of gallic acid in MeOH (0, 50, 100, 150, 200 and 250 µg/mL), were mixed with 2.0 mL Folin-Ciocalteu reagent (diluted 10-fold with deionised water). After 6 min, 0.8 mL of sodium carbonate (7.5%) was added to neutralise the reaction, and the tubes vortexed. The mixture was allowed to stand for 1 h at room temperature in the dark and absorbance at 760 nm was recorded, against a MeOH blank, using a spectrophotometer (Milton Roy Spectronic 1001 Plus UV-visible). A calibration curve was plotted using the data. Each of the sponge extracts (200 µL) was treated in the same manner. The results were expressed as means of triplicate analyses in milligram gallic acid equivalents (GAE) per gram extract (mg GAE/g).

2.5. 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

The antioxidant activity of sponge extracts was assessed using an altered DPPH free radical scavenging *in vitro* model[41]. In capped test tubes, 1 mL of each sponge extract was added to 3 mL of 0.3 mmol/L methanolic solution of DPPH (HiMedia). The tubes were vortexed and allowed to stand for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and a control reading

was obtained using MeOH. Quercetin served as the positive control. The results were expressed as means of triplicate analyses. The percentage of DPPH radical scavenging activity was calculated with the following equation:

$\%\ \text{Inhibition}=[(A_0-A_1)/A_0]\times 100$

Where A_0 is the absorbance of the control; A_1 is the absorbance of test samples.

2.6. Antibacterial assay

Minimum inhibitory concentrations (MICs) of the crude and fractionated extracts were determined using the Mueller-Hinton broth microdilution method as previously described[42]. The reference strains of human pathogens used to test for antibacterial activity included a Gram-positive bacterium, *Staphylococcus aureus* ATCC 25313 (*S. aureus*) and two Gram-negative bacteria, *Escherichia coli* ATCC 25922 (*E. coli*) and *Proteus mirabilis* ATCC 12453 *(P. mirabilis)*. Overnight bacterial cultures were standardised with sterile Mueller-Hinton broth (HiMedia) to achieve an absorbance of 0.4-0.6 at 600 nm. Each test sample (100 µL) was 2-fold serially diluted with sterile distilled water (100 µL) in U-bottom sterile 96-well microtitre plates and bacterial suspension (100 µL) added to each well. Chloramphenicol (Sigma) (0.01 mg/mL) was used as a positive control while MeOH was used as a negative control. Following overnight incubation at 37 ˚C, the MIC, the lowest concentration at which bacterial growth inhibition occurred, was determined by an indicator dye. Ergo, 50 µL of *p*-iodonitrotetrazolium violet (INT) (0.2 mg/mL) was added to wells preceding incubation at 37 ˚C for 30 min; clear wells indicated growth inhibition. All measurements of MIC values were repeated in triplicates.

2.7. Statistical analysis

The experimental results were expressed as means±SD. All measurements were replicated three times (n=3). Statistical analysis was performed using Minitab software version 16.1.0 for Windows at a 5% significance level. One-way analysis of variance (ANOVA, Fisher's test) was carried out to test for any significant differences among the TPCs of different extracts and the DPPH scavenging activities of different extracts. Significant difference was statistically considered at level of P<0.05. Linear regression analysis was used to calculate the standard curve of gallic acid and correlations (Microsoft Excel 14.0 software).

3. Results

3.1. Chemical screening

The detected secondary metabolites are summarized in Table 1. Condensed tannins were present in considerable amounts in the hexane and EtOAc fractions of *P. nigra* extracts. Small amounts of coumarins were found only in the crude extract and EtOAc fraction. Globally, the presence of alkaloids, phenols and steroids/terpenes in different proportions were detected in the extracts. Eventually, the *P. nigra* was found to contain saponins.

Table 1

Chemical constituents of the *P. nigra*.

Chemical group	Test sample				
	Crude extract	Hexane fraction	EtOAc fraction	BuOH fraction	Freeze dried
Sponge					
Alkaloids	+	++	++	+	NT
Anthraquinones	-	-	-	-	NT
Coumarins	+	-	+	-	NT
Leucoanthocyanins	-	-	-	-	NT
Flavonols	-	-	-	-	NT
Phenols	+	++	++	+	NT
Saponins	NT	NT	NT	NT	++
Steroids/Terpenes	+	++	++	-	NT
Tannins	+	++	++	+	NT

+: Present in trace amount; ++: Present; -: Absent; NT: Not tested.

3.2. TPC

The gallic acid standard curve was established by plotting concentration versus absorbance (y=0.008 1x, R^2=0.991 3), where y is absorbance and x is concentration (Figure 1). The sponge extracts contained levels of total phenolics, ranging from 2.28 mg GAE/g extract to 12.79 mg GAE/g extract. The hexane fraction contained the most phenolics with a value of (12.790±0.236) mg GAE/g extract, followed by the crude extract (10.670±0.345) mg GAE/g extract). Meanwhile, the EtOAc and BuOH fractions contained the least phenolics with (4.04±0.29) mg GAE/g extract and (2.280±0.072) mg GAE/g extract respectively.

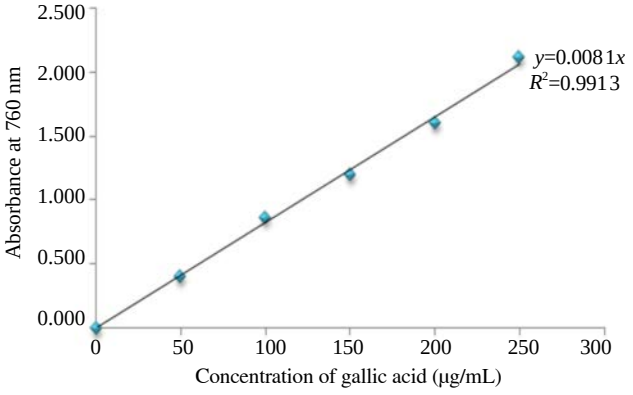

Figure 1. Standard curve for gallic acid.

3.3. Antioxidant activity

High antiradical activity (>95%) was recorded for the positive control, quercetin. The least polar hexane fraction displayed the highest free

radical scavenging activity at (27.50±1.85)%, followed by the crude extract (16.60±1.05)%, while the polar EtOAc and BuOH fractions exhibited lower free radical scavenging activity at (12.90±2.92)% and (4.170±0.444)% respectively.

3.4. Antibacterial activity

The EtOAc and BuOH fractions of *P. nigra* provided the lowest MIC values ranging from 0.103 mg/mL to 0.211 mg/mL and performed better than the reference antibiotic chloramphenicol against all pathogens (Table 2).

Table 2
MIC of *P. nigra* extracts and positive control against test organisms.

Sample	MIC against test organisms (mg/mL)		
	E. coli	*S. aureus*	*P. mirabilis*
Crude extract	2.460	2.460	ND
Hexane fraction	1.280	1.280	1.280
EtOAc fraction	0.103	0.206	0.206
BuOH fraction	0.106	0.211	0.211
Chloramphenicol	0.625	0.313	1.250

n=3. ND: No antibacterial activity detected at highest concentration of sample; Chloramphenicol: Reference antibiotic used as positive control.

3.5. Correlation between antioxidant activity and TPC

An obvious correlation (r=0.918, R^2=0.843) was noted between *in vitro* antioxidant activity and TPC of the sponge extracts (Figure 2). It revealed that *in vitro* antibacterial activity was strongly positively associated to the TPC.

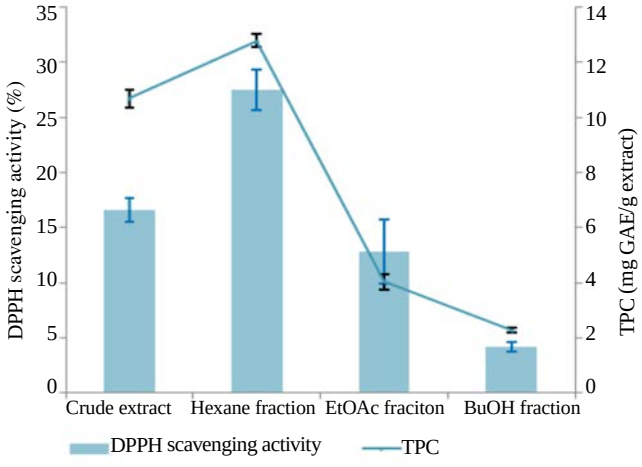

Figure 2. Relationship between antioxidant activity and TPC of *P. nigra* extracts (bars represent SD).

3.6. Correlation between antibacterial activity and TPC

The investigated correlations between MICs and TPCs of the sponge extracts, when tested against *E. coli* (r=0.809, R^2=0.654), *S. aureus* (r=0.795, R^2=0.632) and *P. mirabilis* (r=0.987, R^2=0.974), revealed that the *in vitro* antibacterial activity was strongly inversely associated to the TPC (Figure 3).

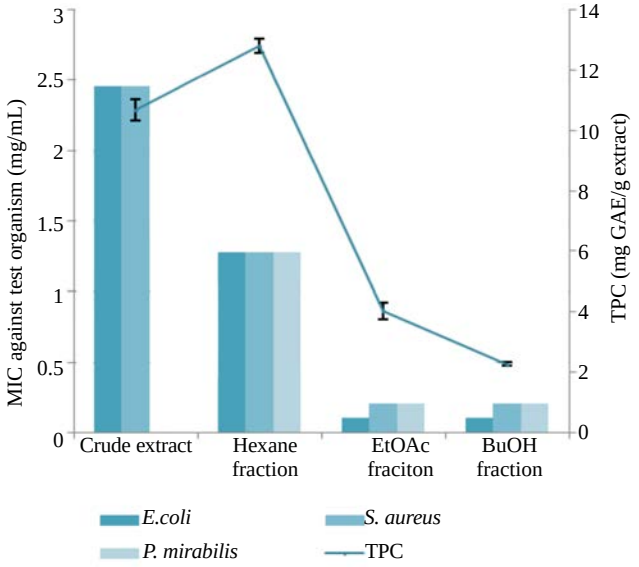

Figure 3. Relationship between antibacterial activity and TPC of *P. nigra* extracts (bars represent SD).

4. Discussion

4.1. Chemical screening

Through preliminary chemical screening, *P. nigra* was revealed as a source of bioactive components, namely alkaloids, coumarins, phenols, tannins, steroids, terpenoids and saponins. Sponges are assumed to produce secondary metabolites to compete for space with other organisms, prevent fouling by other organisms and keep predators away[43]. Consequently, the flourishing biodiversity of the Mauritian lagoons and the competitive environment of the latter may account for the production of an arsenal of secondary metabolites in the *P. nigra* currently under study. Likewise, a sheet coral was reported to be susceptible to allelochemicals released by its neighbour, the *Plakortis halichondroides*[44]. The Mauritian *P. nigra* under investigation might have accumulated chemicals in an attempt to maximise its space-capture abilities.

The presence of alkaloids in the probed *P. nigra* was foreseen since perusal of the literature revealed that plakinidines, most cytotoxic alkaloids, have been isolated from sponges of the Plakinidae family in Ireland, Fiji and Vanuatu, while plakinamines A and B, steroidal alkaloids, were isolated from Micronesian *Plakina* sp.[12,30,45]. The presence of phenols in the *P. nigra* is in keeping with sponges being capable of synthesising phenolics, such as quinones, hydroquinones, and halogenated phenols[45]. Furthermore, the unique monohydric phenol plakinidone was isolated from a member of the genus, to which the *P. nigra* belongs[46]. Also, our findings corroborate the lately reported occurrence of phenols and tannins in Mauritian *Stylissa* sp. and *Biemna tubulosa* extracts of different polarities[38]. Depending on the polarity of a solvent system used during extraction, a mixture of phenolics will be extracted from materials and polar solvents have been reported to extract polyphenolics[47,48].

Although the presence of saponins in sponges was once considered as an infrequent occurrence[49], the present study has revealed the *P. nigra* as a source of such metabolites. This sustains recent findings whereby acanthifoliosides, novel steroidal saponins, were obtained from the *Pandaros acanthifolium* sponges belonging to the same class as the *P. nigra*[50]. Besides, the presence of steroids or terpenoids in the crude, hexane and ethyl acetate extracts of the *P. nigra* is in line with the preceding claims about the widespread incidence of terpenoids in sponges[45]. Alkaloids, steroids, terpenoids, phenols and saponins present in marine sponges, have been associated with various bioactivities, among which antibacterial and antioxidant attributes[2,13,45,49-53].

4.2. TPC

In our study, the range of TPC of different *P. nigra* extracts varied (2.28-12.79 mg GAE/g extract). The hexane fraction contained the highest amount of phenolic compounds [(12.790±0.236) mg GAE/g extract] and the BuOH fraction contained the least amount of phenolics [(2.280±0.072) GAE/g extract).

4.3. Antioxidant activity

Antioxidants are free-radical scavengers which can provide protection to living organisms from damage caused by uncontrolled production of reactive oxygen species. There is a need for identifying natural antioxidants having less or no side effects since commonly used as synthetic antioxidants like butylated hydroxyanisole and butylated hydroxytoluene, may cause liver damage and carcinogenesis[41].

The free radical scavenging efficiency of the *P. nigra* extracts was (27.50±1.85)% for the hexane fraction, (16.60±1.05)% for the crude extract, (12.90±2.92)% for the EtOAc fraction and (4.170±0.444)% for the BuOH fraction. Hence, none of the expressed bioactivity was better than that of quercetin, the positive control (>95%). This concurs with past findings whereby the sponge *Dendrilla nigras* lowered antiradical activity (50.83%) as compared to the standard butylated hydroxytoluene (100.00%) when investigated through the DPPH model[8]. Likewise, metabolites from the marine sponge *Psammocinia* sp. were reported as being weak in scavenging the stable DPPH free radical[54].

While potent antioxidants, including avarol, have been revealed in sponges[9], this investigation portrayed the *P. nigra* extracts moderate ability to scavenge free radicals. Still, a significant positive correlation was observed between the antioxidant activity and TPC of the extracts (P<0.05) through the linear regression obtained (R^2=0.843), pointing at the detected phenolics as antioxidant activity promoters. Therefore, the hexane extract, with the highest TPC displayed the highest free radical scavenging activity, and the butanol fraction, with the lowest TPC furnished the least antioxidant effect. Antioxidant defenses are essential in diverse tropical marine taxa to maintain the steady-state concentration of ROS at low levels in order to prevent subsequent oxidative damage[55]. Factors, such as exposure to moderate temperatures, low irradiance and absence of photosymbionts, have been previously hypothesized to modulate low antioxidant response in sea sponges[10,56,57] and may further account for the low ability of the *P. nigra* extracts to trap free radicals.

4.4. Antibacterial activity

The resistance of several human pathogens to antibiotics has geared researchers towards marine organisms for the development of new antibiotics[16]. The bioassay revealed the *P. nigra* extracts' ability to inhibit equally the Gram-positive bacterium and Gram-negative bacteria. Emphasis was previously laid on sponges' ethyl acetate extracts superior antibacterial activity as compared to aqueous extracts[25]. The EtOAc and BuOH fractions were the most efficient bacterial growth inhibitors, when tested against *S. aureus*, *E. coli*, *P. mirabilis*, with MICs lower than the reference antibiotic chloramphenicol, hence qualifying *P. nigra* as a potential source of novel substances for future drug discovery. Additionally, the MICs of ethyl acetate and methanolic extracts of *Gelliodes* sp. against *S. aureus* were previously reported at 500 and 250 μg/mL respectively[22]. In comparison, the polar EtOAc and BuOH extracts of the *P. nigra* performed better against the Gram-positive bacterial strain with MIC values of 206 and 211 μg/mL respectively. This is most promising when considering the havoc caused by *S. aureus*, which is responsible for half of the hospital-associated infections[58].

The bioactive compounds detected in the *P. nigra* extracts, namely alkaloids, terpenes and tannins, may be actual contributors to the descried significant antimicrobial activities. Steroidal alkaloids from *Plakina* sp., of the same family as the *P. nigra*, inhibited the growth of *S. aureus*. *Agelas* species from the Pacific and the Caribbean have provided diterpenoids which demonstrated antimicrobial activity against *Bacillus subtilis*, *S. aureus* and *Candida albicans*. Two diterpenoids, exhibiting antimicrobial activity, were obtained from the Okinawan sponge *Agelas nakamurai* while *Luffariella variabilis* has furnished four sesterterpenoid antibiotics[45]. Also this study has disclosed a reverse relationship between antibacterial activity and TPC in *P. nigra* extracts. Hence, the fractions with the least TPCs (EtOAc and BuOH), were the most efficient bacterial growth inhibitors. The detected presence of other chemical components in these fractions may indicate a possible synergy. This phenomenon is characterised as the effect of two or more components, applied together, being greater than the effect when each constituent are used separately[59]. Chemically defended organisms produce multiple secondary metabolites, opening up the possibility of synergistic or additive effects among various metabolites[60]. So, a positive correlation could exist between antibacterial activity and the diverse secondary metabolites detected within sponge extracts in this investigation.

This work constitutes the first report revealing the antibacterial and antioxidant activities of the marine sponge *P. nigra*, collected from the coastline of Mauritius. The *P. nigra* was coined as a source of bioactive metabolites, among which saponins are medicinally important metabolites. While displaying moderate antioxidant activity, the investigated sponge was most importantly revealed as a potential source of natural antioxidants. Via this investigation, the *P.*

nigra made an outright demonstration of broad spectrum antibacterial activity through its polar ethyl acetate and butanol extracts, associated to metabolites detected therein and which could lead to the discovery of new chemical classes of antibiotic substances in the future. Phenolics were putatively identified as major contributors to bioactivity following the confirmation of considerable positive correlation between detected *in vitro* antioxidant activity and TPCs of extracts. This study is of considerable importance since it highlights the Mauritian *P. nigra* as a candidate for future research work on natural antioxidants and antibacterial agents. Most importantly, this work conveys further information about the unexploited treasures of the Mauritian lagoons which, considering the emergence of resistant bacteria and side effects engendered by synthetic antioxidative molecules, embody a niche for lead discovery.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are grateful to the technical staff of the Faculty of Agriculture, especially Mr. Junhye L and Mrs. Sobhee A of the Biotechnology Laboratory, for their kind cooperation. The authors are thankful to Mr. Ramanjooloo A, Associate Research Scientist at the Mauritius Oceanography Institute, for his invaluable assistance during the extraction procedures and to Dr. Van Soest, Head Curator of the Zoology Museum, University ofAmsterdam, Netherlands, for the identification of the marine sponge.

Comments

Background

Due to the emergence of multi-drug resistant bacteria and the exhaustion of terrestrial sources of antibiotics, this paper highlights the need and convenience of using marine animals, specifically *P. nigra*, as potential bioactive-rich sources. Notably, little research has been conducted in this area of marine bio-prospecting in and around Mauritius.

Research frontiers

The presented work details crude and basic metabolite, mainly phenolic, profiles of the chosen marine sponge. The authors have identified some of the components to trace level. Three different solvents have been used and resulted in different amounts of phenols obtained. Extracts demonstrated some anti-oxidant activity and antibacterial activity.

Related reports

The authors have included a number of related publications that have conducted similar work but in different species of sponges and other marine fauna. Little literature appears available for sponge-derived saponins, to which the authors' declare is rare.

Innovations and breakthroughs

This manuscript provides novel insight into the potentially important metabolites of a Mauritian sea sponge. Extracts show anti-oxidant activity and anti-bacterial activity against both Gram-positive and Gram-negative bacteria. Total phenolic content correlated positively with anti-oxidant potential. The authors conclude that the sponge, *P. nigra*, is potentially rich in novel bioactives that can be exploited commercially.

Applications

Extracts appeared to kill *S. aureus*, one of the most troublesome nosocomial infections in biomedicine. Although this is a preliminary study and significant more investigation is needed. Hexane appeared to be the best solvent for extracting phenols from this sponge.

Peer review

This is an interesting study in which the authors provided novel insight into the potentially important metabolites of a Mauritian sea sponge. Data in this paper appear novel and it is worth publishing.

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