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BIOACTIVE ASSESSMENT OF SELECTED MARINE RED ALGAE AGAINST *LEISHMANIA MAJOR* AND CHEMICAL CONSTITUENTS OF *OSMUNDEA PINNATIFIDA*

H. SABINA AND R. ALIYA*

Department of Botany,
University of Karachi, Karachi-75270, Pakistan
**Corresponding author E-mail: aliyaal@hotmail.com, Phone: + 92 21 4521974*

Abstract

The antileishmanial activities of the seven different species of red algal extracts were evaluated against the promastigote form of *Leishmania major*. The ethanol extracts of *Osmundea pinnatifida* scored the highest activity with IC_{50} value 6.25 µg/mL comparable to other algae such as *Scinaia hatei* with IC_{50} value 14.10 µg/mL, *Melanothamnus afaqhusainii* with IC_{50} value 32.6 µg/mL and *Gracilaria corticata* with IC_{50} value 37.5 µg/mL, which exhibited significant inhibitory effect on the viability of *Leishmania major*. While other few species, like *Scinaia fascicularis*, *Centroceras clavulatum* and *Botryocladia leptopoda* were found to show good activity and gave lethal effect *In vitro*, as conducted for the first time using the Pakistani seaweeds. Current report describes the isolation and structural elucidation of flavone Scutellarein 4'-methyl ether (1) being a second report on the presence of flavone in algae and 4-Methoxy pyran (2) from *O. pinnatifida* as reported for the first time from the genus *Osmundea*.

Introduction

The rapid emergence and spread of resistant pathogens have generated a great threat to public health world wide. The parasitic disease Leishmaniasis causes disabling and sometime highly mutilating lesions. Present therapeutic regimes for Leishmanial diseases rely on pentavalent antimonials such as Pentamidine, Amphotericine B shows high toxicity at the effective therapeutic doses (Sereno *et al*., 2000; Croft & Coombs, 2003; Deniz *et al*., 2006), it has also lent to the additional urgency to antileishmanial research from other natural resources. Seaweeds have not been receiving appropriate attention in the past and the availability of seaweed pharmaceutical data is still scarce compared to that of terrestrial plants (Mayer *et al*., 2007). Previous investigation in order to measure the antileishmanial potential from marine algae is extremely limited, being restricted to some species with the test organisms *Leishmania donovani* (Orhan *et al*., 2006) and *L. maxicana* (Freile-Plegrina *et al*., 2008). In this context there is a lack of such study, hence the present work provides a result of some local species of Karachi Coast to assess their antileishmanial significance, which is being reported for the first time in Pakistan. This study could be extended to isolation of flavone [1] and 4-Methoxy pyran [2] being reported as a new source from *Osmundea pinnatifida*.

Materials and Methods

Algal material: The entire thalli of *Osmundea pinnatifida* (Hudson) Stackhouse [=*Laurencia pinnatifida* (Hudson) Lamouroux] (Rhodomelaceae, Ceramiales, Ceramophyceae, Rhodophycota; *fide* Shameel, 2008), were collected from the coast of Buleji, Karachi, (Pakistan) during September 2004. It was identified by the second author *Osmundea pinnatifida* and other seaweeds were collected in attached form from the Buleji Coast of Karachi, Pakistan as drift. The voucher specimens (fixed in 4% formaldehyde) and prepared herbarium sheets were deposited in the Department of Botany, University of Karachi. The collected specimens were drained of seawater and epiphytes and the calcareous deposits removed. The material was rinsed carefully in freshwater and 100 g of the material was used and percolated with 500 mL EtOH for two weeks under room temperature. The extract obtained was filtered and concentrated to a syrupy residue under reduced pressure.

Antileishmanial assay (*In vitro*): The 96 well microtiterplate assay was used for antileishmanial screening as previously determined (Ash & Orithel, 1987). The culture was examined microscopically on an improved Neubauer counting chamber and IC_{50} values of the extracts possessing antileishmanial activity were calculated by Software Ezfit 5.03 Perella Scientific. All assays were run in duplicate.

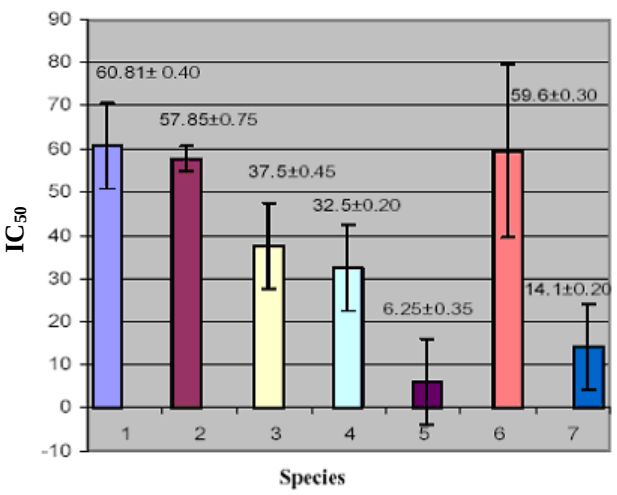
Extraction and isolation: 10 kg fresh material of *O. pinnatifida* was chopped and soaked in ethanol at room temperature for 15 days. After filtration, ethanol extract was concentrated in vacuum to obtain a gummy material which was dissolved in (1 L) methanol and defatted with petrolium ether (1 L), to obtain etherial extract. The defatted methanol extract was evaporated in vacuum to yield a gum (40 kg) which was dissolved in distilled H_2O and extracted with CH_2Cl_2 to afford dichloromethane fraction. The aqueous portion was extracted with ethyl acetate (2 L) to obtain ethylacetate fraction. The remaining aqueous portion was extracted with (2 L) butanol to obtain butanol extract. The fractions having the similar TLC profiles were mixed and individually subjected to repeated column chromatography in order to obtain the metabolites in pure form. The corresponding crude extract was subjected to liquid–liquid fraction method through separating funnel.

General experimental procedures: The melting point of the isolated compound was recorded on a YANACO apparatus, rotation was measured on a digital polarimeter JASCO DIP-360 in methanol. Infrared spectra were obtained on vector 22 Brukner spectrophotometer. High resolution mass spectrum (HR-EIMS) were recorded with a Jeol HX 110 mass spectrometer in *m/*z (rel %). The ^{1}H-

NMR, ^{13}C-NMR, Cosy, HMBC spectra were obtained in Bruker AV-400 specrometer operating at MHz. The chemical shift values were reported in δ (ppm), referenced with respect to the residual solvent signals of CD_3OD and $CDCl_3$, and coupling constants (J) were measured in Hz. Column chromatography (CC) was performed using Diaion HP-20 (Mitsubishi Chem. Ind., Tokyo, Japan), polyamide-6 DF (Riedel-De Haen AG, Germany). Thin-layer chromatography (TLC) was carried out on precoated silica gel plates (DC-Alugram 60 UV_{254} of E. Merck) by using Ceric sulphate spraying reagent.

Results and Discussion

This study established that the extract did influence the metabolic activity of parasite as measured by the ability to inhibit /reduce its growth *In vitro*. Our data showed that among the tested species ethanol crude extracts of *Osmundea pinnatifida* with an IC_{50}/72 = 6.25 ± 0.35 µg/mL exhibited potent inhibitory effect and *Scinaia fascicularis* with an IC_{50}/72h = 14.10 ± 0.20 µg/mL, *Melanothamnus afaqhusainii* with an IC_{50}/72h = 32.6 ± 0.20µg/mL and *Gracilaria corticata* with an IC_{50}/72h = 37.5 ± 0.45 µg/mL were found to induce significantly reduced growth of *Leishmania major*, and few of the species like *Centroceras clavulatum* with an IC_{50}/72h = 57.89 ± 0.75 µg/mL, *Scinaia indica* with an IC_{50}/72h = 59.6 ± 0.30 µg/mL and *Botryocladia leptopoda* with an IC_{50}/72h = 60.81 ± 0.40 µg/mL displayed good activity (Fig. 1). No previous record on antileismanial properties of any seaweed from Pakistani Coast have appeared in the literature. Furthermore isolation of the chemical constituents from the BuOH fraction of the red alga *O. pinnatifida* by spectroscopic methods was chemically elucidated for the first time from the genus *Osmundea*.



1 = *Botryocladia leptopoda*, 2 = *Centroceras clavulatum*, 3 = *Gracilaria corticata* , 4 = *Melanothamnus afaqhusainii*, 5 = *Osmundea pinnatifida*, 6 = *Scinaia fascicularis*, 7 = *Scinaia hatei.*

Fig 1. *In vitro* activity of red algae toward promastigote form of *Leishmania major* ± S.D.

Compound 1, *i.e*. butanol extract (20 g) was fractionated by CC on Diaion HP-20 and eluted with the mixture of H_2O-MeOH to obtain various sub-fractions. A sub-fraction (2:1) eluted with 25:50 MeOH-H_2O was subjected to polyamide column chromatography by eluting with $CHCl_3$-MeOH in a gradient manner to afford various fractions F_1-F_8. Fraction F_4-F_6 were combined on TLC behavior and subjected to polyamide column chromatography by using $CHCl_3$/MeOH/H_2O (8:2.8:0.2) as eluting solvents, which yielded compound 1 (10 mg). Due to its yellow appearance on silica gel plate after spraying with Ceric sulphate Ce $(SO_4)_2$ reagent suggested flavone. The high resolution mass spectrum (HREI-MS) of compound showed molecular ion peak at *m/z* 300.27 [M+H]+ (calcd. for $C_{16}H_{12}O_6$ 300.0634) corresponding to the molecular formula $C_{16}H_{12}O_6$. The infrared spectrum (IR) displayed intense absorption bands revealed the presence of hydroxyl (3418), carbonyl (1738) and other olefinic functionalities (2923).

The ^{1}H-NMR (CD_3OD, 300 MHz) of compound [1] (Table 1) showed the presence of a singlet at δ 3.88 having three proton integrations indicated the presence of an OCH_3, whereas the two doublets appeared at δ 7.11 (d, 1H, J = 7.8 Hz , H-2, H-5') and 7.97 (d, 2H, J = 7.8 Hz , H-2',H-6'). The two protons appeared as singlet at δ 6.50 (s, 1H, H-8) and δ 6.69 (s, 1H, H-3). The HMQC experiment revealed the similar magnitude of coupling constants of the protons 7.11 and 7.97, showed position of three protons which were found to be linked with 114.6 (C-3',C-5') and 127.9 (C-2', C-6') respectively. The HMQC experiment revealed that two proton integrations of each doublet were actually due to aromatic methines having similar environment (AA'BB') system. The ^{1}H-NMR spectra showed two doublets each integrated for two protons with the same coupling constants (7.8 Hz) at δ 7.09 (H-3', H-5') and 7.97 (H-2', H-6'). The similar magnitude of coupling constants showed that carbons corresponding to both the doublets are adjacent to each other. By HMBC correlation, the protons at δ 7.09 and 7.97 were found to be linked with the carbons at δ 114.6 (C-3', C-5') and 127.9 (C-2', C-6') respectively. The HMQC experiment revealed that two proton doublets were actually due to aromatic methines having similar environment (AA'BB') system. The signal at δ 3.88 with a quaternary carbon at δ 152.6 in the HMBC experiment confirmed that methoxy group was connected to the carbon. Another quaternary signal resonating at δ 134 in the 13C-NMR was assigned to C-1' by its HMBC interaction ($_3J$CH) with protons at δ 6.65 (H-3) and 7.11 (C-3', C-5').

Table 1. The ^{1}H-, ^{13}C- NMR COSY, ROSY (300 MHz, $CDCl_3$, CD_3OD) Soln. in δ ppm.

Position	^{1}H (δ = ppm)	^{13}C (δ =ppm)
1	3.60	65.4
2	-	104.9
3	5.45	79.8
4	4.37	74.5
5	3.93	84.2
6	3.83	64.9
1'	5.43	93.3
2'	2.5	73.1
3'	3.65	75.0
4'	4.10	71.3
5'	-	73.9
6'	3.78	62.4

The HMBC correlation ($_3J$CH) of the spectrum at δ 123 assigned this shift (114) to C-1'. The interactions ($_3J$CH) of H-2' and H-6' with C-2 and H- 3 with C-1' in the HMBC experiment confirmed the connection of

fragment B with C-2. On the bases of HMBC experiment all of the carbons were assigned. The C-7 and C-9 carbon assignments could not be obtained due to the lack of full HMBC interactions. It showed only the interaction with H-8. The coupling interaction was established by COSY-45° experiment. The doublet of H-3' (δ 7.11) was coupled with H- 5' (7.08), whereas the doublet of H- 6' at δ 7.96 was coupled with H- 2' (7.98). Similarly the doublet of H-2 had a cross peak with H-3' (7.11), and H-6' (7.96). On the basis of above evidences and comparison with literature (Horie *et al*.,1998) the structure of compound 1 was deduced to be as 5, 6, 7-trihydroxy-4'-methoxy flavone commonly known as Scutellarein-4-methyl ether (Fig. 2). In the present study, this ether is reported for the first time in algae, which was isolated from *O. pinnatifida*. According to the literature survey it has been reported for its anti-allergic (Masaru *et al*., 1994), anti-cancer and anti-cytotoxic (Shan *et al*., 2006) activities *In vitro* and *In vivo*.

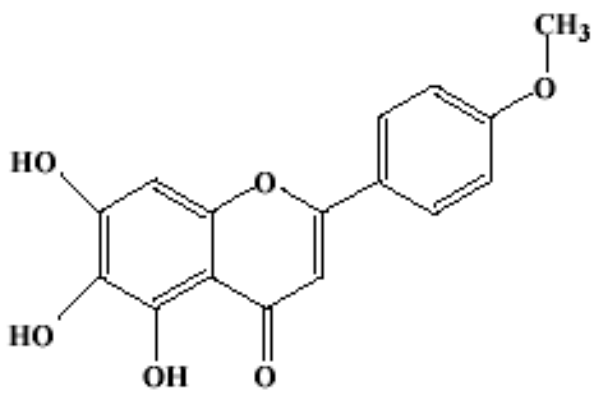


Fig. 2. Scutellarein 4'-methyl ether [1].

Compound 2, *i.e.* crude extract of dichloromethane fraction (35 g) was subjected to silica gel column. The elution of the column was done by increasing polarities of petroleum ether and $CHCl_3$ which yielded a mixture of compounds. The 15 mg mixture was then purified by solvent – solvent fractionation with a different solvent. The compound (10 mg) was obtained as a white powder. The high resolution mass spectrum (EI-MS) of the compound showed peaks at *m/z* 112 [M+1] +, 97 [M-1] +, 69 [M-1] +, 55 [M-1] + suggesting the molecular formula $C_7H_8O_2$. Its infrared spectrum (IR) showed the presence of ketonic (1715) and other olefinic functionalities (1646) respectively. The ^{1}H-NMR ($CDCl_3$ + CD_3OD, 300 MHz) data (Table 2) indicated that doublets were resonating and appeared at δ 6.94 (d, 2H, J = 7.5 Hz, H-4) and δ 5.30. (d, 2H, J = 7.5 Hz, H- 4). The presence of one singlet at δ 3.88 (s) of three proton integrations indicated the presence of one OCH_3 group in the molecule. The ^{13}C-NMR ($CDCl_3$ + CD_3OD, 300 MHz) data showed 5 signals consisting of 4 methines and one quaternary carbon. The associated carbon at 63.27 in the ^{13}C-NMR spectrum showed the presence of only one methoxy group in this compound. The two doublets appeared at δ 7.11 (d, 2H, *J* = 7.8 Hz, H-3', H-5') and 7.97 (d, 2H, *J*=7.8 Hz, H-2',H-6'), with corresponding carbons as appeared at (C-3, C-5) resonating in the spectrum δ 141 and (C-2, C-6) at δ 101 respectively. On the basis of above evidences the structure of compound 2 was deduced to be 4-Methoxy pyran (Fig. 3). The 2H- pyrans are quite reactive, showing tendency to undergo subsequent cycloadditions, often with dimerization. These repeating units are linked to form agarose polymer. It is used for immunological therapy (Schneider *et al*., 1977).

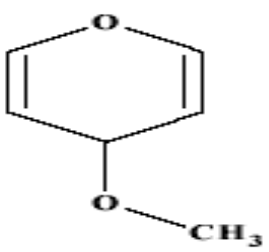


Fig. 3. 4-Methoxy pyran [2].

Table 2. The ^{1}H-, ^{13}C- NMR (300 MHz, $CDCl_3$, CD_3OD) Soln. in δ ppm.

Position	^{1}H (δ = ppm)	^{13}C (δ =ppm)	Multiplicity DEPT
H-3,H-5	(2H, d, 6.94 *J* = 7.5 Hz)	141.24	(CH) 141.26
H-2, H-6	(2H, d, 5.30 *J* = 7.5 Hz)	101.13	(CH) 101.11
OCH_3	3.88 s	63.27	OCH_3

The red algal species *O. pinnatifida* has been reported to be a source of halogenated metabolites, particularly of C_{15} acetogenin and sesquiterpenes (Bano *et al*., 1982, 1988; Gonzalez, *et al*., 1982, 1984; Ahmad & Ali, 1991) and has long been known as a reliable and prolific producer of diverse secondary metabolites. On the basis of earlier reports, presence of flavonoids remained questionable in marine algae (Markham, 1988). Evidence of flavonoid has only once been reported from *Acanthophora spicifera* (Vahl) Børgesen (Wang *et al*., 1998; Zeng *et al*., 2001). Our search for bioactive substances from this species resulted in the isolation of a flavone (Scutellarein 4'-methyl ether [1] and 4-Methoxy pyran [2], which are for the first time reported from *O. pinnatifida*. The lead obtained from the search of antileishmanial activity of selected red algae and their reputed bioactive chemical compounds from the species gives a new impetus seeming to validate their use in medicinal therapy.

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