ISSN : 0974 - 7508 Volume 11 Issue 2

Natural Products

An Indian Journal

Full Paper

NPAIJ, 11(2), 2015 [059-068]

Pure form bulk isolation of rare and bioactive triterpenoid 3-*o*-(4-hydroxy cinnamoyl)-taraxerol from mangrove plant *rhizophora mucronata* through classical chemistry, and biological activities of *rhizophora mucronata*

Ateeq Ahmad[1,*], Pirzada Jamal Siddiqui[1], Anila Naz[1], Shaheen Faizi[2]

[1]Center of Excellence in Marine Biology, University of Karachi, Karachi, (PAKISTAN)

[2]H.E.J.Research Institute of Chemistry, International Center of Chemical and Biological Sciences, University of Karachi, Karachi, (PAKISTAN)

E-mail: ateeqkhanbbt@yahoo.com; jamal.siddiqui@yahoo.com

ABSTRACT

Biological and phytochemical investigations on *Rhizophora mucronata* revealed its remarkable pharmacological significance due to the presence of important secondary plant metabolites in its various parts. In the present studies various extracts and fractions of different parts of *R. mucronata* were subjected to bioassay directed isolation studies for the evaluation of antioxidant, cytotoxic and antifungal activities. The ethyl acetate extract of fruits showed moderately good antioxidant activity, with IC_{50} value of 26.28 µg/mL (87.8 %). The methanol extract of fruits, ethyl acetate extract of leaves and ethyl acetate extract of stem and twigs showed low antioxidant activity, with IC_{50} values of 62.30 (83.2 %), 66.87 (86.1 %) and 79.53 µg/mL (87.8 %), respectively. Antioxidant activity from the fruits of this plant is reported for the first time in present studies. The lower layer of dichloromethane extract of stem and twigs showed cytotoxic activity against nauplii of brine shrimp (*Artemia salina*), with LD_{50} value of 556.92 µg/mL (60 % mortality) at higher dose level of 1000 µg/mL. Extraction and fractionation with polar and non polar solvents resulted in the isolation of bulk quantity of pure 3-*O*-(4-hydroxy cinnamoyl)-taraxerol (I) (6.13 % of wet plant material) from the petroleum ether insoluble fraction of leaves of *R. mucronata* through classical chemistry, without applying any chromatographic technique.

KEYWORDS

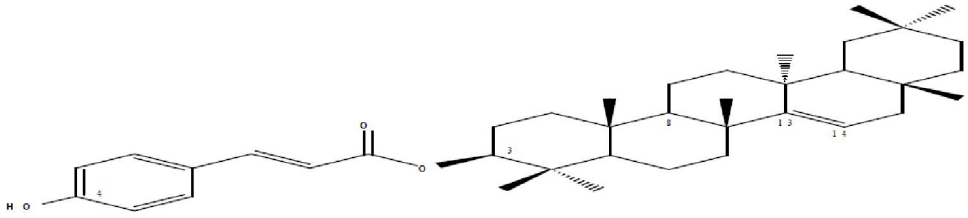
Mangrove;
Rhizophora mucronata;
Classical chemistry;
TLC;
Triterpenoid.

INTRODUCTION

Mangrove plants have been used in folk medicines to cure diseases like paralysis, earache, ulcers, typhoid, dysentery, asthma, diabetes, malaria, leprosy, hepatitis and skin diseases[1-3]. *Rhizophora mucronata* Lam. (Rhizophoraceae) is an evergreen mangrove tree, which grows in the marine intertidal zone along the coasts of Asia, Australia, Africa, Madagascar, Micronesia, Mauritius, Marshall Islands and Pacific tropical regions[4,5]. In Pakistan, *R. mucronata* is found naturally in Miani Hor along

Full Paper

Balochistan coast, while through replantation it has been introduced in the Indus delta along Sindh coast[6, 7]. The seeds and bark of *R. mucronata* have been used for the treatment of haematuria, angina, diabetes, diarrhea and haemorrhagic conditions in China, India and Burma[8]. Its roots have been used for the cure of hemorrhage and angina in Indo-Chinese populations, old leaves and roots for obstetric conditions in Malayans, bark for bloody urine in Burma and for vomiting, nausea, hemorrhage and diarrhea in Thailand[9, 10]. Chemical constituents identified from its different parts include diterpenoids, triterpenoids, sesquiterpenoids, alkaloids, flavonoids, inositols, anthocyanidins, proteins, steroids, carotenoids and tannins[2, 3]. We present herein the isolation of a rare and bioactive triterpenoid, 3-*O*-(4-hydroxy cinnamoyl)-taraxerol (I) in pure form in bulk quantity from the leaves of *R. mucronata* through classical chemistry, and antioxidant, cytotoxic and antifungal activities of extracts and fractions of different parts of *R. mucronata*.



3-*O*-(4-Hydroxy cinnamoyl)-taraxerol (I)

EXPERIMENTAL

General experimental conditions

Ultraviolet (UV) spectra were measured in methanol on Hitachi U-3200 spectrophotometer. Infrared (IR) spectra were obtained in KBr on Bruker Vector 22 spectrophotometer. The EIMS spectra were recorded on Finnigan MAT-312 spectrometer. The ^{1}H NMR spectra were recorded in $CDCl_3$ on Bruker Aspect AV-500 spectrometer at frequency of 500 MHz. The coupling constants (*J*) were recorded in Hz, while the chemical shifts were measured in ppm (δ). Kieselgel 60 GF_{254} precoated cards (0.2 mm thick, E.Merck) were used for analytical thin layer chromatography. For filtration Whatman No 1 filter paper was used.

Plant material

The leaves, stem, twigs and fruits of *Rhizophora mucronata* Lam. was collected from Miani Hor (Sonmiani Bay, Balochistan) in February 2012 and authenticated by plant taxonomist Prof. Dr. Surayia Khatoon of the Department of Botany, University of Karachi. A voucher specimen (G.H. # 86466) was deposited in the herbarium of the same department for future reference.

Extraction and isolation from the leaves

The fresh, undried and uncrushed leaves (4.41 Kg) were successively extracted with petroleum ether (10 L), dichloromethane (10 L), ethyl acetate (10 L) and methanol (10 L) at room temperature. The petroleum ether extract was filtered with Whatman No. 1 filter paper and evaporated under reduced pressure to give a residue RLP (317 g), a portion (17 g) of which was taken and dissolved again in 250 mL petroleum ether. The solution was filtered to give petroleum ether soluble fraction RLP-S (2.5 g) and an insoluble residue RLP-I (white solid, 14.5 g), which showed single spot on TLC (silica gel, PE:EA, 8:2). Spectral studies (UV, IR, EIMS and ^{1}H NMR) of RLP-I disclosed its structure as pure 3-*O*-(4-hydroxy cinnamoyl)-taraxerol (I). The dichloromethane extract was filtered and evaporated under reduced pressure giving a residue RLDC (300 g), a portion (12 g) of which was taken and dissolved in 250 mL petroleum ether. The solution was filtered to furnish petroleum ether soluble fraction RLDC-P-S (1.5 g) and an insoluble residue RLDC-P-I. RLDC-P-I was dissolved in 250 mL chloroform. The solution was filtered to give chloroform soluble fraction RLDC-C-S (1.1 g) and an insoluble residue RLDC-C-I. RLDC-C-I was dissolved in 250 mL ethyl acetate. The solution was filtered to yield ethyl acetate soluble fraction RLDC-EA-S (420 mg) and an

insoluble residue RLDC-EA-I, which was dissolved in 250 mL methanol. The solution was filtered to give methanol soluble fraction RLDC-M-S (2.2 g) and an insoluble residue RLDC-M-I (1 g). The ethyl acetate and methanol extracts were filtered and evaporated under reduced pressure to give residues RLEA (230.5 g) and RLM (480 g), respectively.

Characterization of 3-*O*-(4-Hydroxy cinnamoyl)-taraxerol (I)

Color and state: white solid; UV λ: 204, 221, 310, 365; IR: 3055, 1710, 1600; EIMS *m/z* (%): 572 (M^+, $C_{39}H_{56}O_3$, 52), 557 (12), 448 (55), 396 (21), 204 (100), 147 (99); ^{1}H NMR ($CDCl_3$, 500 M Hz) (δ/Hz); 7.57 (d, *J*=16, H-32), 7.41 (d, *J*=8.5, H-52 , H-92), 6.81 (d, *J*=8.5, H-62 , H-82), 6.28 (d, *J*=16, H-22), 5.50 (nm, H-15), 4.58 (dd, *J*=5.0,11.0, H-3).

Extraction and Isolation from the Stem and twigs

The fresh and undried stem and twigs (0.503 Kg) were successively extracted with petroleum ether (1.7 L), dichloromethane (1.7 L), ethyl acetate (1.7 L) and methanol (1.7 L) at room temperature. The first petroleum ether extract was filtered and evaporated under reduced pressure to give residue RSP1 (641 mg), while the second petroleum ether extract, containing two layers were separated to give upper yellowish layer and lower reddish oily layer. The two layers obtained were evaporated under reduced pressure yielding respective residues RSP2U-S (415 mg) and RSP2L-I (3.3 g). The dichloromethane extract, containing two layers were separated to give upper brownish oily layer and lower yellowish layer. The two layers were separated and evaporated under reduced pressure furnishing respective residues RSDCU-I (5.1 g) and RSDCL-S (1.2 g). The ethyl acetate and methanol extracts were filtered and evaporated under reduced pressure to give respective residues RSEA (437 mg) and RSM (15.2 g).

Extraction and isolation from the fruits

The fresh, undried and uncrushed fruits (0.268 Kg) were successively extracted thrice with petroleum ether (600 mL), twice with dichloromethane (600 mL), ethyl acetate (600 mL) and methanol (600 mL) at room temperature. The first petroleum ether extract was filtered and evaporated under reduced pressure to give residue RFr.P1 (633 mg), which was again dissolved in 100 mL petroleum ether. The solution was filtered to give petroleum ether soluble fraction RFr.P1-S (381 mg) and an insoluble residue RFr.P1-I (white solid). RFr.P1-I was taken in 100 mL chloroform and the solution was filtered to give chloroform soluble fraction RFr.P1-C-S (111 mg) and an insoluble residue RFr.P1-C-I. The later was dissolved in 100 mL ethyl acetate and the solution was filtered to give ethyl acetate soluble fraction RFr.P1-EA-S (16 mg) and an insoluble residue RFr.P1-EA-I. RFr.P1-EA-I was dissolved in 100 mL methanol. The solution was filtered to give methanol soluble fraction RFr.P1-M-S (25 mg) and an insoluble residue RFr.P1-M-I (80 mg). The second and third petroleum ether extracts were filtered and evaporated under reduced pressure to yield respective residues RFr.P2 (315 mg) and RFr.P3 (120 mg). The first dichloromethane extract, containing two layers were separated to give upper reddish oily layer and lower yellowish layer. The two layers were separated and evaporated under reduced pressure to give respective residues RFr.DC1U-I (27.3 g) and RFr.DC1L-S (2.1 g). The residue RFr.DC1U-I was dissolved in methanol and the solution was filtered to furnish methanol soluble fraction RFr.DC1U-M-S (16.2 g) and an insoluble residue RFr.DC1U-M-I (whitish suspension, 11.1 g). The second dichloromethane extract was filtered and evaporated under reduced pressure to yield the respective residue RFr.DC2 (4.1 g). The ethyl acetate extracts (RFr.EA1 and RFr.EA2) were filtered, combined and evaporated under reduced pressure to give a residue RFr.EA (1.3 g). The methanol extracts (RFr.M1 and RFr.M2) were filtered, combined and evaporated under reduced pressure affording a residue RFr.M (18.2 g).

EVALUATION OF ANTIOXIDANT, CYTOTOXIC AND ANTIFUNGAL ACTIVITIES

Antioxidant activity

Extracts and fractions of different parts of *R. mucronata* were evaluated for antioxidant activity,

Full Paper

using DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay[11, 12]. The samples were mixed with stable free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and allowed to react at 37º C for 30 minutes. The dark colored crystalline powder of DPPH was dissolved in ethanol at concentration of 300 µM, while the samples were dissolved in DMSO or respective solvents. Gallic acid was used as standard and DMSO treated control group or respective solvent was used as negative control. Optical density (decrease in absorption) was measured at 515 nm by multiplate reader (Spectra MAX-340) after the incubation. Radical scavenging activity (%) of samples was decided by comparing with a DMSO treated control group. Percent radical scavenging activity was calculated using the formula:

% RSA= 100 – {(OD test sample / OD control) X 100}

The IC_{50} values were determined by plotting % RSA against the various concentrations of test sample and determined the best regression line which fit for the data. IC_{50} values were calculated from the best fit regression line.

Cytotoxic activity

Extracts and fractions of different parts of *R. mucronata* were evaluated for cytotoxic activity, using brine shrimp lethality assay[13, 14]. The samples were incubated with 2 days old nauplii of brine shrimp (*Artemia salina*) at around room temperature (28º ± 1º C), for 24 hours under illumination. The concentration of each sample was kept as 10, 100 and 1000 (µg/mL), while the total volume was made 5 mL using seawater. The respective solvent was used as negative control while the cytotoxic drug Etoposide was used as standard. At the end of 24 hours incubation period the surviving shrimps were counted and recorded, using 3x magnifying glass. The data was analyzed with Finney computer program (Probit analysis) and the LD_{50}/LC_{50} values were determined with 95 % confidence intervals. The assay was run in triplicate.

Percentage of death was calculated using the following formula:

Percentage of Death (%): (Total nauplii – Alive nauplii) × 100/Total nauplii

Antifungal activity

Extracts and fractions of different parts of *R. mucronata* were evaluated for antifungal activity against pathogenic fungal species *Candida albicans, C. glabrata*, *Microsporum canis*, *Aspergillus flavus* and *Fusarium solani*, using agar tube dilution method[13, 15]. Sabouraud dextrose agar (SDA) media (pH 5.5-5.6) loaded with respective sample (66.6 µl, dissolved in DMSO) was inoculated with 4 mm diameter piece of seven-day-old culture of fungus and incubated at 27º C for seven days. The concentration of sample was kept as 400 µg/mL of media. DMSO was used as negative control while reference antifungal drugs (Miconazole and Amphotericin-B) were used as positive control. Growth of the fungus in the media (amended with sample) was determined by measuring linear growth (mm). Percent (%) inhibition of fungal growth was determined with reference to the negative control using the formula:

% Inhibition = 100 – linear growth in test (mm) X 100

Linear growth in control (mm)

RESULTS

Antioxidant activity

The preliminary results revealed that the ethyl acetate extract of fruits (RFr.EA) showed moderately good antioxidant activity, with IC_{50} (EC_{50}) value of 26.28 µg/mL and percent DPPH radical scavenging activity of 87.8 % TABLE-1. The methanol extract of fruits (RFr.M) exhibited low antioxidant activity, with IC_{50} value of 62.30 µg/mL and percent DPPH radical scavenging activity of 83.20 %. The ethyl acetate extract of leaves (RLEA) showed low antioxidant activity, with IC_{50} value of 66.87 µg/mL and percent DPPH radical scavenging activity of 86.1 %. The ethyl acetate extract of stem and twigs (RSEA) showed low antioxidant activity, with IC_{50} value of 79.53 µg/mL and percent DPPH radical scavenging activity of 87.8 %. The dichloromethane extract of fruits (RFr.DC2) and methanol extract of stem and twigs (RSM) exhibited very weak

Antioxidant activity, with IC_{50} (EC_{50}) values of 226.5 and 245.7 µg/mL and percent DPPH radical

Full Paper

TABLE 1 : Antioxidant activity (IC_{50}/EC_{50} values and % radical scavenging activity) of extracts and fractions of *Rhizophora mucronata*

Sr. No.	Sample Code	Concentration (mg/mL)	$IC_{50}(EC_{50})$ ± SEM (µg/mL)	% Radical Scavenging Activity (RSA)
1.	RFr.EA	0.5	26.283±0.2	87.8
2.	RFr.M	0.5	62.303±1.01	83.2
3.	RLEA	0.5	66.87±1.7	86.161
4.	RSEA	0.5	79.531±2.45	87.882
5.	RFr.DC2	0.5	226.5±2.6	60.213
6.	RSM	0.5	245.75±1.789	66.539
7.	RLM	0.5	305.99±3.6	72.750
8.	RFr.DC1L-S	0.5	N.D	22.6
9.	RFr.P1-S (F)	0.5	N.D	20.442
10.	RLDC	0.03	N.D	13.042
11.	RSP1	0.03	N.D	3.439
12.	RFr.P2	0.5	N.D	3.279
13.	RSDCL-S	0.5	N.D	2.374
14.	RSP2U-S	0.5	N.D	0.502
15.	RLP-S (F)	0.03	N.D	0.42
16.	RSDCU-I	0.5	---	---
17.	RSP2L-I	0.5	---	---
18.	RLP-I (F)	0.5	---	---
19.	RFr.DC1U-I	0.03125	IS	---
20.	Standard (Gallic acid)	0.094	4.3±0.43	93.13

IC_{50}/EC_{50} = Inhibitor concentration which gives 50% inhibition/Efficient (substrate) concentration which gives 50 % DPPH radical scavenging activity, SEM = Standard error of mean, N.D = Not determined, — = Inactive; IS = Insoluble

scavenging activity of 60.2 % and 66.5 %, respectively. The methanol extract of leaves (RLM) showed very weak antioxidant activity, with IC_{50} (EC_{50}) value of 305.9 µg/mL and percent DPPH radical scavenging activity of 72.75 %. The lower layer of the dichloromethane extract (RFr.DC1L-S) of fruits and petroleum ether soluble fraction of petroleum ether extract of fruits (RFr.P1-S) displayed 22.6 % and 20.4 % activity, respectively. The rest of the extracts and fractions showed negligibly small or no activity at all, while RFr.DC1U-I was insoluble.

Cytotoxic activity

The preliminary results TABLE-2 revealed that the lower layer of the dichloromethane extract of stem and twigs (RSDCL-S) showed cytotoxic activity (lethality) against nauplii of brine shrimp (*Artemia salina*), with LD_{50}/LC_{50} value of 556.92 µg/mL at higher dose of 1000 µg/mL. The percent mortality of brine shrimp nauplii was 60 %, 23 % and 6.6 % at the concentration of 1000, 100 and 10 µg/mL, respectively. The rest of the extracts and fractions caused mortality below 50 %, having LD_{50} values greater than 1000 µg/mL, which are non-cytotoxic.

Antifungal activity

The preliminary results revealed that petroleum ether extract of fruits (RFr.P2) showed low antifungal activity against *Microsporum canis* and *Aspergillus flavus*, with 35 % and 25 % growth inhibition, respectively (TABLE-3). The lower layer of petroleum ether extract of stem and twigs (RSP2L-I) exhibited low activity against *M. canis* and *F. solani*, with 35 % and 20 % growth inhibition, respectively. Both the methanol extract (RSM) and upper layer of dichloromethane extract (RSDCU-I) of stem and twigs displayed weak activity against *M. canis* and *F. solani*, with 20 % and 10 % growth inhibition, respectively. Both the ethyl acetate ex-

Full Paper

TABLE 2 : Cytotoxic activity (% mortality of brine shrimp nauplii and LD_{50}/LC_{50} values) of extracts and fractions of *Rhizophora mucronata*

Sr. No.	Sample Code	Dose(µg/mL)	No. of Shrimps	No. of Survivors	% Mortality	LD_{50} (LC_{50})(µg/mL)
		1000	30	12	60	
1.	RSDCL-S	100	30	23	23.3	556.92
		10	30	28	6.6	
		1000	30	16	46.6	
2.	RSEA	100	30	21	30	N.D
		10	30	26	13.3	
		1000	30	22	26.6	
3.	RSM	100	30	29	3.3	N.D
		10	30	30	0	
		1000	30	24	20	
4.	RFr.DC2	100	30	25	16.6	N.D
		10	30	27	10	
		1000	30	24	20	
5.	RLM	100	30	28	6.6	N.D
		10	30	30	0	
		1000	30	25	16.6	
6.	RSP2L-I	100	30	27	10	N.D
		10	30	29	3.3	
		1000	30	26	13.3	
7.	RSP1	100	30	28	6.6	N.D
		10	30	29	3.3	
		1000	30	26	13.3	
8.	RFr.DC1U-I	100	30	29	3.3	N.D
		10	30	30	0	
		1000	30	27	10	
9.	RSDCU-I	100	30	29	3.3	N.D
		10	30	30	0	
		1000	30	28	6.6	
10.	RFr.P2	100	30	29	3.3	N.D
		10	30	30	0	
		1000	30	28	6.6	
11.	RFr.DC1L-S	100	30	29	3.3	N.D
		10	30	30	0	
		1000	30	28	6.6	
12.	RFr.DC1U-M-S (F)	100	30	29	3.3	N.D
		10	30	30	0	
		1000	30	28	6.6	
13.	RLP-I (F)	100	30	30	0	N.D
		10	30	30	0	
		1000	30	29	3.3	
14.	RLDC	100	30	30	0	N.D
		10	30	30	0	
		1000	30	29	3.3	
15.	RLEA	100	30	30	0	N.D
		10	30	30	0	
		1000	30	29	3.3	
16.	RFr.EA	100	30	30	0	N.D
		10	30	30	0	
		1000	30	29	3.3	
17.	RSP2U-S	100	30	30	0	N.D
		10	30	30	0	
		1000	30	30	0	
18.	RFr.M	100	30	30	0	---
		10	30	30	0	
		1000	30	30	0	
19.	RLP-S (F)	100	30	30	0	---
		10	30	30	0	
20.	Standard Drug (Etoposide)					7.4625

N.D = Not determined, — = inactive

Full Paper

TABLE 3 : Antifungal activity (linear growth in mm and % inhibition) of extracts and fractions of *Rhizophora mucronata*

Sr. No.	Fungal Species	RFr.P2	RSP2 L-I	RSDC U-I	RSM	RLEA	RFr.DC1 U-I	RFr.M	RSP2 U-S	RFr.DC1 U-M-S (F)	Standard Drug	MIC (µg/mL)
1.	*Candida albicans*	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	Miconazole	110.8
2.	*Aspergillus flavus*	75/25 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	Amphotericin-B	20.20
3.	*Microsporum canis*	65/35 %	65/35 %	80/20 %	80/20 %	80/20 %	80/20 %	90/10 %	70/30 %	70/30 %	Miconazole	98.4
4.	*Fusarium solani*	100/0 %	80/20 %	90/10 %	90/10 %	90/10 %	90/10 %	90/10 %	100/0 %	100/0 %	Miconazole	73.25
5.	*Canadida glabrata*	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	Miconazole	110.8

TABLE 4 : Antifungal activity (linear growth in mm and % inhibition) of extract and fractions of *Rhizophora mucronata*

Sr. No.	Fungal Species	RFr. DC2	RLP-I (F)	RFr.DC1 L-S	RLP-S (F)	RLM	RSDC L-S	RFr.EA	RSEA	RLDC	RSP1	Standard Drug	MIC (µg/mL)
1.	*Candida albicans*	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/ %	100/0 %	100/0 %	Miconazole	110.8
2.	*Aspergillus flavus*	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	Amphotericin-B	20.20
3.	*Microsporum canis*	75/25 %	80/20 %	90/10 %	100/0 %	90/10 %	100/0 %	100/0 %	100/0%	100/0 %	100/0 %	Miconazole	98.4
4.	*Fusarium solani*	100/0 %	100/0 %	100/0 %	90/10 %	100/0 %	100/0 %	100/0 %	100/0%	100/0 %	100/0 %	Miconazole	73.25
5.	*Canadida glabrata*	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0 %	100/0%	100/0 %	100/0 %	Miconazole	110.8

tract of leaves (RLEA) and upper layer of the dichloromethane extract of fruits (RFr.DC1U-I) showed

Weak activity against *M. canis* and *F. solani*, with 20 % and 10 % growth inhibition, respectively. The methanol extract of fruits (RFr.M) showed weak activity against *Microsporum canis* and *Fusarium solani*, with 10 % growth inhibition. The upper layer of petroleum ether extract (RSP2U-S) of stem and twigs exhibited low activity against *M. canis*, with 30 % growth inhibition. The methanol soluble fraction of dichloromethane extract (upper layer) of fruits (RFr.DC1U-M-S) displayed low activity against *M. canis*, with 30 % growth inhibition. The dichloromethane extract of fruits (RFr.DC2) showed low activity against *M. canis*, with 25 % growth inhibition TABLE-4. The petroleum ether insoluble fraction of petroleum ether extract of leaves (RLP-I) exhibited weak activity against *M. canis*, with 20 % growth inhibition. The lower layer of the dichloromethane extract of fruits (RFr.DC1L-S) & methanol extract of leaves (RLM)

Exhibited weak activity against *M. canis*, with 10 % growth inhibition. The petroleum ether soluble fraction of petroleum ether extract of leaves (RLP-S) showed weak activity against *F. solani*, with 10 % growth inhibition. The rest of the extracts were found to be inactive.

DISCUSSION

The plant material of *R. mucronata* was used fresh, undried and uncrushed in order to protect it from possible oxidation through air and degradation by endogenous enzymes due to the drying and crushing process that may result in artifacts formation. Another reason was to prevent the loss of residual water in order to preserve and extract the water soluble polar chemical constituents. Different parts of the plant were sequentially extracted with petroleum ether, dichloromethane, ethyl acetate and methanol in order to isolate both non polar and polar chemical constituents, providing with an insight of their morphological composition and physiological importance. Extraction and fractionation using classical solvent-solvent separation resulted in the isolation of large quantity of a pure compound (I) (6.13 % of wet plant material), which appeared as a single

Full Paper

spot on TLC chromatogram. Spectral analysis determined its structure as a triterpenoid, 3-*O*-(4-hydroxy cinnamoyl)-taraxerol (I). It is a rare triterpenoid and has been previously isolated from the fruits of this plant along with its parent compound taraxerol[9]. Taraxerol have been shown to possess anti-diabetic, anti-inflammatory, analgesic, anticancer and antigiardial activities[16-18]. The rare triterpenoid (I) isolated in present studies displayed antifungal activity against the dermatophyte *Microsporum canis*. It is important to note that *Rhizophora* species are very rich in taraxerol, which can be used as a lipid biomarker for mangrove biomass input to the Southeast Atlantic[19]. For tracing *Rhizophora'* derived organic matter in marine sediments and peats taraxerol is considered as a well established lipid biomarker[20]. Leaves of *Rhizophora mangle*, *R. stylosa*, *R. racemosa* and *R. apiculata* contain significantly large amount of taraxerol, while from the leaves of *R. mucronata* taraxerol is reported as a minor common lipid[20, 21]. In present investigation the rare triterpenoid 3-*O*-(4-hydroxy cinnamoyl)-taraxerol (I) was isolated in bulk quantity from the leaves of *R. mucronata*, which may serve as a signature triterpenoid for the presence of organic matter derived from *R. mucronata* in marine peats and sediments.

Antioxidant substances have the ability to protect body from free radical induced tissue damage by neutralizing free radicals or their actions during the conditions of oxidative stress. Search for antioxidant phytochemicals is intensively carried out and a variety of antioxidants have been identified from plants[22, 23]. Mangrove plants have been shown to possess antioxidant potential, and several mangrove species such as *Rhizophora apiculata*, *R. mucronata*, *R. lamarkii* and *Bruguiera cylindrica* have high polyphenols content, which have antioxidant properties[22-25]. Polyphenols are considered important antioxidants due to their redox potential, and phenolic content of mangrove extracts is linearly correlated with their antioxidant potential. Plant phenolics have been classically considered to play role in the protection of plants from herbivores, while in contrast now their main role have been suggested as antioxidants in the protection of leaves from photodamege and their levels may vary according to the ecological conditions in order to anticipate the possible photodamege. The polyphenols particularly flavonoids have been reported to protect mangroves from UV radiation[22]. The results of present studies TABLE-1 showed that polar (methanol and ethyl acetate) extracts displayed good antioxidant activity as compared to non polar extracts, reflecting high phenolic content of these extracts, and the results obtained in our studies are consistent with the past investigations on *R. mucronata*[22-25]. The IC_{50} value of ethyl acetate extract of leaves RLEA (66.87 µg/mL) is lower than the reported value in literature (79.7 µg/mL) for the leaves[24], and the percent DPPH radical scavenging activity (86.1 %) is high enough, reflecting higher polyphenols content of the extract (RLEA) obtained in our studies. Mangrove species *R. mucronata* survives the harsh ecological conditions such as high wave action, elevated temperature, high salinity, overweening radiation, limited nutrients and frequent herbivory of coastal habitat by synthesizing secondary metabolites such as phenolic compounds in excessively high amount in order to morphologically and physiologically anticipate the adverse outcomes (including production of reactive oxygen species) of such stressful conditions. Ultimately gene expression and regulation of the enzymes responsible for metabolic and catabolic processes play essential role in the survival of this plant in such devastating ecological conditions.

Cytotoxic substances are usually toxic to cultured cells, but may or may not show any specific toxicity to cancerous cells. Those substances which exhibit selective *in vivo* cytotoxicity against tumor cells are designated as antitumor, while those substances which are active against cancers in humans are tagged as anticancer. Brine shrimp lethality assay is a simple, cheap, fast, general purpose bioassay used during natural products isolation studies for screening and monitoring of cytotoxicity of natural products[13]. The toxicity to brine shrimp nauplii is regarded as a useful preliminary screen to current cytotoxicity and antitumor testing as usually it is correlated with pharmacological properties[26]. From the stand point of monitoring it can be concluded from

the results of present studies (TABLE-2) that all of the extracts and fractions of *R. mucronata* have safety profile regarding cytotoxic activity to normal cells of brine shrimp nauplii, except the lower layer of dichloromethane extract of stem and twigs (RSDCL-S) which is cytotoxic (LD_{50}/LC_{50} = 556.92 µg/mL) to normal cells of brine shrimp nauplii, leading to mortality of brine shrimp nauplii. Conversely as the cytotoxicity to brine shrimp nauplii is usually associated with antitumor and anticancer properties, so further studies will require evaluating *in vivo* antitumor and anticancer activity of this extract (RSDCL-S), as this extract may prove positive for antitumor and anticancer activity.

Many mangrove species have been used in traditional medicine as antimicrobial agents, and in recent years mangrove plants extracts proved to be biologically active against human, animal and plant pathogens[1, 2]. A number of mangrove plants synthesize biologically active compounds which control microbial growth[27]. The results of present studies TABLE-3 & TABLE-4 demonstrated that all the plant parts i.e. fruits, leaves, stem and twigs exhibited antifungal activity; fruits exhibited antifungal activity against *A. flavus* (plant pathogen, as well as opportunistic human pathogen), *M. canis* (human pathogen) and *F. solani* (plant pathogen), while leaves, stem and twigs exhibited antifungal activity against *M. canis* and *F. solani*. The activity was found in all the four polar and non polar solvents used for extraction and fractionation. Eleven out of the total sixteen extracts displayed antifungal activity: seven extracts displayed activities against two pathogenic fungal species; RFr.P2 against *M. canis* and *A. flavus*, RSP2L-I, RSDCU-I, RSM, RLEA, RFr.DC1U-I and RFr.M against *M. canis* and *F. solani*, while the remaining four extracts showed activity only against one fungal species; RSP2U-S, RFr.DC2, RFr.DC1L-S and RLM against *M. canis*. All the three fractions displayed activity against only one species; RFr.DC1U-M-S and RLP-I against *M. canis*, while RLP-S against *F. solani*. As a whole the most antifungal activity was found against *M. canis*; 12 out of 14 (active) samples showed activity against *M. canis*, followed by *F. solani*; 7 out of 14 (active) samples showed activity against *F. solani*, while only one sample showed activity against *A. flavus*. Though the antifungal activity reported in present studies is low, but it is amongst one of the very few reports of antifungal activity from this plant. Moreover, here the antifungal activity is reported from leaves, fruits, stem and twigs, while Sakaki *et al.*, in 1993 for the first time reported fungicidal activity from the supercritical carbon dioxide extract of the roots against *Penicellium* spores[28].

CONCLUSION

Present bioassay directed isolation and purification studies revealed that mangrove plant *R. mucronata* exhibits antioxidant, cyctotoxic and antifungal properties due to the presence of physiologically active secondary plant metabolites in its various parts. In present investigation the rare triterpenoid 3-*O*-(4-hydroxy cinnamoyl)-taraxerol (I) was isolated from the leaves of *R. mucronata* in pure form in bulk quantity through a fast, simple and cheap classical chemical method that can be applied for large scale production of this rare and bioactive triterpenoid for drug development. This compound (I) can be also used as signature compound for tracing and locating organic matter derived from *R. mucronata* in marine sediments.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

ACKNOWLEDGMENTS

We are very much grateful to the Higher Education Commission (HEC) Govt. of Pakistan for funding the research project.

REFERENCES

[1] W.M.Bandaranayke; Mang. & Salt M., **2**, 133-148 **(1998)**.

[2] W.M.Bandaranayake; Wet.Eco. & Manag., **10**, 421-452 **(2002)**.

[3] J.K.Patra, H.N.Thatoi; Acta Phy.Plant, **33**, 1051-1061 **(2011)**.

Full Paper

[4] J.A.Duke; Handbook of energy crops, **(1983)**.

[5] www.hort.purdue.edu/newcrop/duke_energy../Rhizophora_mucronata.html.

[6] N.C.Duke; Spec.Prof.for Pacific Island Agr., ver., www.traditionaltree.org, **2(1)**, 1-21 **(2006)**.

[7] A.A.Shah, I.Kasawani, K.Jusoff; Int.J.of Geo., **1(3)**, 27-34 **(2007)**.

[8] P.J.A.Siddiqui, S.Farooq, S.Shafique, Z.Burhan, Z.Farooqi; Ocean and Coastal Manag., **51(5)**, 377-382 **(2008)**.

[9] R.M.Rohini, A.K.Das; Nat.Prod.Res., **24(2)**, 197-202 **(2010)**.

[10] S.Laphookhieo, C.Karalai, C.Ponglimanont; Chem.Pharm.Bull., **52(7)**, 883-885 **(2004)**.

[11] M.A.Rahman, S.N.Hasan, K.S.Sampad, A.K.Das; Pharmacologyonline, **1**, 921-929 **(2011)**.

[12] S.K.Lee, Z.H.Mbwambo, H.Chung, L.Luyengi, E.J.Gamez, R.G.Mehta, A.D.Kinghorn, J.M.Pezzuto; Comb.Chem.& High Thr.Scr., **1(1)**, 35-46 **(1998)**.

[13] P.Molyneux; Songklanakarin J.Sci.Tech., **26(2)**, 211-219 **(2004)**.

[14] Atta-Ur-Rahman, M.I.Choudhary, W.J.Thomsen; 'Bioassay techniques for drug development', Harwood academic publishers, 6-25 **(2005)**.

[15] G.Sahgal, S.Ramanathan, S.Sasidharan, M.N.Mordi, S.Ismail, S.M.Mansor; Pharm.Res., **2(4)**, 215-220 **(2010)**.

[16] Z.F.Rizvi, R.Mukhtar, M.F.Chaudhary, M.Zia; Pak.J.Bot., **45(1)**, 275-278 **(2013)**.

[17] K.N.Sangeetha, S.Sujatha, V.S.Muthusamy, S.Anand, N.Nithya, D.Velmurugan, A.Balakrishnan, B.S.Lakshmi; Biochim.*et* Biophy.Acta, **1800**, 359–366 **(2010)**.

[18] S.S.Swain, K.K.Rout, P.K.Chand; Appl.Biochem. Biotech., **168(3)**, 487-503 **(2012)**.

[19] I.H.Chavez, L.W.T.Tapia, P.S.Polanco, R.C.Rivera, R.M.Puc, S.R.P.Sanchez; J.Mex.Chem.Soc., **56(2)**, 105-108 **(2012)**.

[20] G.J.M.Versteegh, E.Schefub, L.Dupont, F.Marret, J.S.S.Damste, J.H.F.Jansen; Geo.*et* Cosm.Acta, **68(3)**, 411–422 **(2004)**.

[21] R.Wöstmann, G.Liebezeit; Mires and Peat, **10(2)**, 1-13 **(2012)**.

[22] M.Basyuni, H.Oku, S.Baba, K.Takara, H.Iwasaki; Isoprenoids of Okinawan mangroves as lipid input into estuarine ecosystem.J.of Ocean., **63**, 601-608 **(2007)**.

[23] D.Banerjee, S.Chakrabarti, A.K.Hazra, S.Banerjee, J.Ray, B.Mukherjee; African J.of Biotech., **7(6)**, 805-810 **(2008)**.

[24] V.Vadlapudi, K.C.Naidu; J.of Pharm.Res., **2(11)**, 1745-1750 **(2009)**.

[25] G.Agoramoorthy, F.A.Chen, V.Venkatesalu, D.H.Kuo, P.C.Shea; Asian J.of Chem., **20(2)**, 1311-1322 **(2008)**.

[26] S.Gurudeeban, T.Ramanathan, K.Satyavani; Pharm.Chem.J., **47(1)**, 50-53 **(2013)**.

[27] F.K.Sigaroodi, M.Ahvazi, A.Hadjiakhoondi, M.Taghizadeh, D.Yazdani, S.K.Sigaroodi, S.Bidel; Iranian J.of Pharm.Res., **11(1)**, 295-302 **(2012)**.

[28] N.Arivuselvan, D.Silambarasan, T.Govindan, K.Kathiresan; Adv.in Bio.Res., **5(5)**, 251-254 **(2011)**.

[29] T.Sakaki, M.Shibata, Y.Inukai, H.Hirosue, Y.Ikema; Kyu.Kog.Gij.Ken.Hok., **51**, 3185-3190 **(1993)**.