International Journal of Phytomedicine 9 (2017) 213-218
http://www.arjournals.org/index.php/ijpm/index

Short Communication

ISSN: 0975-0185

The phytochemical investigation and biological activities of *Berberis orthobotrys*

Akber Dad[1], Iftikhar Ali*[1], Nadja Engel[2], Muhammad Atif[3], Hidayat Hussain[4], Viqar Uddin Ahmad[5], Peter Langer[6], Ahmed Al-Harrasi[4], Ivan R. Green[7]

***Corresponding author:**

Iftikhar Ali

[1]Department of Chemistry, Karakoram International University, 15100-Gilgit, Gilgit-Baltistan, Pakistan
[2]Department of Cell Biology, University Medical Center Rostock, Schillingallee 69, 18059-Rostock, Germany
[3]Division of Science & Technology, University of Education Vehari Campus, Punjab, Pakistan
[4]UoN Chair of Oman's Medicinal Plants and Marine Natural Products, University of Nizwa, P.O Box 33, Postal Code 616, Birkat Al Mauz, Nizwa, Sultanate of Oman
[5]HEJ Research Institute of Chemistry, ICCBS, University of Karachi, Karachi-75270, Pakistan
[6]Institut für Chemie, Universität Rostock, Albert-Einstein-Str. 3a, 18059 Rostock, Germany
[7]Department of Chemistry and Polymer Science, University of Stellenbosch, P/Bag X1, Matieland, 7602, South Africa

Abstract

The leaf extract of *Berberis orthobotrys* Bien. ex Aitch. (B.o.) afforded three compounds viz., β-sitosterol (1), sesamin (2) and 10-eicosanol (3) which were identified by spectroscopic methods including 1D and 2D NMR, GC-MS, IR and comparison of their spectral data with the published data. To the best of our knowledge these three compounds are reported here for the first time to be isolated from *Berberis orthobotrys* Bien. ex Aitch. (B.o.). Moreover the root extract exhibited good antilieshmanial activity and root and fruit extracts demonstrated minor antifungal activity against *Fusarium solani*.

Keywords: *Berberis orthobotrys*, Bioassays, Sesamin, 10-Eicosanol.

Introduction

The genus *Berberis* belongs to the family Berberidaceae [1]. The term *Berberis* was derived from the Arabic word berberys because of its fruit type [2]. The genus *Berberis* is represented by 3 genera and 20 species in Pakistan [3,4]. *Berberis orthobotrys* Bien ex Aitch. is a shrub that is commonly known as ishkeen/churka in the Shina language. It is an indigenous plant to Pakistan which is mainly found in Gilgit-Baltistan [3,4]. The inhabitants of the mountain areas have been aware of the medicinal importance of this plant for centuries. The leaves, bark, stem as well as the roots are used in the treatment of various ailments such as uterine tumor, ulcer, stomach problems, kidney stones [5], gastrointestinal diseases [6], blood purification, jaundice, urine problems, chronic diarrhea [7], cough, bone injuries including fractures, malaria, asthma, backache and sexually transmitted infections [8]. Various biological activities including antihypertention [9], cardiac depressant [3], antihyperlipidemic [4] have also been reported. The fruit of *B. orthobotrys* has been reported to alleviate the consequences cardiovascular diseases in people [10]. In addition, the berries of this plant are used to color certain food items and the root extracts are used to give a specific yellow color to the locally manufactured butter. The first two alkaloids reported to be isolated from *B. orthobotrys* are pakistamine and 1-O-methylepakistanine. Moreover the alkaloids aporphine-benzylisoquinolines, chitraline, kalashine, khyberine, berbamine and oxyacanthine have been reported to be present in the species [11,12].

DOI:10.5138/09750185.1899

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The main aim of this study was to investigate the extracts of the plant species for various biological activities and isolate the chemical constituents responsible for these activities. This investigation includes the study of phytotoxic, antifungal, brine shrimp lethality, insecticidal, antibacterial and antilieshmanial properties of leaf, root and fruit extracts of *B. orthobotrys*. As a result of the investigation β-sitosterol (1), sesamin (3) and 10-eicosanol (3) were isolated and identified in the methanolic leaf extract of the plant species. A particular focus by the group is to better understand the therapeutic nature of *B. orthobotrys* to thus explain the indigenous use the plant enjoys within the context of natural product resources.

Material and Methods

Plant material

The plant *Berberis orthobotrys* (Berberideaceae) was collected in September 2012 and identified by Dr. Sher Wali Khan (taxanomist), Assistant Professor, Department of Biological Sciences, Karakoram International University, Gilgit. All three parts i.e. root, leaves and fruit were collected separately and were dried in the shade at room temperature. The roots and leaves of the plant were powdered separately and the fruit transformed into a semi liquid paste by using a grinder. The paste was allowed to dry naturally over several days.

Extraction and Isolation

The air-dried crushed parts of the plant *Berberis orthobotrys* including leaves (745 g), fruit (700 g) and root (652 g) were separately macerated with 100% MeOH (5 L) for 7 days at room temperature in separate bottles. The extract of each part was evaporated to dryness using a rotary evaporator at 40 ºC under reduced pressure to yield residues of each respective extract.

Thus the methanolic extracts of leaves (BOL, 325 g), fruit (BOF, 250 g) and root (BOR, 200 g) were obtained. In addition and for completeness, the flower part was also extracted with 30% H_2O:MeOH to get a BOFW residue. All the samples were submitted for various biological activities. However only the BOL extract was investigated for phytochemical isolation in the present study. (The BOF, BOFW and BOR extracts were not considered for phytochemical isolation.)

Thus the leaf extract (BOL, 300 g) was suspended in distilled water (250 mL) and extracted successively with *n*-hexane (3x500 mL), CH_2Cl_2 (3x500 mL) and EtOAc (3x500 mL). Each extract was evaporated by a rotary evaporator to yield the following residues; *n*-hexane (75 g), CH_2Cl_2 (26 g) and EtOAc (47 g) and a water residue.

The entire *n*-hexane fraction (75 g) was subjected to column chromatography (70-230 mesh, Merck) using 10% EtOAc:*n*-hexane as eluent to afford 105 fractions i.e. F1-F105. Fractions F34 and F59 were then further repeatedly chromatographed separately on silica gel columns (70-230 mesh, Merck) using 30% EtOAc:*n*-hexane as eluent. In this way F34 afforded 60 sub-fractions i.e. SF1-SF60. Sub-fraction SF12 was purified by preparative TLC using 15% EtOAc:*n*-hexane as eluent which afforded compound 1 (49 mg). Sub-fraction SF24 was purified by preparative TLC using 30% EtOAc:*n*-hexane as eluent to afford compound 2 (12 mg). Finally, F59 on preparative TLC and using 30% EtOAc:*n*-hexane as eluent afforded compound 3 (9 mg).

The isolated compounds were identified as β-sitosterol (1), 10-Eicosanol (2), and Sesamin (3) (Fig. 1) on the basis of their respective ^{1}H-NMR, ^{13}C-NMR, 2D, GC-MS, and IR spectra analysis and by comparison with those reported data in the related literature.

β-Sitosterol (1)

White crystalline solid; IR (KBr) υ_{max} cm^{-1}: 3348.5 (br, O-H stretching), 2957.2 (s) and 2864.4 (s, aliphatic C-H stretching), 1711.3 (w), 1667.8 (w, C=C absorption peak), 1462.9 (s, CH_2), 1442.8 (w), 1375.5 (s), 1365.8 (s), 1333.1 (w), 1303.7 (w), 1259.7 (s), 1236.2 (w), 1193.4 (w), 1156.9 (w), 1131.3 (w), 1057.0 (s), 1021.2 (s), 987.3 (w), 956.9 (s), 925.7 (w), 882.5 (w), 799.8 (s), 754.2 (s). ^{1}H-NMR ($CDCl_3$, 300 MHz) (ppm): 5.27-5.29 (1H, m), 3.39-3.51 (1H, m), 2.10-2.27 (2H, m), 1.85-1.98 (2H, m), 1.70-1.82 (3H), 1.35-1.64 (10H), 0.99-1.31 (13H), 0.94 (3H, s), 0.85 (3H, d, *J* = 6.48 Hz), 0.73-0.80 (9H, 3 Me), 0.61 (3H, s). ^{13}C-NMR ($CDCl_3$, 100 MHz) (ppm): 139.74 (C-5), 120.70 (C-6), 70.80 (C-3), 55.75 (C-14), 55.04 (C-17), 49.12 (C-9), 44.83 (C-25), 41.31 (C-13), 41.29 (C-4), 38.76 (C-12), 36.24 (C-1), 35.49 (C-10), 35.13 (C-20), 32.93 (C-22), 30.90 (C-7), 30.89 (C-8), 30.65 (C-2), 28.14 (C-24), 27.23 (C-16), 25.06 (C-23), 23.29 (C-15), 22.05 (C-28), 20.07 (C-11), 18.80 (C-27), 18.38 (C-26), 18.02 (C-19), 17.76 (C-21), 10.96 (C-29), 10.84 (C-18). GC/MS m/z: 414, 381, 329, 303, 273, 255, 213, 159, 145, 119, 107, 81, 55, 43.

Sesamin (2)

White solid; IR (KBr) υ_{max} cm^{-1}: 3079.6, 3008.4, 2956, 2919, 2849.9, 2783.3, 1729.7, 1562.5, 1499.3, 1442.1, 1366, 1248.9, 1035.2. ^{1}H-NMR ($CDCl_3$, 250 MHz) (, ppm): 2.96-3.04 (1H, H-3/H-7, m), 4.64 (1H, H-4/H-8, d, *J* = 4.30 Hz), 4.13-4.19 (1H, H-2a/H-6a, m), 3.77-3.82 (1H, H-2b/H-6b, m), 6.86-6.74 (2H, H-16/H-25, H-17/H-26, m), 6.77-6.78 (1H, H-10/H-19, m), 5.88 (2H, H-13/H-22, OCH_2O, s). ^{13}C-NMR ($CDCl_3$, 100 MHz) MHz) (, ppm): 54.31 (C-3, C-7), 71.69 (C-2, C-6), 85.77 (C-4, C-8), 106.47 (C-10, C-19), 108.17 (C-16, C-25), 119.33 (C-17, C-26), 135.03 (C-9, C-18), 147.09 (C-15, C-24), 147.95 (C-11, C-20). GC/MS m/z: 354, 279, 167, 149, 132, 104, 93, 71, 57 and 41.

10-Eicosanol (3)

White solid; IR (KBr) υ_{max} cm^{-1}: 3309.5 (br), 3214.8 (w), 2954.6 (w), 2915.0 (s), 2847.8 (s), 1469.1 (w), 1464.0 (w), 1411.1 (w),

1376.8 (w), 1260.9 (w), 1133.7 (w), 111.7 (w), 1105.4 (w), 1091.3 (w), 1067.8 (w), 1041.6 (w), 1031.2 (w), 1022.2 (w), 721.1 (s). ^{1}H-NMR ($CDCl_3$, 250 MHz) (, ppm): 3.55 (1H, s, H-10), 1.47 (1H, s, OH) 1.35 (8H, br s, H-8, 9, 11, 12), 1.18 (26H, br s, H- 2-7, 13- 19), 0.78-0.83 (6H, t, J = 6.92 Hz H- 1, 20), 3.48- 3.55 (1 H, m, H-10). ^{13}C-NMR ($CDCl_3$, 100 MHz) (ppm): 72.02 (C-10), 37.48 (C-9, 11), 31.91 (C-3), 31.88 (C-18), 29.68 (C-7, 13, 14) 29.56 (C-6, 15), 29.34 (C-5, 16), 29.30 (C-4, 17), 25.64 (C-8, 12), 22.66 (C-2, 19), 14.09 (C-1, 20).GC/MS m/z: 297 [M-H], 157, 139, 125, 111, 97, 83, 69.

Determination of phytotoxicity test

Phytotoxicity evaluation of the crude extracts was performed according to the modified protocol of McLaughlin (1988) [13] against the *Lemna minor* L. The test fractions were incorporated with sterilized E-medium at different concentrations i.e. 10, 100, 1000 µg/mL in 100% MeOH. Sterilized conical flasks were inoculated with fractions of the desired concentrations prepared from the stock solution and allowed to evaporate overnight. Each flask was inoculated with 20 mL of sterilized E-medium and then ten *L. minor,* each containing a rosette of three fronds were placed on the media. Other flasks were supplemented with methanol serving as negative control and reference inhibitor i.e. Parquet serving as positive control. Treatment was replicated three times and the flasks incubated at 28 C in a Fisons Fi-Totron 600 H growth cabinet for 12 days at 9000 lux intensity and a 56% ±10 relative humidity. Growth of *L. minor* for each fraction was determined by counting the number of fronds per dose and growth inhibition was calculated with reference to the negative control [13].

Determination of antifungal activity

The antifungal activity was determined by the Agar Tube Dilution Method against *Trichophylon longifusus*, *Candida albicans*, *Aspergillus flavus*, *Microsporum canis* and *Fusarium solani*. The methanolic extract was dissolved in DMSO. Sterile Sabouraud's dextrose agar medium (5 mL) was placed in a test tube and inoculated with the sample solution (400 µg/mL) kept in a slanting position at room temperature overnight. The fungal culture was then inoculated on the slant. The samples were incubated for 7 days at 27 ^{o}C and growth inhibition was observed and the percentage growth inhibition was calculated with reference to the negative control by applying the formula:

$$\% \text{ Inhibition} = 100 - \frac{\text{Linear growth and test (mm)}}{\text{Linear growth and control (mm)}} \times 100$$

Determination of brine shrimp lethality test

Brine shrimp (*Artemia salina* larvae) eggs were hatched in a shallow rectangular plastic dish filled with artificial seawater, prepared by mixing a commercial salt mixture (Instant Ocean, Aquarium System, Inc., Mentor, OH, USA) with double distilled water [14]. An unequal partition was made in the plastic dish with the help of a perforated device. Then 50 mg of eggs were sprinkled into the larger compartment, which was darkened whilst keeping the smaller compartment was open to ordinary light. After two days, naupils were collected. A sample of the test fraction was prepared by dissolving 20 mg of each fraction in 2 mL of methanol. From this stock solution, 1000, 100 and 10 µg/mL was transferred to 12 vials; three for each dilution and three vials were kept as control having 2 mL of methanol only. The solvent was allowed to evaporate overnight. When shrimp larvae were ready, 1 mL of sea water was added to each vial along with 10 shrimps and the volume was adjusted with sea water to 5 ml per vial. After 24 h, the number of surviving shrimps was counted. Data were analyzed by the Finney computer program [15] to determine the LD_{50}. Each experiment was replicated thrice.

Determination of insecticidal activity

Each of the three crude extracts were evaluated against different insects viz., *Tribolium castaneum*, *Sitophilus oryzae*, *Rhyzopertha dominica* and *Callosbruchus analis*. After 24 hours, 10 test insects were placed in each plate and incubated at room temperature for 24 hours with 50% relative humidity in a growth chamber. The results were analyzed as percentage mortality, calculated with reference to the positive and negative controls. Permethrin was used as the standard drug. Permethrin, acetone and test insects were used as positive and negative controls [16].

Determination of antibacterial activity

The antibacterial activity was evaluated by the agar-well diffusion method [17,18]. In this method one full loop of a 24 h old culture containing approximately 104-106 CFU was spread on the surface of Mueller-Hinton Agar plates. Wells were dug in the medium with the help of sterile metallic Cork borer. Stock solutions of the test samples in the concentration of 3 mg/mL were prepared in dimethyl sulfoxide (DMSO) and 100 µL dilutions were added in their respective wells. The antibacterial activity of extracts was compared with the standard drug imepinem which together with DMSO were used as positive and negative control respectively. Zones of inhibition (in mm) were recorded visually. The amount of growth in each well was measured visually by comparing with the growth in the control wells.

Results and Discussion

Phytochemical investigation of the hexane fraction of the crude methanolic leaf extract of *B. orthobotrys* resulted in the isolation of three known compounds viz., β-sitosterol (1), sesamin (2), and 10-eicosanol (3) and their structures were determined by means 1D and 2D NMR spectra (Figure 1).

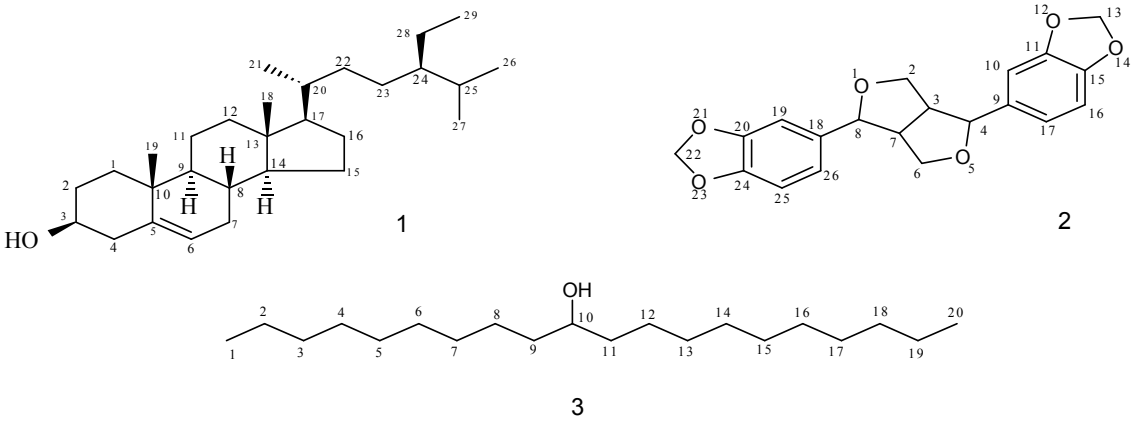

Figure 1. Structures of chemical constituents of the leaf extract of *Berberis orthobotrys*

Phytotoxicity test

The phytotoxicity of the methanolic extracts of leaves (BOL), root (BOR) and fruit (BOF) and flower (BOFW) of *B. orthobotrys* was carried out by using the modified protocol of Prof. McLaughline. This assay was performed at three different concentrations i.e. 1000, 100 and 10 µg/mL. The BOL, BOR and BOF extracts did not demonstrate any significant phytotoxic activity. However, the BOFW extract showed a moderate phytotoxic activity but only at the highest dose (Table 1).

Table 1. Phytotoxicity activity of different extracts of *B. orthobotrys*

Plant	Conc. (µg/mL)	No. of Fronds					% Growth Regulation			Conc. of Std. Drug (µg/mL)	
		BOL	BOR	BOF	BOFW	Control	BOL	BOR	BOF	BOFW	
Lemna minor L.	1000	14	16	16	11	20	30	20	20	45	0.015
	100	16	18	18	17		20	20	20	15	
	10	17	18	18	18		15	20	20	10	

BOL: *B. orthobotrys* leaves extract; BOR: *B. orthobotrys* root extract; BOF: *B. orthobotrys* fruit (100%) methanolic extract; BOFW: *B. orthobotrys* fruit (30% H_2O:MeOH) extract

Antifungal activity

The antifungal activity of the samples was determined by using the Agar Tube Dilution Method against fungi *viz. Trichophylon longifusus*, *Candida albicans, Aspergillus flavus, Microsporum canis, Candida glabirata* and *Fusarium solani.* Miconazole and amphotericin B were the standard drugs used. The BOL did not show any signifcant antifungal activity. However, the BOR and BOF extracts showed a minor antifungal activity against *Fusarium solani* (Table 2).

Table 2. Antifungal activities of different extracts of *B. orthobotrys*

Name of the fungus	Linear growth (mm)					100% Inhibition				Std. Drugs	MIC µg/mL
	Sample				Control						
	BOL	BOR	BOF	BOFW		BOL	BOR	BOF	BOFW		
Trichophylon longifusus	-				-	-				Miconazole	
Candida albicans	100	100	100	100	100	0%	0%	0%	0%	Miconazole	110.8
Aspergillus flavus	100	100	100	100	100	0%	0%	0%	0%	Amphotericin B	20.20
Microsporum canis	100	100	100	100	100	0%	0%	0%	0%	Miconazole	98.4
Fusarium solani	100	80	90	90	100	0%	20%	20%	20%	Miconazole	73.25
Candida glabirata	100	100	100	100	100	0%	0%	0%	0%	Miconazole	110.8

Antilieshmanial activity

Antileshmanial activity of all four parts of *B. orthobotrys* was determined by using amphotericine/petamidine as standard drugs. IC_{50} values indicate the effective concentration of a compound in µg/mL necessary to achive 50% growth inhibition. The root extract (BOR) exhibited good leishmanicidal activity having an IC_{50} value of 41±0.1 while BOL, BOF and BOFW did not show any significant Leishmancidal activity (Table 3).

Table 3: Antileishmanial activity of different extracts of *B. orthobotrys*

Leishmanicidal Activity		IC_{50} (µg/mL) ± S.D
Test samples	BOL	>100
	BOR	41±0.1
	BOF	>100
	BOFW	>100
Std. Drug Amphotericine B/Petamidine		0.29±0.05/5.09± 0.09

Miscellaneous activities

A sample of the test fractions was prepared by dissolving 20 mg of each fraction in 2 mL of MeOH. From this stock solution, 1000, 100 and 10 µg/mL were transferred to 12 vials; three for each dilution, and three vials were kept as control having 2 mL of MeOH only. The solvent was allowed to evaporate overnight. When shrimp larvae were ready, 1 mL of sea water was added to each vial along with 30 shrimps and the volume was adjusted with sea water to 5 mL per vial. The standerd drug used was etopside having a LD_{50} of 7.4625. After 24 hours, the number of surviving shrimps was counted, however the tested samples did not show any significant activity.

Furthermore the extracts were evaluated against different insects *viz*., *Tribolium castaneum*, *Sitophilus oryzae*, *Rhyzopertha dominica*, and *Callos bruchusanalis*. The test insects were incubated for 24 hours at 27 ^{0}C and 50% humidity in growth chamber. Permethrin was used as a standard drug, while acetone and test insects were used as positive and negative controls. However none of the test extracts showed any significant insecticidal activity.

Similarly stock solutions of the test samples in the concentration of 3 mg/mL were prepared in DMSO and 100 µL dilutions were added in their respective wells. The antibacterial activity of extracts was tested against the bacteria *viz. Escherichia coli, Bacillism subtilis, Shigella flexenari, Staphylococcus aureus, Pseudomonas aeruginosa and Salmonella typhi.* The activity of each each extract was compared with standard drug imepinem; the standard drug Imepinem and DMSO were used as positive and negative control. Zone of inhibition (in mm) was recorded visually. The amount of growth in each well was determined visually by comparing with the growth in the control wells but the test samples did not show any significant antibacterial activity.

Conclusion

In this study all four parts i.e. leaves, root, fruit and flower of the local medicinal plant *Berberis orthobotrys* were investigated for their biological activities viz., antibacterial, antilieshmanial, antifungal, phytotoxicity and brine shrimp lethality among others. Three known compounds viz., β-sitosterol (1), Sesamin (2) and 10-Eicosanol (3) were isolated from the hexane fraction of the leaf extract. Some of the extracts demonstrated good biological activities like the flower extract (BOFW) which exhibited moderate phytotoxic activity at the highest dose. The root and fruit extracts showed minor antifungal activity while the root extract showed good lieshmanicidal activity. Thus taken as a whole, use of the plant as a traditional medicine has a scientific base.

Conflict of Interest

None to declare

Acknowledgement

The authors thank HEC (Pakistan) for the financial support.

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