

## Bioassay-directed isolation of quaternary benzyloquinolines from *Berberis integerrima* with bactericidal activity against *Brucella abortus*

Gholamhassan Azimi<sup>1</sup>, Ali Hakakian<sup>2</sup>, Mustafa Ghanadian<sup>3,\*</sup>, Ali Joumaa<sup>3</sup>,  
and Saeed Alamian<sup>4</sup>

<sup>1</sup>Department of Chemistry, University of Isfahan, Isfahan, I.R. Iran.

<sup>2</sup>Department of Research and Development, Production and Research Complex, Pasteur Institute of Iran, Tehran, I.R. Iran.

<sup>3</sup>Department of Pharmacognosy and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

<sup>4</sup>Brucellosis Department, Razi Vaccine and Serum Research Institute, Karaj, I.R. Iran.

### Abstract

*Berberis integerrima* Bonge. (Syn: *Berberis densiflora* Boiss. & Buhse) is a shrub widely distributed in Middle East and central part of Asia. An ethnobotanical study revealed that indigenous and tribal people in Iran use *B. integerrima* root decoction for treatment of brucellosis. Therefore, the aim of this study was bioassay directed isolation of antibacterial compounds from this plant based on their *in vitro* bactericidal activity against *Brucella abortus*. Briefly, the ethanol extract of *B. integerrima* was fractioned and subjected to preliminary antibacterial screening tests against *Brucella*. The more active fraction (Fr.3) was subjected to purification by repeated chromatography systems. Quaternary benzyloquinoline alkaloids including columbamine, palmatine, berberine, and jatrorrhizine were four main components identified in the selected active fraction. Except for berberine which is reported before, palmatine, columbamine and jatrorrhizine are isolated for the first time from this plant. Anti-brucellosis properties of isolated compounds **1-4** were studied against *B. abortus* under different test conditions. In minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results, jatrorrhizine (**4**) showed more antibacterial activity with MIC and MBC of 0.78 and 1.56 µg/mL, respectively. In both agar well diffusion and disk diffusion ANOVA results showed that there were statistically significant differences between compounds **1-4** versus placebo in all of the tested concentration ( $P < 0.001$ ). In conclusion, all of four alkaloids showed potent antibacterial activity against *B. abortus* but jatrorrhizine and columbamine with free hydroxyl group on C-3 or C-2 showed more activity than palmatine and berberine without any free hydroxyl group on their structures. The antibacterial effects of columbamine (15 µg/mL) and jatrorrhizine (15 µg/mL) were comparative to streptomycin (10 µg/mL) as standard drug which candidate them for more pharmacological researches to find new antibacterial agents against brucellosis.

**Keywords:** *Berberis integerrima*; *Brucella abortus*; Brucellosis

### INTRODUCTION

Brucellosis is one of the most common diseases between human and mammals like sheep, goats, cows, cattle, dogs, and pig with more than 500 000 new cases each year (1). The Middle East is one of the brucellosis endemic areas and Syria has the highest annual incidence following with Iran, Iraq, and Saudi Arabia (1).

*Brucella* species are types of intracellular bacteria lives in the human lymph

nodes macrophages, mammary gland and reproductive system. Location of these microorganisms in macrophages protects them from the host defense systems and makes treatment with high failure and relapses rates either because of the inability of penetration of the most antibacterial drugs through the human macrophage cell membranes, or inability of innate and specific immunity system for removing infected phagocytic cells (2,3).

\*Corresponding author: M. Ghanadian  
Tel: +98-3137927131, Fax: +98-3136680011  
Email: ghanadian@pharm.mui.ac.ir

Access this article online



Website: <http://rps.mui.ac.ir>

DOI: 10.4103/1735-5362.223797

Today, standard drug regimen is limited to doxycycline, streptomycin, and rifampicin or immunomodulation with levamisole (4,5). But because of their high relapses and also the bacterial resistance newer treatment or combinational regimens are urgently needed. In ethnomedicine, *Berberis integerrima* by indigenous people of Iran is long been considered for the treatment of brucellosis (6,7).

*B. integerrima* Bonge. (Syn: *Berberis densiflora* Boiss.& Buhse) is a deciduous shrub up to 5 meters tall with obovate leaves, yellow wood and yellow flowers and red fruits. This plant belongs to the Berberidaceae family and grows widely in Middle East and central part of Asia (8). Berberry shrubs have ornamental uses in the parks and city and the fruit is used in food industries in preparation of jellies, concentrates, candies, juices, and marmalades (9). Karimov, et al. isolated interbrinine and intebrinine and reported from its root barks in 1993 (10).

Recently, biological and pharmacological studies on different *Berberis* species showed their antimicrobial, antioxidant, anti-diabetic, hepatoprotective, and antihypertensive effects (11). They are also used in traditional medicine for many years for treatment of infectious fevers, plagues, typhus, diarrhea, rheumatism and fractures (11). An ethnobotanical study found that indigenous and tribal peoples in provinces of Chaharmahal and Bakhtiari and Kohgiluyeh Boyer Ahmad in Iran are using a decoction of *B. integerrima* roots called "Zereshke Koohi" for treatment of brucellosis. (6,7). Therefore, this study was planned based on a bioassay directed method for isolation of major bioactive compounds possessing bactericidal activity against *Brucella abortus*.

## MATERIALS AND METHODS

### General methods

The structures of bioactive compounds were identified with  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ , and electrospray mass spectra (ESI-MS) data. The NMR spectra were acquired on a Bruker Avance AV400 (Germany). ( $^1\text{H}$  at 400 MHz and  $^{13}\text{C}$  at 100 MHz) using  $\text{CD}_3\text{OD}$

( $\delta_{\text{H}}$  3.31,  $\delta_{\text{C}}$  49.0 ppm) as solvent. The  $^{13}\text{C-NMR}$  multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) pulse sequences (DEPT 135 and DEPT 90). one-bond  $^1\text{H-}^{13}\text{C}$  connectivities were detected with heteronuclear single quantum correlation (HSQC), two and three bond heteronuclear  $^1\text{H-}^{13}\text{C}$  correlations by heteronuclear multiple bond correlation (HMBC) experiments, and  $^1\text{H-}^1\text{H}$  correlation of all protons which have J-J coupling by  $^1\text{H-}^1\text{H}$  correlation spectroscopy (COSY) spectra. ESI-MS spectra were measured on Shimadzu 2010EV liquid chromatography–mass spectrometry (LC-MS) system (Shimadzu, Japan). Thin layer chromatography (TLC) detection was achieved by spraying TLC silica gel aluminum foil (60 F254, Merck) with Dragendorff's and 1% cerium (IV) sulfate solution as TLC reagents followed by heating at 100 °C for about 2-10 min. Medium-pressure liquid chromatography (MPLC) was done by a Büchi 861 instrument and pump module C-601(Switzerland) on MPLC glass column dry packing with silica gel (25 – 40  $\mu\text{m}$ ) as the stationary phase. Preparative thin layer chromatography (PLC) was performed in chloroform:methanol:ammonia (79.5:20:0.5) on pre-activated (110 °C) silica gel plate (60 F254, 20 × 20 cm). In minimum inhibitory concentration (MIC) test wells optical density (OD) were examined by microplate reader (Tecan Sunrise, Reading, UK).

### Plant materials

*B. integerrima* roots were collected in autumn, from Zagros Mountains, in Shamsabad valley (besides Borujen-Lordegan road) about 60 km S, Chaharmahal va Bakhtiari province, in elevation of 2300 meters above the sea level. It was identified by Prof. Hojjatollah Saeidi, at Department of Biology, Faculty of Science, University of Isfahan, Iran. Its voucher specimen (no: 3537) was deposited in Pharmacognosy department, Isfahan faculty of Pharmacy, Isfahan University of Medical Sciences, Iran.

### Extraction and preliminary fractionation

Air-dried roots (2000 g) were powdered using an electrical mill (mesh number 100),

and extracted by percolation method with methanol. Percolated extract was filtered and evaporated by rotary evaporator in reduced pressure at 40 °C. The concentrated extract (85 g) was treated by diluted HCl (5 %, 2 L) and dichloromethane was added to acidic solution and partitioned in a separating funnel (three consecutive times). Organic layers were combined and concentrated to afford Fraction 1 (16 g). Aqueous residue was made alkaline by ammonia to pH 9 and stand up for 30 min. Dichloromethane was added and partitioned in a separating funnel (three consecutive times). Organic layer was evaporated and afforded fraction 2 (13 g). Aqueous layer was adjusted to pH 7 by dilute HCl (0.2 N) and evaporated in reduced pressure, and yielded Fraction 3 (4 g) (12).

#### Microbial strain and growth media

*B. abortus* strain RB-51 was obtained from the Department of Bacterial Vaccine and Antigen Production Pasteur Institute of Iran. Bacterial species was taken based on ethical clearance approval from the ethical committee. For inoculum preparation, *B. abortus* suspension was grown to the exponential phase in Mueller-Hinton Broth at 37 °C and adjusted to a density of  $10^8$  cfu/mL.

#### Determination of MIC and MBC of extract and fractions

Antimicrobial activities of total extract and fractions (Fr.1 - Fr.3) of *B. integerrima* were assessed using the standard minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Briefly, one row line in each plate was used for tobramycin (BioMerieux, Marcy-L'Etoile, France) as

positive control in a serial dilution of 32–0.015 µg/mL.

The stock sample solutions were diluted and transferred into the next wells, and serial dilutions were made so that concentrations in the range of 2000, 1000, 500, 250, 125, 62.50, 31.25, 15.62, 7.81, 3.90, 1.95 and 0.97 µg/mL were obtained. Then 5 µL of the inoculum density equivalent to 0.5 McFarland of *Brucella* suspension was added to each well and incubated in shaker-incubator at 37 °C for 17 h, and the wells optical density (OD) were examined for turbidity, indicating growth of the bacteria. The lowest concentration of samples that inhibited bacterial growth (lack of visual turbidity), was regarded as the MIC. Then, 100 µL of cell suspensions of MIC and 4 next dilutions showing no bacterial growth were cultured again on Mueller-Hinton agar plates and incubated for 24 h. MBC was regarded as the first lowest concentration after MIC which showed no bacterial growth on plates. Each concentration was repeated three times to ensure reproducibility (13).

#### Isolation and spectral data of bioactive compounds

According to the MIC and MBC results, the most bioactive fraction (Fr.3) was subjected to chromatography on a silicagel (25-40 µm) MPLC packed column using  $\text{CHCl}_3$ :MeOH in a stepwise gradient manner (5→20 %). Based on TLC analyses, fractions eluted by  $\text{CHCl}_3$ :MeOH (95:5), (93:7), (90:10), and (85:15) were purified on preparative layer chromatography (PLC) using  $\text{CHCl}_3$ :MeOH:NH<sub>3</sub> (79.5:20:0.5) solvent system and yielded compounds **1** (154 mg), **2** (250 mg), **3** (15 g), and **4** (110 mg), respectively (Fig. 1).

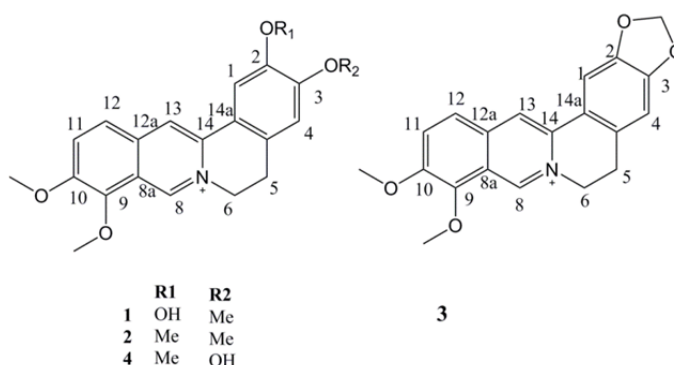


Fig. 1. Quaternary benzylisoquinolines isolated from *Berberis integerrima*.

**Table 1.**  $^{13}\text{C}$  NMR data of compounds **1-4** at 100 MHz, in  $\text{CD}_3\text{OD}$ .

Atom	1	2	3	4
1	109.4	109.9	106.6	109.9
2	157.5	153.8	152.2	151.6
3	151.4	152	151.7	156.4
4	116.9	120.5	121.5	116.2
4a	116.1	112.2	109.4	116.2
5	27.9	27.8	28.2	27.7
6	57.5	57.4	57.6	57.5
8	145.6	146.5	146.4	146.0
8a	135.8	135.3	135.2	135.6
9	145.6	145.8	145.7	143.8
10	151.2	150.9	150	150.6
11	124.2	124.6	124.5	124.4
12	128.1	128.1	128	128.1
12a	122.8	123.3	123.3	123.1
13	119.9	121.3	121.9	120.7
14	139.9	139.8	139.8	140.6
14a	130.7	130.1	131.9	130.4
2-MeO	-	57.0	-	56.9
3-MeO	56.6	56.7	-	-
9-MeO	57.7	57.7	57.6	57.7
10-MeO	62.5	62.6	62.5	64.4
-O-CH <sub>2</sub> -O-	-	-	103.7	-

**Columbamine (1, 0.008 %)**

Orange needles (MeOH);  $^1\text{H-NMR}$  ( $\text{CD}_3\text{OD}$ ): 3.15 (2H, t,  $J = 6.2$  Hz, H-5), 3.97 (3H, s, 3-OCH<sub>3</sub>), 4.09 (3H, s, 9-OCH<sub>3</sub>), 4.19 (3H, s, 10-OCH<sub>3</sub>), 4.90 (overlapped with solvent peak, H-6), 6.72 (1H, s, H-4), 7.53 (1H, s, H-1), 7.94 (1H, d,  $J = 9.17$  Hz, H-11), 8.04 (1H, d,  $J = 9.17$  Hz, H-12), 8.63 (1H, s, H-13), 9.64 (1H, s, H-8);  $^{13}\text{C-NMR}$  ( $\text{CD}_3\text{OD}$ ): (Table 1). Positive ESI-MS  $m/z$ : 338, 323, 294, 280, 265.

**Palmatine (2, 0.013 %)**

Yellow needles (MeOH);  $^1\text{H-NMR}$  ( $\text{CD}_3\text{OD}$ ): 3.27 (2H, t,  $J = 6.4$  Hz, H-5), 3.95 (3H, s, 2-OCH<sub>3</sub>), 4.00 (3H, s, 3-OCH<sub>3</sub>), 4.11 (3H, s, 9-OCH<sub>3</sub>), 4.21 (3H, s, 10-OCH<sub>3</sub>), 4.94 (2H, t,  $J = 6.34$ , H-6), 7.06 (1H, s, H-4), 7.67 (1H, s, H-1), 8.03 (1H, d,  $J = 9.12$  Hz, H-11), 8.12 (1H, d,  $J = 9.12$  Hz, H-12), 8.83 (1H, s, H-13), 9.78 (1H, s, H-8);  $^{13}\text{C-NMR}$  ( $\text{CD}_3\text{OD}$ ): (Table 1). Positive ESI-MS  $m/z$ : 352, 337, 322, 307, 292.

**Berberine (3, 0.75 %)**

Yellow needles (MeOH);  $^1\text{H-NMR}$  ( $\text{CD}_3\text{OD}$ ): 3.26 (2H, t,  $J = 6.5$  Hz, H-5), 4.21 (3H, s, 9-OCH<sub>3</sub>), 4.11 (3H, s, 10-OCH<sub>3</sub>), 4.85 (overlapped with solvent peak, H-6), 6.11 (O-CH<sub>2</sub>-O), 6.97 (1H, s, H-4), 7.67 (1H, s, H-1), 8.01 (1H, d,  $J = 9.10$  Hz, H-11), 8.12 (1H, d,  $J$

= 9.10 Hz, H-12), 8.71 (1H, s, H-13), 9.77 (1H, s, H-8);  $^{13}\text{C-NMR}$  ( $\text{CD}_3\text{OD}$ ): (Table 1). Positive ESI-MS  $m/z$ : 336, 320, 292, 278.

**Jatrorrhizine (4, 0.006 %)**

Orange needles (MeOH);  $^1\text{H-NMR}$  ( $\text{CD}_3\text{OD}$ ): 3.15 (2H, t,  $J = 6.2$  Hz, H-5), 3.96 (3H, s, 2-OCH<sub>3</sub>), 4.09 (3H, s, 9-OCH<sub>3</sub>), 4.19 (3H, s, 10-OCH<sub>3</sub>), 4.90 (overlapped with solvent peak, H-6), 6.70 (1H, s, H-4), 7.51 (1H, s, H-1), 7.94 (1H, d,  $J = 9.16$  Hz, H-11), 8.05 (1H, d,  $J = 9.16$  Hz, H-12), 8.63 (1H, s, H-13), 9.63 (1H, s, H-8);  $^{13}\text{C-NMR}$  ( $\text{CD}_3\text{OD}$ ): (Table 1). Positive ESI-MS  $m/z$ : 338, 323, 294, 280, 265.

**Determination of MIC and MBC of isolated compounds**

Briefly, the stock sample solutions (1000  $\mu\text{g/mL}$ ) of isolated compounds **1-4** were diluted and transferred into the wells at concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, and 0.19  $\mu\text{g/mL}$ . 5  $\mu\text{L}$  of the inoculum density equivalent to 0.5 McFarland of *B. abortus* suspension was added to each well and incubated at 37 °C for 17 h, and then the wells OD were examined for turbidity. MBC was regarded as the first lowest concentration after MIC which showed no bacterial growth on plates (12,14).

### Antibacterial activity of isolated compounds by agar well diffusion method

*B. abortus* inoculum (McFarland turbidity standard 0.5) was plated onto Mueller–Hinton agar by sterile swabs. Wells (6 mm diameter) were created in the plates using a sterile Pasteur pipette. The wells were filled with different concentrations of isolated compounds (1, 3, 6, 8, 10, and 15  $\mu\text{g/mL}$  per well) and streptomycin (10  $\mu\text{g/mL}$  per well) as positive control. Then plates were incubated at 37 °C for 24 h. The diameters of the inhibitory zones were measured in millimeters and compared based on clinical and laboratory standards institute (CLSI) criteria in comparison with positive control (14,15).

### Antibacterial activity of isolated compounds by agar disc diffusion method

5  $\mu\text{L}$  of different concentrations of isolated compounds (1, 3, 6, 8, 10, and 15  $\mu\text{g/mL}$ ) and streptomycin (10  $\mu\text{g/mL}$ ) as positive control were added to paper disks. The sample discs were left overnight to dry their moisture. *B. abortus* colonies were suspended in Mueller–Hinton broth and adjusted to a density equal to McFarland turbidity standard 0.5. Suspensions were spread onto the plates with sterile cotton swabs and then the discs were added. The plates were incubated at 37 °C for 24 h. The diameters of the inhibitory zones were measured in millimetres (16).

### Statistical analysis

The results are presented as mean  $\pm$  standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by Dunnett's post hoc comparison was used for multiple between-group comparisons. Within-group comparisons were determined using paired sample t-test.

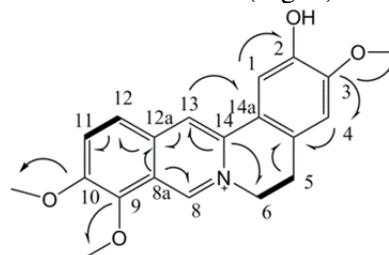
## RESULTS

### Identification of bioactive compounds

The methanol extract of *B. integerrima* was partitioned based on its polarity and basicity to three different fractions. Determination of MIC and MBC showed that MIC values of total extract, Fr.1, Fr.2, and Fr.3 against *B. abortus* were 620, 500, 250, and

120  $\mu\text{g/mL}$ , and MBC values were 1250, 1000, 500, and 250  $\mu\text{g/mL}$ , respectively. Based on antibacterial screening results, the most active fraction, Fr.3 was subjected to more purification by MPLC and PLC chromatography and yielded **1** to **4** as pure compounds (Fig. 1).

Compound **1** was afforded as an orange powder with positive reaction to Dragendroff's reagent (an orange coloration).  $^1\text{H-NMR}$  spectrum showed four one proton singlets at 6.72 (s, H-4), 7.53 (s, H-1), 8.63 (s, H-13), 9.64 (s, H-8), two aromatic AB doublets resonating at 7.94 (d,  $J = 9.17$ , H-11), and 8.04 (d,  $J = 9.17$ , H-12), two methylene at 3.15 (t,  $J = 6.2$ , H-5), and 4.90 ppm overlapped on solvent peak (H-6) and three methoxy singlets resonating at 3.97, 4.09, and 4.19 ppm. In addition to three methoxy groups,  $^{13}\text{C-NMR}$  and DEPT spectra of the main alkaloid core showed seventeen carbons comprising of two  $\text{sp}_3$  methylenes, six olefinic methines, and nine quaternary  $\text{sp}_2$  carbones similar to that reported for berberine type quaternary benzylisoquinoline alkaloids. The  $^1\text{H-}$  and  $^{13}\text{C-NMR}$  data (experimental section) were assigned based on HSQC, double quantum filter (DQF-COSY) and HMBC data. Methoxy groups were located at C-9, C-10, and C-3 based on HMBC correlations of  $\delta_{\text{H}}$  4.09 with C-9 ( $\delta_{\text{C}}$  145.6),  $\delta_{\text{H}}$  4.19 with C-10 ( $\delta_{\text{C}}$  151.2), and  $\delta_{\text{H}}$  3.97 with C-3 ( $\delta_{\text{C}}$  151.4). Based on these data and C-NMR downfield shifts of C-2 at  $\delta_{\text{C}}$  157.5, as well as HMBC correlation of H-4 ( $\delta_{\text{H}}$  6.72) with C-3 bearing methoxy group at  $\delta_{\text{C}}$  151.4, H-4 with C-4a, and H-1 with C-2 compound **1** was identified as columbamine in agreement with literature data (15). Surprisingly  $^{13}\text{C-NMR}$  data is not published in the literature and to the best of our knowledge  $^{13}\text{C-NMR}$  and its HSQC assignment is reported for the first time (Fig. 2).



**Fig. 2.**  $^1\text{H-}^1\text{H}$  correlation spectroscopy (COSY) (in bold), and selected heteronuclear multiple bond correlation ( $\rightarrow$ ) detected for compound **1**.

Compound **2** was obtained as a yellow powder with positive reaction to Dragendorff's reagent (an orange coloration).

<sup>1</sup>H-NMR spectrum showed four one proton singlets at 7.06, 7.67, 8.83, and 9.78, two aromatic AB doublets resonating at 8.03, and 8.12 (each 1H,  $J = 9.12$ ), two triplets at 3.27, 4.94 (each 2H with  $J = 6.4$  Hz), and four sharp methoxy peaks resonating at 3.95, 4.00, 4.11, 4.21.

<sup>13</sup>C-NMR and DEPT spectra of the main alkaloid core showed seventeen carbons similar to those reported for compound **1**, in addition to four methoxy group attached to the main core. High resolution EI mass of 352.1523 suggests its molecular formula to be C<sub>21</sub>H<sub>22</sub>O<sub>4</sub>N<sub>1</sub>, and comparison of <sup>1</sup>H- and <sup>13</sup>C-NMR chemical shifts with compound **1**, it was identified as palmatine cation (15,17).

Compound **3** was a yellow powder with positive ESI Mass of 336 ( $m/z$ ). Inspection of its NMR spectral data (experimental data) showed distinct similarity resonances with compound **2** except for the appearance of a methylenedioxy (O-CH<sub>2</sub>-O) function at  $\delta_C$  103.7 ( $\delta_H$  6.11, 2H, s) and loss of two methoxy groups which was in agreement with berberine cation in the literature (18).

Compound **4** was obtained as an orange powder and showed resonances similar to compound **2** except for the loss of one methoxy group at C-3 and a downfield chemical shift resonated at  $\delta_C$  156.4 related to the presence of a phenolic group and identified as jatrorrhizine (12).

To the best of our knowledge, jatrorrhizine <sup>13</sup>C-NMR data is being reported for the first

time and differs from **1** in the resonances of ring 1 especially resonances of C-2 ( $\delta_C$  156.4), C-3 ( $\delta_C$  151.6), and methoxy group attached to C-2 ( $\delta_C$  56.9).

To check the anti-brucellosis properties of isolated compounds, the *in vitro* bacteriostatic and bactericidal activities of compounds **1-4** were studied against *B. abortus* under different test conditions including determination of MIC and MBC, agar well diffusion, and agar disk diffusion which are presented in Table 2, Fig. 3, and Fig.4.

As presented in Table 2, in MIC and MBC results, jatrorrhizine (**4**) showed more antibacterial activity with MIC and MBC of 0.78 and 1.56  $\mu\text{g/mL}$ , respectively comparable with tobramycin as standard drug (MIC = 1.00  $\mu\text{g/mL}$ , and MBC = 2.00  $\mu\text{g/mL}$ ).

In agar well diffusion statistically significant differences between compounds **1-4** versus placebo in all tested concentrations ( $P < 0.001$ ) were observed. Also significant between-group differences at lower concentration 1  $\mu\text{g/mL}$  ( $P < 0.05$ ) between columbamine and palmatine were observed (Fig. 3).

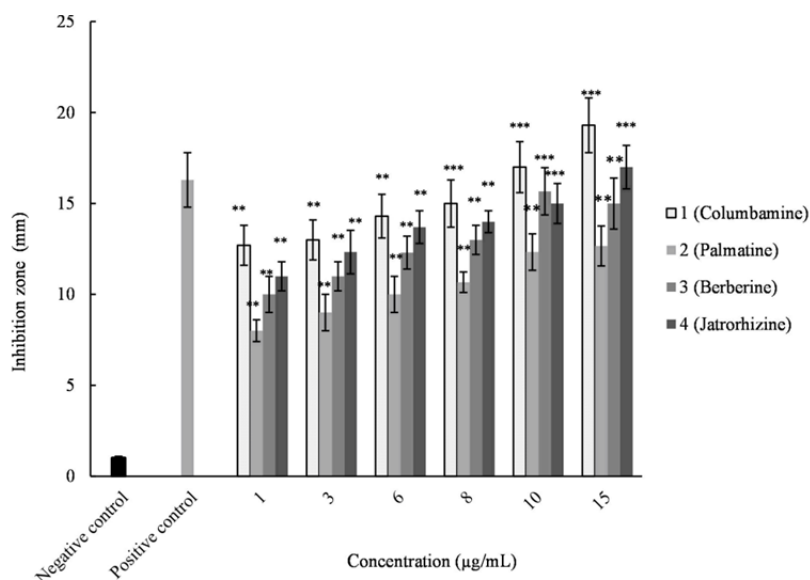
In disk well diffusion there were also significant differences between compounds **1-4** versus placebo in all tested concentrations ( $P < 0.001$ ). Significant between-group differences between compounds **1-4** in all tested concentrations ( $P > 0.05$ ) were not observed.

However, columbamine seems to have more activity at higher concentration of 15  $\mu\text{g/mL}$  compared with streptomycin (10  $\mu\text{g/mL}$ ) as the standard drug (Fig. 4).

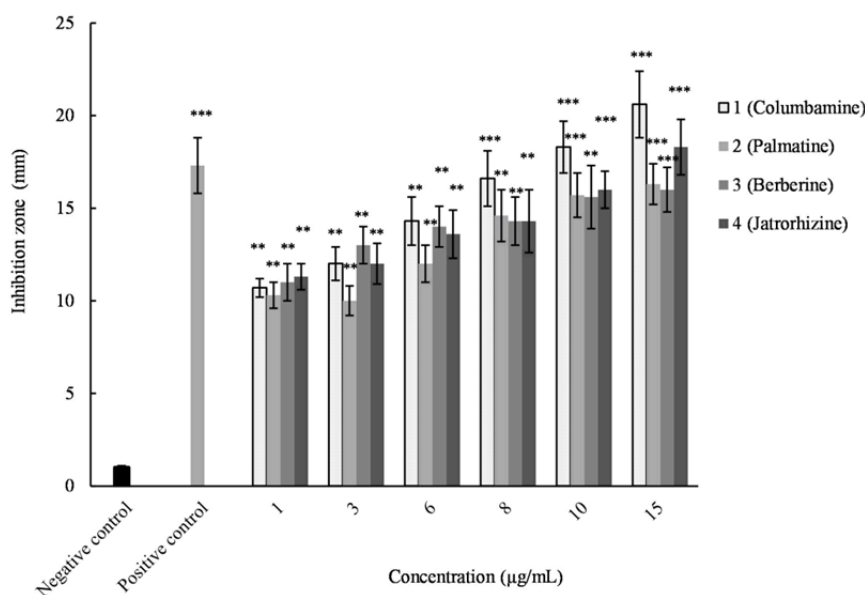
**Table 2.** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of isolated compounds from *Berberis integerrima*.

Compounds	MIC ( $\mu\text{g/mL}$ )	MBC ( $\mu\text{g/mL}$ )
1 (Palmatine)	6.25	12.50
2 (Berberine)	1.56	3.12
3 (Columbamine)	3.12	6.25
4 (Jatrorrhizine)	0.78	1.56
*Tobramycine	1.00	2.00

\*The clinical and laboratory standards institute (CLSI) minimum inhibitory concentration interpretive criteria for tobramycin = 4  $\mu\text{g/mL}$ , susceptible; 8  $\mu\text{g/mL}$ , intermediate; and 16  $\mu\text{g/mL}$ , resistant.



**Fig. 3.** Antibacterial activity by agar well diffusion in six different concentrations. Clinical and laboratory standards institute (CLSI) zone diameter interpretive criteria for streptomycin (10 µg/mL) as positive control.  $\geq 15$ , susceptible; 12-14, intermediate;  $\leq 11$ , resistant. (\* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus negative control).



**Fig. 4.** Antibacterial effect by agar disk diffusion in six different concentrations. The clinical and laboratory standards institute (CLSI) zone diameter interpretive criteria for streptomycin (10 µg/mL) as positive control.  $\geq 15$ , susceptible; 12-14, intermediate;  $\leq 11$ , resistant. (\* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus negative control).

## DISCUSSION

Based on preliminary MIC and MBC screening, the most active fraction, Fr.3 was subjected to phytochemical analysis and yielded four pure alkaloids as palmatine, berberine, columbamine, and jatrorhizine. These compounds showed potent anti-brucellosis properties *in vitro* against

*B. abortus* as determined by MIC, MBC, agar well diffusion, and agar disk diffusion tests. The antimicrobial effects of total extract and isolated isoquinoline alkaloid were in agreement with antimicrobial properties reported for other *Berberis* species and close to isoquinoline alkaloids in the literature. Previously, Ghasemi Pirbalouti, *et al.* reported that *B. integerrima* root bark extract had

significant anti-brucellosis activity *in vivo* (7). Alimirzaee, *et al.* also reported *B. integerrima* fruits showed a synergistic effect on ampicillin bactericidal activity against *Staphylococcus aureus* (19). In a study designed by Jacquelyn Villinski, *et al.* on antibacterial activity of Japanese berberry (*B. thunbergii*), the root ethanolic extract showed antibacterial activity against five bacteria. It showed more activity against *Streptococcus pyogenes* and *Staphylococcus aureus* (20). In another study by Irshad, *et al.* the methanolic root extract of *B. lycium* showed higher antibacterial activity than other solvent extracts against *Pseudomonas* sp., *Escherichia coli*, *Streptococcus* sp. and *Staphylococcus* sp (21). In another study by Meenakshi Singh, *et al.* the antimicrobial activity of hydroalcoholic extracts of four *Berberis* species including *B. aristata*, *B. asiatica*, *B. chitria* and *B. lycium* were tested against eleven bacterial species. The hydroalcoholic extracts of root and stem of the *Berberis* species showed significant antibacterial activity against most of the tested bacteria. Among them, *B. aristata* root and *B. lycium* stem extracts showed more activity with low MIC values (22). In a study conducted by Maqal de Q. Pauloa, *et al.* on antimicrobial activities of benzyloquinoline alkaloids, bark of *Annona salzmarzii* from Annonaceae family, yielded four benzyloquinoline alkaloids, including reticuline, anonaine, laureilptine and isoboldine of which anonaine, an isoquinoline alkaloid with aporphine core structure, showed moderate antibacterial properties and others showed antifungal activities (23). In a similar study by Villar A, *et al.* antimicrobial activity of 14 benzyloquinoline alkaloids were investigated by agar diffusion and agar dilution methods against different microorganisms. Among them, alkaloids with aporphine core structure specially noraporphine and oxoaporphine groups, showed more activity against *Mycobacterium* and Gram positive bacteria, but none of them had significant activity against Gram negative rods (24). The exact mechanism of antibrucellosis activity of isolated protoberberine alkaloids is unclear and a new molecular approach to find their specific

action is required. However, berberine mechanism of action is known. It is a hydrophobic cation that increases membrane permeability and its positive charge facilitates its effective accumulation in bacterial cells which enhances its antimicrobial activity, i.e. against multi-drug resistant *Escherichia coli* (25). In a structure activity relationship study designed by Iwasa, *et al.* seventeen quaternary protoberberine alkaloids including berberine derivatives were tested against *Plasmodium falciparum*. The antimalarial activity was more influenced by the type of the oxygen substituents on rings A, C and D and especially the position of the oxygen functions on ring D. Briefly, the type of the oxygen substituents at ring D, and especially at the C-13 position (ring C) strongly influenced the activity. Compounds with methylenedioxy group at ring A showed more activity than those which have ortho di methoxy groups on ring A. Replacement of each of the free hydroxy groups at ring A by methoxy groups also reduced the activity (26). A similar structure–activity relationship was also observed in our study. All of four protoberberine alkaloids 1-4 showed potent antibacterial activity against *B. abortus*. Among them jatrorrhizine and columbamine with free hydroxyl group on C-3 or C-2 at ring A showed more activity than palmatine and berberine without any free hydroxyl group on ring A in their structure. Berberine with methylenedioxy group at ring A showed more activity than palmatine with two ortho methoxy.

## CONCLUSION

In this research bioactivity guided technique was successively used for separation of active antibacterial compounds of *B. integerrima* against *B. abortus in-vitro*. The most active fraction Fr.3 inhibited growth of bacteria with MIC and MBC concentrations 12 and 25 µg/mL, respectively. Quaternary benzyloquinoline alkaloids including palmatine, berberine, columbamine and jatrorrhizine were identified in the selected active fraction. All of four alkaloids showed potent antibacterial activity against *B. abortus*



but jatrorrhizine and columbamine with free hydroxyl group on C-3 or C-2 showed greater activity than palmatine and berberine without any free hydroxyl group on their structure.

### ACKNOWLEDGMENTS

This research was supported by the research project No. 99735 from the Research Council of University of Isfahan. The authors also would like to thank the Research Department of Isfahan University of Medical Sciences, Isfahan, I.R. Iran, Pasteur Institute of Iran, and Husein Ebrahim Jamal (HEJ) Research Institute of Chemistry at University of Karachi, Karachi-75270, Pakistan for their supports.

### REFERENCES

- Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. *Lancet Infect Dis.* 2006;6:91-99.
- Franco MP, Mulder M, Gilman RH, Smits HL. Human brucellosis. *Lancet Infect Dis.* 2007;7:775-786.
- Porte F, Naroeni A, Ouahrani-Bettache S, Liautard JP. Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome fusion in murine macrophages. *Infect Immun.* 2003;71:1481-1490.
- Skalsky K, Yahav D, Bishara J, Pitlik S, Leibovici L, Paul M. Treatment of human brucellosis: systematic review and meta-analysis of randomised controlled trials. *BMJ.* 2008;336:701-704.
- Dizer U, Hayat L, Beker CM, Görenek L, Özgüven V, Pahsa A. The effect of the doxycycline-rifampicin and levamisole combination on lymphocyte subgroups and functions of phagocytic cells in patients with chronic brucellosis. *Chemotherapy.* 2005;51:27-31.
- Pirbalouti AG. Medicinal plants used in Chaharmahal and Bakhtyari districts of Iran. *Herba Polonica,* 2009;55:69-77.
- Ghasemi Pirbalouti A, Ghasemi MR, Momtaz H, Golparvar AR, Hamed B, Shahgholian L. The effect of some of the Iranian medicinal plants on *Brucella Abortus* on *In-vitro* and *In-vivo*. *J Herb Med.* 2010;1:21-29.
- Ashraf H, Heidari R, Nejati V. Antihyperglycemic and antihyperlipidemic effects of fruit aqueous extract of *Berberis integerrima* Bge. in streptozotocin-induced diabetic rats. *Iran J Pharm Res.* 2014;13:1313-1318.
- Alemardan A, Asadi W, Rezaei M, Tabrizi L, Mohammadi S. Cultivation of Iranian seedless barberry (*Berberis integerrima* 'Bidaneh'): A medicinal shrub. *Ind Crops Prod.* 2013;50:276-287.
- Karimov A, Vinogradova VI, Shakirov R. *Berberis* alkaloids. XXII. Interbrinine and intebimine-New alkaloids from *Berberis integerrima*. *Chem Nat Compd.* 1993;29:57-60.
- Arayne MS, Sultana N, Bahadur SS. The *berberis* story: *Berberis vulgaris* in therapeutics. *Pak J Pharm Sci.* 2007;20:83-92.
- Suau R, Rico R, López-Romero JM, Nájera F, Cuevas A. Isoquinoline alkaloids from *Berberis vulgaris* subsp. *australis*. *Phytochemistry.* 1998;49(8):2545-2549.
- Reller LB, Weinstein M, Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin Infect Dis.* 2009;49:1749-1755.
- Ayatollahi AM, Ghanadian M, Afsharypuor S, Siddiq S, Pour-Hosseini SM. Biological screening of *Euphorbia aellenii*. *Iran J Pharm Res.* 2010;9:429-436.
- Hsieh TJ, Chia YC, Wu YC, Chen CY. Chemical constituents from the stems of *Mahonia japonica*. *J Chin Chem.* 2004;51:443-446.
- Boyanova L, Gergova G, Nikolov R, Derejian S, Lazarova E, Katsarov N, Mitov I, Krastev Z. Activity of *Bulgarian propolis* against 94 *Helicobacter pylori* strains *in vitro* by agar-well diffusion, agar dilution and disc diffusion methods. *J Med Microbiol.* 2005;54:481-483.
- Li CY, Lu HJ, Lin CH, Wu TS. A rapid and simple determination of protoberberine alkaloids in cortex phellodendri by <sup>1</sup>H-NMR and its application for quality control of commercial traditional Chinese medicine prescriptions. *J Pharm Biomed Anal.* 2006;40:173-178.
- Leyva-Peralta MA, Robles-Zepeda RE, Garibay-Escobar A, Ruiz-Bustos E, Alvarez-Berber LP, Gálvez-Ruiz JC. *In vitro* anti-proliferative activity of *Argemone gracilentia* and identification of some active components. *Complement Altern Med.* 2015;15:13-19.
- Alimirzaee P, Gohari AR, Hajiaghvae R, Mirzaee S, Jamalifar H, Monsef Esfahani HR, *et al.* 1-methyl malate from *Berberis integerrima* fruits enhances the antibacterial activity of ampicillin against *Staphylococcus aureus*. *Phytother Res.* 2009;23:797-800.
- Villinski J, Dumas E, Chai HB, Pezzuto J, Angerhofer C, Gafner S. Antibacterial activity and alkaloid content of *Berberis thunbergii*, *Berberis vulgaris* and *Hydrastis canadensis*. *Pharm Biol.* 2003;41:551-557.
- Irshad AH, Pervaiz AH, Abrar YB, Fahelboum I, Awen BZ. Antibacterial activity of *Berberis lycium* root extract. *Trakia J Sci.* 2013;1:88-90.
- Singh M, Srivastava S, Rawat AK. Antimicrobial activities of Indian *Berberis* species. *Fitoterapia.* 2007;78(7-8):574-576.
- Paulo MD, Barbosa-Filho J, Lima EO, Maia RF, de Cassia R, Barbosa BB, *et al.* Antimicrobial activity of benzylisoquinoline alkaloids from *Annona salzmanii* DC. *J Ethnopharmacol.* 1992;36:39-41.

24. Villar A, Mares M, Rios JL, Canton E, Gobernado M. Antimicrobial activity of benzyloquinoline alkaloids. *Die Pharmazie*. 1987;42:248-250.
25. Bandyopadhyay S, Patra PH, Mahanti A, Mondal DK, Dandapat P, Bandyopadhyay S, *et al.* Potential antibacterial activity of berberine against multi drug resistant enterovirulent *Escherichia coli* isolated from yaks (*Poephagus grunniens*) with haemorrhagic diarrhoea. *Asian Pac J Trop Dis*. 2013;6:315-319.
26. Iwasa K, Nishiyama Y, Ichimaru M, Moriyasu M, Kim HS, Wataya Y, *et al.* Structure-activity relationships of quaternary protoberberine alkaloids having an antimalarial activity. *Eur J Med Chem*. 1999;34:1077-1083.