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Activity Guided Isolation of Nematicidal Constituents from the Roots of *Berberis brevissima* Jafri and *Berberis parkeriana* Schneid

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ABSTRACT

Biological screening of different parts of the selected *Berberis* species (*B. brevissima* Jafri and *B. parkeriana* Schneid) showed that methanolic root extract possessed significant efficacy against *Meloidogyne javanica* (a root knot nematode). From root methanolic extracts of selected *Berberis* species four isoquinoline alkaloids; jatrorrhizine, dehydrocheilnthifoline, berberine and berberrubine were isolated. Structures of the isolated compounds were determined by using EIMS, ^{1}H and ^{13}C NMR, and other 2D spectroscopic techniques. Percentage juveniles mortality of *M. javanica* was determined at various concentrations (100, 200 and 300 µg mL^{-1}) using carbofuran as control. Berberine possessed the highest nematicidal activity (71.33%) followed by jatrorrhizine (59.50%). The *in vitro* results suggested that these compounds from *Berberis* species could be potential novel nematicides against *M. javanica*.

Keywords: Nematicidal activity; *Berberis brevissima*; *Berberis parkeriana*; *Meloidogyne javanica* and isoquinoline alkaloids

1. Introduction

All over the world, in the field of agriculture root knot nematodes (*Meloidognye* species) are mainly responsible for huge economic losses (Liu et al 2011). For several decades the use of various chemical nematicides is an important tool to control root knot nematodes. But due to its negative impact on environment and after use resistances, have either reduced or totally banned its use, therefore these nematicides must be replaced with safe and more effective chemical nematicides (Zuckerman & Esnard 1994). In the area of vegetables and fruits production, approximately 70 billion U.S. dollar crop damage is due to these root knot nematodes (*Meloidognye* species) annually (Reynolds et al 2011). Amongst the all probable strategies for controlling these pests, the biocontrol agents obtained from plant or microorganisms could be used to lower non-target contact of harmful pesticides and to face resistance growth (Isman 2006; Tian et al 2007). Different types of plants, constituents and

metabolites have been screened for efficacy against various plant nematodes (Hong et al 2007; Thoden et al 2009; Ntalli et al 2010; Bai et al 2011).

Berberis (Berberidaceae) posses more than 500 species and is the only genus of the family in the southern hemisphere (Bai et al 2011). The genus *Berberis* is full of isoquinoline alkaloids having high potential in the treatments of many ailments and insects control (Baird et al 1997; Wright et al 2000; Quevedo et al 2008). The hydro ethanolic extract of *Berberis* species (*B. aristata*, *B. asiatica*, *B. chitria* and *B. lyceum*) have shown very good antimicrobial efficacy against bacterial (eleven) and fungal (eight) strains (Küpeli et al 2002). In scurvy angina, sore throat and dysentery its leaves decoction has been used as antiscorbutic. Berries of the genus could be used as a tonic and be used in the form of a dye (Singh et al 2007). Various Chinese folk remedies have reported use of different species of the genus *Berberis* (*B. aquifolium*, *B. aristata* and *B. vulgaris*) for inflammations and rheumatic problems (Li et al 1989; Ju et al 1990; Teh et al 1990; Kondo et al 1992; Saied & Begum 2004). *B. aristata* have shown a very high anticancer efficacy against colon cancer cell line (HT29) (Seow et al 1992). In the current study, we have isolated four isoquinoline alkaloids through activity guided fractionation of methanolic crude extract of roots of *Berberis* species (*B. brevissima* and *B. parkeriana)* and studied their efficacy against root knot nematode *M. javanica.*

2. Material and Methods

2.1. General

Silica gel 60 (0.063-0.200 mm) was used for Column Chromatography (CC) while silica gel 60 PF254 was used for preparative Thin Layer Chromatography (TLC). The melting points of isolated compounds were determined by the melting point apparatus (Bibby Scientific Limited, Stone Staffordshire ST15 0SA, UK). UV spectra were taken by Thermo Spectronic UNICAM UV 300. IR spectra were recorded using JAESCO FT/IR-4200/A. Spectral characterizations of the compounds were performed by using Bruker AVANCE 500 and 400 MHz instruments. ^{13}C Nuclear Magnetic Resonance spectra (^{13}C NMR) were recorded at 100 MHz while ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectra at 500 MHz and 400 MHz using deutrated chloroform and methanol as solvents. EIMS (JEOL MSRoute) was determined by using direct insertion probe.

2.2. Plant material

B. brevissima roots (2.5 kg) were collected from Tirah (Khyber Agency, Khyber Pakhtunkhwa, Pakistan) and *B. parkeriana* roots (2 kg) from Dir (Lower) (Khyber Pakhtunkhwa). The species were identified by Prof. Dr. Jandar SHAH (Ex. Voice Chancellor Benazir Bhutto University, Sherengal, Khyber Pakhtunkhwa, Pakistan). The voucher specimens (No. Bot/10710 and 8719) were deposited in herbarium (Department of Botany, University of Peshawar).

2.3. Extraction and chromatography

The plants material were socked in 95% methanol for 7 days and the solvent was then evaporated at 40 ^{o}C (reduced pressure), using rotary evaporator. The residue obtained (*B. brevissima* root methanolic extract (BBR-MeOH= 225.7 g) and *B. parkeriana* root methanolic extract (BPR-MeOH= 186.5 g)) were dissolved in 6.5 L of 5% acidic water, filtered and left over night at room temperature. The yellow precipitate was filtered to obtained fraction A (*B. brevissima* root fraction A (BBR-FA= 86.3 g) and *B. parkeriana* root fraction A (BPR-FA= 77.5 g)). The filtrate was then extracted with CH_2Cl_2 (3x300 mL). The organic layer was separated and evaporated to afford fraction B (*B. brevissima* root fraction B (BBR-FB= 8.5 g) and *B. parkeriana* root fraction B (BPR-FB= 6.0 g)). The aqueous (acidic) layer was basified (pH 9-10) with concentrated aqueous NH_3 and then extracted with $CHCl_3$ (3×300 mL). Which was evaporated to afford fraction C (*B. brevissima* root fraction C (BBR-FC= 17.5 g) and *B. parkeriana* root fraction C (BPR-FC= 14.8 g)). The remaining aqueous layer was dried to obtained fraction D (*B. brevissima* root fraction D (BBR-FD= 53.5 g) and *B. parkeriana* root fraction D (BPR-FD= 45.8 g)).

2.3.1. Fraction A

On recrystalization of fraction A (BBR-FA and BPR-FA) almost pure berberine (3) (56 g) was obtained, mp 207-209 °C (lit 208-210 °C) (Suau et al 1998).

2.3.2. Fraction C

BBR-FC was further put to column chromatography (Silica gel, 180 g) and eluted with $CHCl_3$-MeOH with increasing polarity. The fractions so obtained were further subjected to preparative TLC using $CHCl_3$-MeOH-NH_3 (90:10:0.5 and 85:15:0.5) to give dehydrocheilanthifoline (2) (15.3 mg) and Jatrorrhizine (1) (21.6 mg) respectively. BPR-FC was also separated on CC (Silica gel, 150 g) eluted with $CHCl_3$-MeOH (10:90) followed by preparative TLC using $CHCl_3$-MeOH-NH_3 (10:90:0.5) to gave berberrubine (4) (7.5 mg).

2.4. Nematicidal assay

Pure culture of *M. javanica* was obtained from Department of Plant Pathology, The University of agriculture, Peshawar, Khyber Pakhtunkhwa and was maintained on tomato cultivar Rio-grande through single egg mass inoculation. For 60 days the tomato plants were grown inside the glass house. M. Javanica eggs were extracted (1% NaOCl solution) rinsed with on 25 µm aperture sieve (distilled water). Juveniles of second stage (J2s) were obtained from surface sterilized eggs placed in sterile water in a cavity block, which hatched after 3-4 days (Hussey & Barker 1973). Nematicidal assay of the various fractions and pure compounds were determined against M. javanica (J2s) using microwell assay (Naz et al 2012). All fractions and pure compound's stock solutions were prepared by dissolving them in 1% DMSO and was further diluted using distilled water. From the slandered solutions (300 µg mL^{-1}) final concentrations (100, 200 and 300 µg mL^{-1}) were prepared (Naz et al 2012). Second stage juveniles (100) were transferred to 24 microwell plate (Multiwell, TM 24, Becton Dickinson, USA) in final volume of 1 mL in various concentrations (100, 200 and 300 µg mL^{-1}) of the fractions and pure compounds, carbofuran (with DMSO; 1% v/v) was used as a positive control. At room temperature (25 °C), the experiment was performed twice and repeated four times for each concentrations. After 24 hours of incubation, total number of active or inactive J2s were recorded. Finally to find out the mobility or mortality after 24 hours, the J2s was transferred to distilled water. The J2s were considered as dead if they did not move even after mechanical prodding (Choi et al 2007). Percentages of J2s mortality was calculated for each well and the results obtained were subjected to ANOVA (Analysis of Variance) and means were separated through Fisher's projected least significance difference (LSD) test at P= 0.05 using MSTAT-C software (Gomez & Gomez 1984).

3. Results and Discussion

3.1. Activity guided isolated bioactive compounds

Four isoquinoline alkaloids were isolated through activity guided fractionation of methanolic crude extracts and their structures were characterized by various spectroscopic techniques. The values were compared with the literature data and the structures are given in Figure 1.

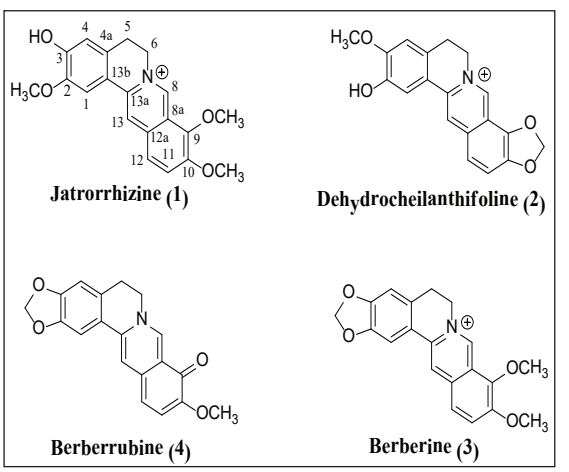

Figure 1- Structures of isolated nematicidal bioactive compounds

3.2. Compound characterization

3.2.1. Jatrorrhizine

Brown crystalline compound (MeOH); melting point: 281-282 °C (lit 280-282 °C) (Hsieh et al 2004). Molecular formula $C_{20}H_{20}N^{+}O_4$, EIMS m/z: 338.1387 (M^{+}). UV λ_{max} nm (MeOH) 226.0, 265.0, 349.0, 435.5; IR ν_{max} cm^{-1} 3340.1, 2942.8, 1600.6. The melting point, EIMS, UV, IR, ^{1}H and ^{13}C NMR data were in agreement with the literature (Hsieh et al 2004).

3.2.2. Dehydrocheilanthifoline

Brown amorphous compound (MeOH), melting point 269-270 °C. Molecular formula $C_{19}H_{16}N^{+}O_4$, deduced from the EIMS m/z 322.1074. UV λ_{max} nm (MeOH) 264.5, 359.0, 464.0; IR ν_{max} cm^{-1} 3361.8, 2924.5, 2358.5, 1601.6 (Santavy 1979).

3.2.3. Berberine

Yellow crystalline compound (MeOH), melting point 207-09 °C (lit 208-210 °C) (Suau et al 1998). Molecular formula $C_{20}H_{18}N^{+}O_4$, EIMS m/z: 336.1230 (M^{+}). UV λ_{max} nm (MeOH) 264.50, 349.0, 427.5; IR ν_{max} cm^{-1} 3047.9, 2925.5, 1596.8. The melting point, EIMS, UV, IR^{1}H and ^{13}C NMR data were in agreement with the literature (Hsieh et al 2004).

3.2.4. Berberrubine

Brown amorphous compound (MeOH), melting point 257-260 °C (lit. 255-259 °C) (Liu et al 2010). Molecular formula $C_{19}H_{15}NO_4$, EIMS m/z: 321.1001 (M^{+}), 306.3 (M^{+}-15), 292.3, 278.3. The EIMS, ^{1}H and ^{13}C NMR values were in close similarity to the reported one (Shamma & Rahimizadech 1986).

3.2.4.1. In vitro nematicidal efficacy of crude extracts and various fractions obtained from the berberis species against J2s mortality of M. javanica

The data obtained (Table 1) revealed significant ($P \leq 0.05$) effect on mortality of J2s at various concentrations i.e. 100, 200 and 300 µg mL^{-1}. The mortality of J2s was increased with the increase in concentration, the highest concentration (300 µg mL^{-1}) was most effective (54.10%). *Berberis brevissima* roots methanolic crude extract (BBR-MeOH) showed 22.33% J2s mortality and *Berberis parkeriana* methanolic roots extract (BPR-MeOH) 31.11%. Amongst the different fractions BBR-FA showed the highest mortality of 62.22% followed by BPR-FA (57.22%) and BBR-FB (54.00%). The BBR-FB and BPR-FB exhibited approximately 50% activity of the standard (carbofuran) but the differences were non-significant (Table 1).

Table 1- *In vitro* effect of different concentrations of plant fractions on juvenile mortality of root knot nematode *Meloidogyne javanica*[a]

Fractions	*Concentrations*			
	100 µg mL^{-1}	*200 µg mL^{-1}*	*300 µg mL^{-1}*	*Mean*
BBR-MeOH	18.33	23.00	25.67	22.33 g
BBR-FA	56.33	59.00	71.33	62.22 b
BBR-FB	41.67	48.67	51.66	47.33 d
BBR-FC	50.67	62.33	78.33	54.00 c
BBR-FD	39.00	43.33	45.67	42.67 e
BPR-MeOH	25.33	31.00	37.00	31.11 f
BPR-FA	35.67	40.67	44.00	40.11 e
BPR-FB	45.00	46.67	47.00	46.22 d
BPR-FC	54.00	58.00	59.00	57.22c
Carbofuran	89.67	89.67	96.67	88.22 a
Mean	44.27 c	49.07 b	54.10 a	

Data are means of five replicate per treatment using the combination of two experiments (Spring and fall, 2011); [a], means followed by the same letters do not differ significantly ($P \leq 0.05$) according to Fisher's protected LSD test. (LSD value for fractions= 3.31, LSD value for concentration= 1.82, LSD value for interaction= 6.62)

3.2.4.2. In vitro nematicidal activity of isoquinoline alkaloids from the two berberis spp. against second stage juvenile mortality of M. javanica

In vitro nematicidal efficacy of the four isolated alkaloids at various concentrations (100, 200 and 300 µg mL^{-1}) and its interaction were determined ($P \leq 0.05$). Increase in mortality of J2s was linear ($R^2 = 0.98$) dose dependent (Figure 2). Second stage juvenile mortality was 76.67% at a concentration of 300 µg mL^{-1}. Figure 3 indicated significant effect of the pure compounds at various concentrations. Amongst the tested compounds berberine (3) exhibited highest potential (97.3%) of the standard carbofuran at a concentration of 300 µg mL^{-1}. Amongst the isolated compounds jatrorrhizine (1) ranked second with efficacy of 59.50% followed by berberrubine (4) with J2s mortality of 49.17% (Figure 3). In the four isolated isoquinoline alkaloids dehydrocheilnthifoline (2) was less effective, nevertheless, showed significant mortality at the tested concentrations. The interaction of isolated compounds were studied at various concentrations and were lightly significant (Figure 4). The data showed that the percentage J2s mortality increased as the concentration of tested compounds were increased (Figure 4).

Literature survey indicated the antibacterial activity of the alkaloids of *B. thunbergii* DC and *B. vulgaris* (L) (Villinski et al 2003). The stem bark of *B. asiatica* L. showed high antimicrobial activity than the standard (Bhandari et al 2000), while the fresh and dried, aqueous as well as methanolic extracts of *B. asiatica* showed good activity against G-positive and G-negative bacteria (Shahid et al 2009). Berberine was suggested to be the main antimicrobial component of the plant. Alkaloids was suggested to have microbiocidal properties (Ghoshal et al 1996) whereas berberine has been found effective against many trypanosomes (Freiburghaus et al 1996), plasmodia (Omulokoli et al 1997) and many invertebrate pests (Rattan 2010). It was suggested that mechanism of action of berberine could be attributed to its ability to intercalate with the DNA synthesis of parasites (Phillipson et al 1987).

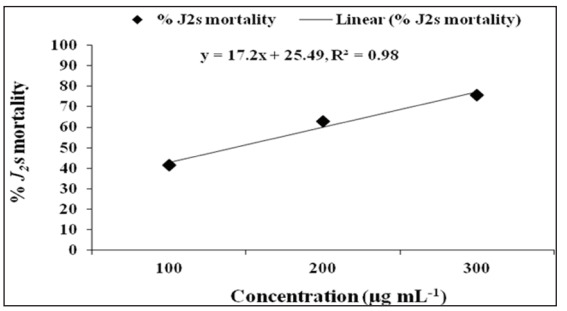

Figure 2- Effect of three different concentrations of pure compounds of *Berberis* spp., on % J2s mortality of *Meloidogyne javanica*

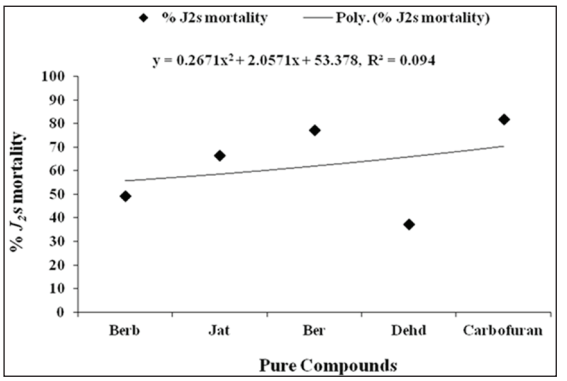

Figure 3- *In vitro* nematicidal effect of different pure compounds of *Berberis* spp., against % J2s mortality of *Meloidogyne javanica* (Berb, Berberrubine; Jat, Jatrorrhizine; Ber, Berberine; Dehd, Dehydrocheilanthifoline)

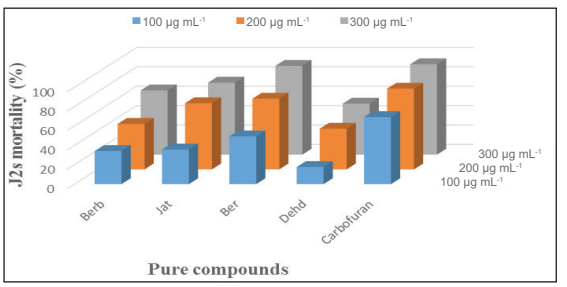

Figure 4- *In vitro* interaction effect between pure compounds of *Berberis* spp., and three different concentrations on % J2s mortality of *Meloidogyne javanica* (Berb, Berberrubine; Jat, Jatrorrhizine; Ber, Berberine; Dehd, Dehydrocheilanthifoline)

4. Conclusions

In the present study we have found that fractions of methanolic extracts of the two *Berberis* species have high potential against the root knot nematodes. Secondary metabolites of plants could be used as defense (toxic), which hinder reproduction and other physiological and biological functions of pests and parasites. These biomolecules could be used for enhancing the effectiveness and specificity in future nematicides design with specific or multiple target sites. These studies suggest that methanolic crude extracts and especially the isoquinoline alkaloids could be used as potential novel nematicides against *M. javanica*. Further research encompassing the isolation and identification of more nematicidal isoquinoline alkaloids from *Berberis* spp. may be carried out and tested against root knot nematodes as well as other plant parasitic nematodes.

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