

Full Length Research Paper

Bioassay guided isolation and characterization of anti-microbial and anti-trypanosomal agents from *Berberis glaucocarpa* Stapf.

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Different fractions of *Berberis glaucocarpa* Stapf were tested for their antibacterial and anti-trypanosomal properties using the well established Alamar blue™ 96 well microplate assays. Then eight known compounds (1 to 8), including five alkaloids (1 to 5), were isolated and characterized from this plant for the first time. All the compounds were tested for their antimicrobial and antitrypanosomal properties. Most of the pure compounds were also found to possess good antibacterial and anti-trypanosomal properties. Berberine (minimum inhibitory concentration (MIC) = 12.5 µg/ml), berberine chloroform (MIC = 25 µg/ml) and syringresinol (MIC = 12.5 µg/ml) were found to have anti-bacterial and anti-trypanosomal properties. The MIC values were determined for only active compounds.

Key words: *Berberis glaucocarpa*, alkaloids, anti-trypanosomal, antibacterial.

INTRODUCTION

Plants are the major source of biologically active compounds that can be used as medicines. In recent years, the interest in the use of medicinal plants have increased rapidly due to the resistance developed by the pathogens against the existing antibiotics (Arias et al., 2004). The genus *Berberis* L. has 500 living species around the world. They are mostly shrubs having 3-5 branched spines and have simple leaves (Ahrendt, 1961). Onion extracts have been found to have anti-oxidant properties. Onion prevents cancer, coronary heart diseases, cataract and diabetes. Fresh onion juice have been found to have analgesic and anti-inflammatory properties (Sima et al., 2012).

Peganum harmala L. extracts have been found to have antiviral, antidiabetic, antifungal, antioxidant, anti-septic,

antitumor, insecticidal, cytotoxic, hepatoprotective and antileishmanial properties (Jinous and Fereshteh, 2012). Different *Berberis* species are important folk medicines for the treatment of allergies, fever, metabolic disorders, eye diseases, osteoporosis, joint pain and menopause problems (Dipti et al., 2012). Berberine is one of the major and most important isoquinoline alkaloid found in all berberis species and has a wide range of pharmacological uses. (Dipti et al., 2012) The alcoholic extracts of *Berberis* have isoquinoline alkaloids and bisbenzyl isoquinoline alkaloids. The alkaloids are mainly responsible for the bioactivities of *berberis* species (Slavik and Slavikova, 1995). Keeping in mind the phytochemical importance of the genus *Berberis*, we decided to explore *Berberis glaucocarpa* Stapf for its

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phytoconstituents and bioactivity.

MATERIALS AND METHODS

General experimental procedures

Ultraviolet (UV) data was collected with Thermo Spectronic Unicam UV-300 spectrophotometer. IR (Infrared) analysis was recorded with JASCO FTIR 4200. ID and 2D NMR was collected with Bruker Avance DRX - 400 operating at a frequency of 400.13 MHz (^1H) and 100.62 MHz (^{13}C) at a temperature of 296.0 K.

Plant material

Berberis glaucocarpa Stapf was collected from Azad Kashmir Pakistan during July 2009 (in blooming period) and was identified by Prof Dr. Tanveer Akhtar (chairperson Botany Department University of Azad Jammu and Kashmir). A voucher specimen number 9615-A was allotted and a specimen was deposited in the herbarium of Botany Department University of Peshawar.

Extraction and isolation

B. glaucocarpa Stapf root wood (4 kg) was dried at room temperature and then grinded with a grinder. The powdered plant material was soaked in commercial grade ethanol (95 %) at room temperature for ten days. The dilute crude extract was concentrated under reduced pressure using rotary evaporator to yield a gummy residue (308 g). The residue was treated with 5 % aqueous HCl solution and filtered. The filtrate was allowed to stand overnight to afford Fraction A (Fr.A) (acidic fraction) (87 g). The acidic filtrate was extracted with dichloromethane several times to afford Fraction B (Fr.B) (dichloromethane fraction) (23 g). The acidic filtrate was basified with NH_3 to pH 10 and then repeatedly extracted with ethyl acetate to afford Fraction C (Fr.C) (basic fraction) (67 g). The remainder aqueous solution was Fraction D (Fr. D) (water fraction).

Fraction A (Fr.A)

Fr. A was subjected to silica gel column chromatography to afford berberine using chloroform as solvent system and gradually increasing the polarity with methanol.

Fraction B (Fr.B)

This fraction was subjected to silica gel column chromatography eluted with n-hexane and ethyl acetate mixture at first and then followed by elution with chloroform and methanol mixture. After repeated column and preparative chromatography, syringaresinol, syringic acid and gallic acid were purified.

Fraction C (Fr.C)

It was subjected to silica gel column chromatography. The column was eluted with different solvents in increasing order of polarity. Palmatine, oxoberberine, berberine chloroform and columbamine were purified using repeated column, prep chromatography and sephadex LH-20.

RESULTS AND DISCUSSION

Ethanol extract of *B. glaucocarpa* Stapf root wood (4 kg) resulted in the isolation of seven known compounds and a known alkaloidal artifact for the first time from this plant. The isolated compounds were found to be berberine [1] (Lenka et al., 2007), palmatine [2] (Radek et al., 2003), columbamine [4] (Tian-Jye et al., 2004), oxoberberine [5] (Jiri et al., 2004), syringaresinol [6] (Wisit et al., 2011), syringic acid [7] (Sheng-Ming et al., 2006) and gallic acid [8] (Eldahshan, 2011) while the isolation artifact was found to be berberine chloroform [3] (Radek et al., 2003).

One of the prominent clinical uses of *Berberis vulgaris* is in bacterial infection such as bacterial diarrhea and parasitic intestinal and ocular infections. (Birdsall and Kelly, 1997) Berberine is a major constituent alkaloid of all *berberis* species. Berberine has shown very significant antimicrobial properties against different strains of bacteria such as *S. aureus*, *Candida* spp (Freile et al., 2003). Subfractions containing berberine and syringaresinol were found to have good activity and hence it can be said that berberine and syringaresinol are mainly responsible for the antibacterial and ant-trypanosomal activities of the sub fractions also. Furthermore, as all the compounds were already known compounds therefore their structures were confirmed by comparing their 1D and 2D NMR data with the literature (Figure 1).

Berberine

Yellow needles; m.p = 208-209°C; UV λ_{max} (MeOH) = 266, 349, 427 nm; IR ($\nu \text{ cm}^{-1}$) = 3020, 1634, 1601, 1458. EI-MS m/z (rel. int. %) = 336 (6, M^+), 335 (7), 321 (100), 306 (38), 292 (32), 278 (75), 263 (10), 248 (6), 219 (7), 191(7), 138(7), 95(3), 44(11). ^1H NMR (MeOD) (Table 1), ^{13}C NMR (MeOD) (Table 3).

Palmatine

Brownish yellow needles, 202-203°C; UV λ_{max} (MeOH) = 266, 347, 430 nm; IR ($\nu \text{ cm}^{-1}$) = 2992, 1624, 1522, 1501, 1390, 1240; EI-MS m/z (rel. int. %) = 352 (11, M^+), 351 (35), 337 (100), 294 (46), 278 (34), 138(5) ^1H NMR (DMSO-d_6) (Table 1), ^{13}C NMR (DMSO-d_6) (Table 3).

Berberine chloroform

Light yellow crystals, m.p = 186-188°C; UV λ_{max} (MeOH) = 231, 285, 381 nm; IR ($\nu \text{ cm}^{-1}$) = 2980, 2400, 1610, 1501, 1290, 1240; EI-MS m/z (rel. int. %) = 420 (78), 383 (82), 368 (75), 336 (100), 321 (26), 292 (24), 278(27)

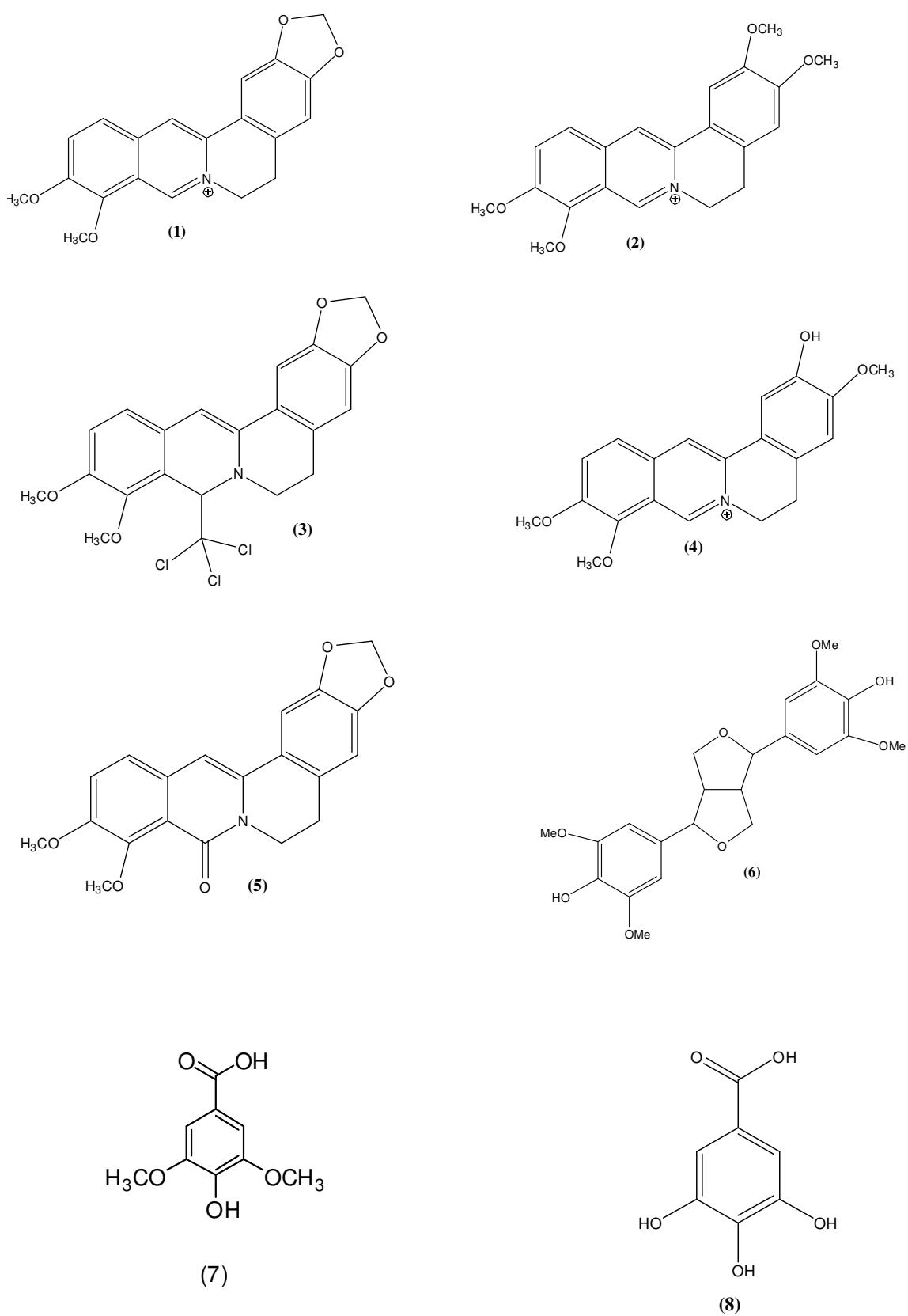


Figure 1. Structures of compound 1 to 8.

Table 1. ^1H Chemical shifts (mult, J in Hz) of compounds 1 to 5.

Atom	Beberine (MeOD)	Palmatine (DMSO- d_6)	B/Chloroform (CDCl $_3$)	Columbamine (MeOD)	Oxoberberine (DMSO- d_6)
H-1	7.62, (s)	7.72, (s)	7.14 (s)	7.35 (s)	7.40 (s)
H-4	6.94, (s)	7.08, (s)	6.59 (s)	6.54 (s)	6.63 (s)
H-5	3.24 (t, 10.0 Hz)	3.20 (t, 6.6 Hz)	2.73 (m); 3.3 (m)	3.20 (m)	2.91 (t, 8.2 Hz)
H-6	4.92 (t, 10.0 Hz)	4.95 (t, 6.4 Hz)	3.71 (m); 3.8 (m)	4.87 (m)	4.13 (t, 8.8 Hz)
H-7	-	-	-	-	-
H-8	9.75, (s)	9.89, (s)	5.62, (s)	9.52, (s)	-
H-9	-	-	-	-	-
H-10	-	-	-	-	-
H-11	8.10 (d, 9.0 Hz)	8.20 (d, 9.2 Hz)	6.97 (d, 8.4 Hz)	7.84 (d, 8.0 Hz)	7.54 (d, 8.4 Hz)
H-12	7.98 (d, 9.0 Hz)	8.04 (d, 9.2 Hz)	6.86 (d, 8.4 Hz)	7.94 (d, 8.0 Hz)	7.23 (d, 8.4 Hz)
H-13	8.67 (s)	9.09 (s)	6.08 (s)	8.45 (s)	7.01 (s)
9-OCH $_3$	4.19 (s)	4.09 (s)	3.92 (s)	4.05 (s)	3.74 (s)
10-OCH $_3$	4.09 (s)	4.06 (s)	3.86 (s)	4.15 (s)	3.89 (s)
2-OCH $_3$	-	3.93 (s)	-	-	-
3-OCH $_3$	-	3.86 (s)	-	3.90 (s)	-
2 -O-CH $_2$ -O-	6.09 (s)	-	5.93 (d, 3.2 Hz)	-	6.03 (s)

Table 2. ^1H Chemical shifts (mult, J in Hz) of compounds 6 to 8.

Atom	Syringaresinol (CDCl $_3$)	Syringic ACID (CDCl $_3$)	Gallic acid (DMSO- d_6)
H-1	3.12 (m)	-	-
H-2	4.76 (d, 4.0 Hz)	7.34 (s)	6.94 (s)
H-3	-	-	-
H-4	-	-	-
H-4a	4.31 (dd, 9.2 and 6.8Hz)	-	-
H-4b	3.94 (m)	-	-
H-5	3.12 (m)	-	-
H-6	4.76 (d, 4.0 Hz)	7.34 (s)	6.94 (s)
H-7	-	-	-
H-8a	4.31 (dd, 9.2 and 6.8Hz)	-	-
H-8b	3.94 (m)	-	-
3-OCH $_3$	-	3.90 (s)	-
5-OCH $_3$	-	3.90 (s)	-
H-1', 1''	-	-	-
H-2', 2''	6.60, (s)	-	-
H-3', 3''	-	-	-
H-4', 4''	-	-	-
H-5', 5''	-	-	-
H-6', 6''	6.60 (s)	-	-
3'-OCH $_3$	3.92 (s)	-	-
4'-OH	5.52 (s)	-	-
5'-OCH $_3$	3.92 (s)	-	-
3''-OCH $_3$	3.92 (s)	-	-
4''-OH	5.52 (s)	-	-
5''-OCH $_3$	3.92 (s)	-	-

Table 3. ^{13}C data of compounds 1 to 5.

Atom	Beberine (MeOD)	Palmatine (DMSO- d_6)	B/Chloroform (CDCl_3)	Columbamine (MeOD)	Oxoberberine (DMSO- d_6)
C-1	106.53	108.78	104.16	115.10	104.69
C-2	149.90	150.20	146.76	144.21	146.98
C-3	152.16	151.46	147.48	150.78	148.11
C-4	109.39	111.27	108.15	110.20	108.01
C-4a	131.88	128.56	128.73	134.01	129.92
C-5	28.19	25.94	30.52	27.12	27.87
C-6	57.19	55.32	51.61	56.11	38.90
C-8	146.40	145.39	73.53	145.11	159.11
C-8a	123.30	121.31	114.89	118.27	118.61
C-9	145.73	143.60	146.41	148.88	148.50
C-10	152.01	149.78	149.69	150.86	150.95
C-11	128.03	126.73	114.62	127.91	119.44
C-12	124.52	123.40	118.62	124.83	122.67
C-12a	135.12	133.10	129.36	129.55	131.92
C-13	121.47	119.91	97.52	121.01	101.33
C-13a	139.63	137.65	137.60	139.33	135.22
C-13b	121.85	118.90	125.07	122.21	123.34
9-OCH ₃	62.56	61.88	60.98	57.94	60.80
10-OCH ₃	57.63	57.02	56.37	62.88	56.71
2-OCH ₃	-	56.17	-	-	-
3-OCH ₃	-	55.83	-	57.01	-
8 (CCl_3)	-	-	105.55	-	-
2-O-CH ₂ -O-	103.68	-	101.07	-	101.89

(27); ^1H NMR (CDCl_3) (Table 1), ^{13}C NMR (CDCl_3) (Table 3).

Columbamine

Yellow needle like crystals, m.p = 281-283°C; UV λ_{max} (MeOH) = 224, 276, 349 nm; IR ($\nu \text{ cm}^{-1}$) = 3310, 1605, 1510, 1456, 1316; EI-MS m/z (rel. int. %) = 338 (7, M^+), 321 (100), 306 (35), 291 (30), 278 (73), 264 (10); ^1H NMR (MeOD) (Table 1), ^{13}C NMR (MeOD) (Table 3).

Oxoberberine

Yellow needles, m.p = 191-192°C; UV λ_{max} (MeOH) = 284, 315, 370 nm; IR ($\nu \text{ cm}^{-1}$) = 3020, 1643, 1501, 1293, 1240; EI-MS m/z (rel. int. %) = 351 (100, M^+), 336 (71), 322 (45), 292 (37), 160 (24); ^1H NMR (DMSO- d_6) (Table 1), ^{13}C NMR (DMSO- d_6) (Table 3).

Syringaresinol

Colorless crystals, m.p = 183-185°C; UV λ_{max} (MeOH) =

240, 276 nm; IR ($\nu \text{ cm}^{-1}$) = 3325, 1612, 1520, 1456, 1378, 1320; EI-MS m/z (rel. int. %) = 418 (100, M^+), 210 (20), 193 (26), 181 (99), 167 (70) ^1H NMR (CDCl_3) (Table 2), ^{13}C NMR (CDCl_3) (Table 4).

Syringic acid

Grey amorphous solid, m.p = 204-205°C; UV λ_{max} (MeOH) = 229, 286 nm; IR ($\nu \text{ cm}^{-1}$) = 3380, 1675, 1564, 1480; EI-MS m/z (rel. int. %) = 198 (100, M^+), 183 (34), 167 (17), 153 (11), 127 (19), 97 (27), 83 (36), 71 (34), 69 (41). ^1H NMR (MeOD) (Table 2), ^{13}C NMR (MeOD) (Table 4).

Gallic acid

Off white amorphous powder; m.p = 269-270°C; UV λ_{max} (MeOH) = 273 nm; IR ($\nu \text{ cm}^{-1}$) = 3370, 2996, 1715, EI-MS m/z (rel. int. %) = 170 (98, M^+), 154 (94), 127 (15), 106 (7), 79 (18); ^1H NMR (DMSO- d_6) (Table 2), ^{13}C NMR (DMSO- d_6) (Table 4).

Table 4. ^{13}C data of compounds 6 to 8.

Atom	Syringaresinol (CDCl_3)	Syringic acid (MeOD)	Gallic acid (DMSO-d_6)
C-1	54.40	120.77	121.56
C-2	86.09	106.77	109.77
C-3	-	140.55	146.13
C-4	71.84	147.34	138.89
C-5	54.40	140.55	146.13
C-6	86.09	106.77	109.77
C-7	-	168.93	169.11
C-8	71.84	-	-
3-OCH ₃	-	55.52	-
5-OCH ₃	-	55.52	-
C-1', 1''	132.13	-	-
C-2', 2''	102.76	-	-
C-3', 3''	147.18	-	-
C-4', 4''	134.36	-	-
C-5', 5''	147.18	-	-
C-6', 6''	102.76	-	-
3'-OCH ₃	56.40	-	-
4'-OH	-	-	-
5'-OCH ₃	56.40	-	-
3''-OCH ₃	56.40	-	-
4''-OH	-	-	-
5''-OCH ₃	56.40	-	-

Table 5. Anti-trypanosomal results.

Compound/crude	<i>T. brucei</i>			
	% D control	% D control	% D control	MIC
	20 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	5 $\mu\text{g/ml}$	$\mu\text{g/ml}$
BGA (Fr.A)	21.0	82.0	109.2	NC
BGSD (Fr.B)	21.6	78.5	93.4	NC
BJSET (Fr.C)	18.7	69.2	89.7	NC
Beberine	1.8	46.5	76.1	25
Berberine chloroform	- 1.1	1.9	86.8	12.5
Syringaresinol	23.7	83.4	98.4	NC
Palmatine	85.0	97.0	93.0	NC
Columbamine	85.0	97.0	93.0	NC
Oxoberberine	8.5	83.2	86.8	NC

%D control, Percentage of DMSO control; NC, not calculated.

Anti-bacterial and anti-trypanosomal activities

All the isolated compounds and their fractions were tested for their anti-bacterial and anti-trypanosomal properties using Alamar blue™ 96 well microplate assays (Tables 5 and 6). Four strains of bacteria namely SMRSA (200 $\mu\text{g/ml}$), EMRSA (200 $\mu\text{g/ml}$), *Mycobacterium*

marinum (100 $\mu\text{g/ml}$) and *Escherichia coli* (500 $\mu\text{g/ml}$) and one strain of *Trypanosoma* namely *Trypanosoma brucei. brucei* (20, 10 and 5 $\mu\text{g/ml}$) were used for assays. Berberine (MIC = 12.5 and 25 $\mu\text{g/ml}$), berberine chloroform (MIC = 25 and 12.5 $\mu\text{g/ml}$) and syringaresinol (12.5 $\mu\text{g/ml}$) were found to be very active against SMRSA, *Mycobacterium marinum* and *T. brucei. brucei*.

Table 6. Anti-bacterial results.

Compound/crude	<i>M. marinum</i>	SMRSA	SMRSA	EMRSA	EMRSA	<i>E. coli</i>
	% D control	% D control	MIC	% D control	MIC	% D control
	100 µg/ml	200 µg/ml	µg/ml	200 µg/ml	µg/ml	500 µg/ml
BGA (Fr.A)	4.0	2.0	50	78.8	NC	49.7
BGSD (Fr.B)	12.2	-2.2	25	14.9	NC	54.0
BJSET (Fr.C)	15.5	1.4	50	86.5	NC	56.5
Beberine	- 2.9	- 0.1	12.5	72.0	NC	51.3
Berberine chloroform	0.6	50.2	25	70.5	NC	19.4
Syringaresinol	12.2	- 0.6	12.5	84.9	NC	52.3
Palmatine	78.1	71.8	NC	75.3	NC	52.0
Columbamine	89.9	86.5	NC	94.5	NC	66.8
Oxoberberine	41.6	85.9	NC	87.9	NC	65.2

% D control, Percentage of DMSO control; NC, not calculated.

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REFERENCES

- Ahrendt LWA (1961). *Berberis* and *Mahonia*, a taxonomic revision, J. Linn. Soc. Bot. 57(369):1-410.
- Arias ME, Gomez JD, Cudmani NM, Vattuone MA, Isla MI (2004). Antibacterial activity of ethanolic and aqueous extracts of *Acacia aroma* Gill. Ex Hook et Arn. Life Sci. 75:191-202.
- Birdsall TC, Kelly GS (1997). Berberine: therapeutic potential of an alkaloid found in several medicinal plants. Altern. Med. Rev. 2:94-103
- Dipti P, Hirwani RR, Sivakami D (2012). Phyto-chemical and pharmacological applications of *Berberis aristata*. Fitoterapia 83:817-830.
- Eldahshan OA (2011). Isolation and Structure Elucidation of Phenolic Compounds of Carob Leaves Grown in Egypt. Curr. Res. J. Biol. Sci. 3(1):52-55.
- Jinous A, Fereshteh R (2012). Chemistry, pharmacology and medicinal properties of *Peganum harmala* L. Afr. J. Pharm. Pharmacol. 6(22):1573-1580
- Freile ML, Giannini F, Pucci G, Sturniolo A, Rodero L, Pucci O, Balzareti V, Enriz RD (2003). Antimicrobial activity of aqueous extracts and of berberine isolated from *Berberis heterophylla*. Fitoterapia 74:702-705.
- Jiri D, Stanislav M, Pavlina S, Dagmar H, Marek N, Milan P, Jaromir T, Roger D, Walter VD, Radek M (2004). Berberine and coptisine free bases. J. Mol. Struct. 687:135-142.
- Lenka G, Jiri D, Radek M (2007). Quaternary protoberberine alkaloids. Phytochemistry 68:150-175
- Radek M, Pavlina S, Dagmar H, Jaromir M, Jiri D, Vladimir S (2003). Palmatine and Berberine Isolation Artifacts. J. Nat. Prod. 66:481-486.
- Sima N, Mahdieh, Anoush, Narges K (2012). Evaluation of analgesic and anti-inflammatory effects of fresh onion juice in experimental animals. Afr. J. Pharm. Pharmacol. 6(23):1679-1684
- Slavik J, Slavikova L (1995). Quaternary isoquinoline alkaloids in plants of Czech Republic. Collect. Czech. Chem. Commun. 60:1034-1041.
- Sheng-Ming P, Hsiou-Yu D, Wen-Liang C, Hang-Ching L (2006). Phenols from the Aerial Parts of *Leonurus sibiricus*, Chin. Pharm. J. 58:35-40.
- Tian-Jye H, Yi-Chen C, Yang-Chang W, Chung-Yi C, (2004). Chemical Constituents from the Stems of *Mahonia japonica*. J. Chin. Chem. Soc. 51:443-446.
- Wisit M, Siripit P, Narong N, Wilart P (2011). (+)-Syringaresinol Lignan from New Species *Magnolia Thailandica*. Am. J. Appl. Sci. 8(12):1268-1271.