INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

HPLC-PDA-MS/MS PROFILE, ANTIMICROBIAL ACTIVITY OF INSTANTLY USED MISWAK (*Salvadora persica* L.) ROOT *VERSUS* ITS AQUEOUS EXTRACT AND BENZYL ISOTHIOCYANATE QUANTIFICATION

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Article Received on: 20/02/19 Approved for publication: 12/04/19

DOI: 10.7897/2230-8407.1005160

ABSTRACT

Salvadora persica L. root (Miswak) is a natural tooth cleaning tool which contains a lot of biologically active metabolites. The aim of this study is to find out the optimal method of using *S. persica* root as a mouth antimicrobial agent and to establish a new method for quantification of benzyl isothiocyanate (BICT) in Miswak dichloromethane extract (MDE) applying HPLC-UV. Non targeted metabolomic analysis *via* high performance liquid chromatography coupled with electro spray ionization tandem mass spectrometry (HPLC-PDA-ESI-MS/MS) was established for analysis of the instantly used powder (IUP) and the aqueous extract (AE). The antiviral and antibacterial activities were evaluated using MTT assay and broth micro dilution methods, respectively. HPLC-PDA-ESI-MS/MS tentatively identified 12 metabolites in IUP (glucotropaeolin, 8 flavonoid glycosides, 1 sulphated sugar derivative and 2 sugars) and 3 metabolites in the AE (2 sugars and 1 sulphated sugar derivative). IUP exhibited more potent antiviral activity against *Herpes simplex* virus type 1 (HSV-I) (34.79 %) than AE (25.23 %). It also demonstrated antibacterial activity against aerobic (*Pseudomonas aeruginosa* MIC of 6.25 mg/ml, and *Bacillus sphaericus* MIC 12.5 mg/ml) and anaerobic (*Porphyromonas gingivalis* MIC of 100 mg/ml) strains. The presence of glucotropaeolin in IUP only is suggested to be responsible for its potent antimicrobial activity in comparison to AE. A simple and validated method for quantification of BITC in MDE was established and 95 µg BITC was found to be in one gram of Miswak powder.

Keywords: Glucotropaeolin; *Salvadora persica* L.; *P. gingivalis; Herpes simplex;* HPLC-PDA-ESI-MS/MS.

INTRODUCTION

Salvadora persica L. root is known to contain several biologically active metabolites, such as glucosinolates, flavonoids, alkaloids and saponins[1]. Among these active metabolites, benzyl glucosinolate (glucotropaeolin) gives upon enzymatic hydrolysis the powerful antibacterial and antiviral benzyl isothiocyanate (BITC) against *Herpes simplex* virus type-I (HSV-I)[2-4]. The antibacterial activity of *S. persica* different organs and extracts was previously studied[5]. However, very little work was performed on the antiviral activity[6] and methods of analysis of BICT in *S. persica*[7]. The objective of this study is to apply metabolomics tools (HPLC-PDA-ESI-MS/MS) and to investigate the difference in metabolites and antimicrobial activity between instantly used powder (IUP) and aqueous extract (AE) of *S. persica* root; as well as to establish a simple and validated method for determination of BITC in miswak dichloromethane extract (MDE) using HPLC-UV.

MATERIAL AND METHODS

Plant material

HPLC-PDA-ESI-MS/MS analysis

The roots of *Salvadora persica* L. were obtained from El-Orman Botanic garden, Giza, Egypt. A voucher sample (No.18-9-2017) is reserved in the museum of Pharmacognosy Department, Faculty of Pharmacy, Cairo University. The roots were air-dried and ground, Five grams was extracted by infusion in distilled water for three hours at temperature not more than 25°C to avoid volatilization of Glucotropaeolin. The extract was filtered and divided into two parts. The first part was used directly for analysis and named instantly used powder (IUP) and the second part was evaporated using a rotary evaporator at 45°C and was referred as aqueous extract (AE).

Autolysis of glucotropaeolin for HPLC-UV analysis

Thirty grams of freshly ground powdered root of *S. persica* was infused with 250 ml distilled water overnight (12-15 h) at (37 ± 2) ºC. Benzyl isothiocyanate (the hydrolyzed product of glucotropaeolin) was extracted by shaking with dichloromethane (3 × 50 ml) to yield Miswak dichloromethane extract [8] (MDE). This extract was evaporated to dryness directly before injection into HPLC. MDE was dissolved in the mobile phase, filtered using 0.45 µl syringe filter and 20 µl was injected into HPLC injector.

Chemicals and Microbes

Sigma Aldrich (USA) supplied the required reference materials BITC, ampicillin, metronidazole and chemicals (acetonitrile, dichloromethane and methanol) of HPLC grade for HPLC analysis. MTT (3-(4, 5 dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide solution was purchased from bio basic Canada inc. The bacterial strains: *Bacillus sphaericus* MTCC 511, *Staphylococcus aureus* MTCC 87, *Enterobacter*

aerogenes MTCC 111, *Pseudomonas aeruginosa* MTCC1034 and *Porphyromonas gingivalis* ATCC 33277 were obtained from the Microbiological Resources Center (Cairo MIRCEN).

HPLC analysis of benzyl isothiocyanate

Agilent, USA (series 1260 infinity) HPLC instrument with quaternary pump and ultraviolet (UV) detector equipped with manual injector TCC was used. Isocratic elution using water and acetonitrile (30:70) was applied. UV detector was set at 226 nm. Reversed phase Hypersil BDS C-18 column (4.6 × 250 mm, 5 µm). Temperature was maintained at room temperature at flow rate of 1 ml/min. BITC standard was dissolved in methanol (10 mg/ml) then serial dilution was prepared to obtain the following concentrations (1, 0.5, 0.25, 0.1, 0.05 mg/ml). Twenty micro liter of each concentration was injected into HPLC. The sample MDE was prepared as under autolysis of glucotropaeolin section. The retention time and the peak area were used to calculate the concentration of BITC in MD by the data analysis of Agilent software.

HPLC-ESI-PDA-MS/MS

Both the IUP and AE were analyzed by HPLC-PDA-ESI-MS/MS. The Thermo Finnigan (Thermo Electron Corporation, USA) LC system was coupled with the mass spectrometer (LCQ-Duo ion trap) having a (Thermo Quest) electro spray ionization (ESI) source. The column (Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 3.5 µm) was used for separation. The mobile phase water and acetonitrile each having 0.1 % formic acid with gradient elution of acetonitrile increased from 5 to 30 % within 60 min (Table 6) in 1 ml/min flow rate and a 1:1 split using the auto sampler for injection of samples was adopted. Negative mode was operated with −10 V as capillary voltage, 200 °C source temperature, and nitrogen as both auxiliary and sheath gas with a flow rate of 40 and 80 (arbitrary units), respectively. Ions were detected within 50–2000 m/z mass range in a full scan mode. The interpretations of the chromatograms were controlled by (Xcalibur TM 2.0.7, Thermo Scientific) software[9].

Antiviral activity

Cell culture and virus

The Vero cell line (*Cercopithecus aethiops* (African green monkey) kidney epithelial cells) ATCC (CCL-81) was purchased from the American Type Culture Collection (ATCC) and preserved in RPMI 1640 medium (Gibco, USA) enhanced with L-Glutamine (2 mM), fetal bovine serum (10 % v/v), streptomycin (100 µg/mL) and penicillin (100 U/ml). Cells were incubated in (5 % CO_2, 37 °C). Clinically isolated *Herpes simplex* virus type-I (HSV-I) was kindly provided by Dr. Mohammed Ali Baser, Faculty of Science, Al-Azhar University, Egypt.

Cytotoxicity evaluation

Cytotoxicity estimation is based on Vero cell mitochondrial succinate dehydrogenase reduction of yellow MTT to give a blue formazan dye[10]. The optical density (OD) was measured at 540 nm using a Perkin-Elmer ELISA reader (HTS 7000 plus). The cytotoxicity percentage was calculated as [(A−B)/A] × 100, where A and B were the optical density of untreated and treated cells, respectively. Cell viability (%) was calculated from the result of Mean OD/Control OD × 100 %[11].

MTT assay

Herpes simplex type I virus was exposed to the maximum nontoxic concentration (MNTC) of tested extracts for an hour at 37°C. Then 100 µl of the mixture was added to the cells cultured in 96-well flat-bottom micro titer plate; after addition of 20 µl MTT solution to each well. It was incubated for 5hours at 37°C and the optical density was measured at 560 nm[12].

Antibacterial activity

Antibacterial activity of IUP and AE (100 mg/ml) was tested by well diffusion method[13,14]. Four aerobic bacteria; *Bacillus sphaericus*, *Staphylococcus aureus*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa* and one anaerobic pathogenic bacteria; *Porphyromonas gingivalis* were used. The tested bacteria were subcultured on Mueller–Hinton agar medium (Oxoid laboratories, UK). Ampicillin and metronidazole were used as a positive control. The inhibition zone diameter was measured, and the experiment was carried out in triplicate. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were evaluated by broth microdilution method[15].

RESULTS AND DISCUSSION

HPLC–PDA–MS/MS characterization of *S. persica* root metabolites

HPLC-PDA-ESI-MS/MS enabled the tentative identification of 12 metabolites in IUP and 3 metabolities in AE. The identified metabolites in IUP were 1 major glucotropeolin (benzyl glucosinolate), 8 flavonoid glycosides and 1 sulphated sugar derivative and 2 sugars. However, 3 metabolites were identified only in AE (2 sugars and 1 sulphated sugar derivative). Ten of the identified metabolites in IUP were reported for the first time in this study. IUP was rich in glucotropeolin (benzyl glucosinolate) and flavonoid glycosides which were absent in AE, while sulphated sugar derivatives and sugars were detected in both IUP and AE (Table 1 and Figures 1- 3).

The major peak (4) in IUP exhibited a molecular ion peak $[M - H]^-$(m/z) at 408.12 was assigned to Glucotropaeolin which is characterized by the loss of benzyl isothiocyanate resulting in the base peak at m/z 259 and 2 daughter ions at 275,328, (Figure S_1a). Peak (5) showed $[M - H]^-$ (m/z) at 816.64 and three fragment ions at 358,418 and 496; it was identified as glucotropaeolin dimer (Figure S_1b). Peak (6) displayed a molecular ion peak $[M - H]^-$ (m/z) at 349.3. In MS2 it produced ion peaks at m/z 241 and at m/z 97 which were characteristic to sulphated hexoside fragmentation. It was assigned to sulphated sugar derivative[16] (Figure S_1c).

Two peaks (7 and 8) were detected in both IUP and AE with a molecular ion of $(M-H)^-$ at m/z 539.19 and 377.09 (Figure S_2a). In MS2, it produced a major ion peak at m/z 503[M-36(dihydrate)], another peak at 377 [M–H–162, loss of a hexose moiety]; it was tentatively identified as trisaccharide dihydrate. In MS3 of peak (8) 377, two daughter ions at m/z 341 [M-36(dihydrate) and 215 [M–H–162, loss of a hexose moiety] were observed; thus, it was identified as disaccharide dihydrate (Figure S_2b).

In the AE, peaks (1 and 3) showed MS2 of a molecular ion peak $[M - H]^-$(m/z) at 366.86 (Figure S_3a) and MS3 of a molecular ion peak $[M - H]^-$(m/z) 287.27 which has two characteristic fragments of sulphated hexoside at m/z 241 and 97 (Figure S_3b) that was identified as sulphated sugar derivative[16].

Table 1: Characterization of *S. persica* IUP and AE metabolites by HPLC-PDA- ESI-MS/MS

Peak NO.	IUP	AE	Rt (min.)	Molecular ion$(M-H)^-$	Molecular formula	MS/MS	Tentatively identified metabolites
1	-	+	0.79-0.92	366	$C_7H_3O_2N_4S_6$	287,241,162	Unknown
2	-	+	0.96-1.09	428	$C_{17}HO_{14}$	349,428,179	Unknown
3	-	+	1.14-1.18	287	$C_{17}H_{37}NS$	241,287,97	Sulphated sugar derivative
4	+	-	0.8-0.99	408	$C_{14}H_{18}O_9NS_2$	259,275,328	Glucotropaeolin
5	+	-	1.08-1.13	816	$C_{28}H_{36}O_{18}N_2S_4$	358,418,496	Dimer of Glucotropaeolin
6	+	-	1.18-1.28	349	$C_{18}H_{37}O_4S$	241,349,97	Sulphated sugar derivative
7	+	+	1.3	539	$C_{18}H_{32}O_{16}$	503,377,341	Trisaccharide dihydrate
8	+	+	1.35-1.43	377	$C_{12}H_{25}O_{13}$	341,363,160	Disaccharide (dihexoside) dihydrate
9	-	+	1.73-1.82	425	$C_{26}H_{33}O_5$	241,242,425	Unknown
10	-	+	1.91-2.00	374	$C_{22}H_{30}O_5$	241,374,375	Unknown
11	+	+	2.03-2.11	451	$C_{25}H_{39}O_7$	451,452,419	Unknown
12	+	+	2.54	456	$C_{21}H_{28}O_{11}$	312,354,161	Unknown
13	+	-	2.77	515	$C_{25}H_{23}O_{12}$	353,399,299	Isochlorogenic acid C or b
14	+	+	2.82	617	$C_{31}H_{37}O_{13}$	581,599,567	Unknown
15		+	3.02	504	$C_{28}H_{24}O_9$	468,440,469	Unknown
16	+	-	3.29-3.39	609	$C_{27}H_{45}O_{15}$	301,343,271	Rutin
17	-	+	3.56-3.66	287	$C_{17}H_{35}O_3$	269,227,251	Unknown
18	-	+	3.76-3.81	644	$C_{24}H_{52}O_{19}$	537,467,300	Unknown
19	+	-	3.95-3.99	463	$C_{21}H_{19}O_{12}$	301,300,343	Quercetin-O-hexoside (Hyperoside)
20	+	-	4.04-4.14	447	$C_{21}H_{19}O_{11}$	315,285,300	Kaempferol-7-O-hexoside
21	+	-	4.35-4.4	593	$C_{27}H_{19}O_{16}$	285,327,257	Luteolin 7-rutinoside
22	+	-	4.45-4.54	623	$C_{28}H_{31}O_{16}$	315,300,271	Rhamnosyl hexosyl methyl quercetin
23	-	+	4.52-4.6	586	$C_{28}H_{58}O_{12}$	586,587,241	Unknown
24	+	-	5.00-5.10	433	$C_{20}H_{17}O_{11}$	300,301,307	Quercetin 3-alpha-L-Arabinofuranoside Or Quercetin-O-pentoside
25	+	-	5.15-5.3	477	$C_{22}H_{21}O_{12}$	314,315,356	Isorhamnetin-3-O-glucoside
26	+	-	5.85-5.95	445	$C_{17}H_{17}O_{14}$	323,161,179	Unknown
27	+	+	5.72-6.00	534	$C_{25}H_{26}O_{13}$	498,373,337	Unknown
28	+	-	6.14-6.21	433	$C_{16}H_{33}O_{13}$	269,287,259	Unknown
29	+	-	8.56-8.66	369	$C_{13}H_6O_{13}$	176,148,174	Unknown
30	+	-	8.70-8.71	176	$C_{10}H_{24}O_2$	176,174,162	Unknown
31	+	-	16.5-16.6	220	$C_4H_{12}O_{10}$	205,220,221	Unknown

Rt : retention time, IUP : Instantly used powder, AE : Aqueous extract, + : present, - : absent

Table 2: HPLC validation parameters

linear regression equation	r^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
y = 9.7644x - 196.86	0.991	50-1000	15	45

LOD: limit of detection, LOQ: limit of quantification

Table 3: Determination of maximum nontoxic concentration (MNTC) and antiviral activity for *S. persica* IUP and AE

Name	Conc. (mg/ml)	Mean (O.D ± S.E)	Viability %	Antiviral activity%	CC_{50} (mg/ml)
Vero cell		0.25	100		
IUP	**10**	0.24 ± 0.001	96.4	34.79	48.3
AE	**5.65**	0.23 ± 0.007	100	25.23	16.84
Acyclovir	**10**	0.24 ± 0.0072	98.8	91.23	

O.D: Optical density, S.E: Standard error

Table 4: Antibacterial activity of *S. persica* IUP and AE as indicated by growth-inhibition zone

Bacteria		Growth inhibition zone in mm			
		IUP 100 mg/ml	AE 100 mg/ml	St.	
Gram positive aerobic bacteria	*Staphylococcus aureus*	12	12	45	Ampicillin (30 mcg/ml)
	Bacillus sphaericus	16	10	34	
Gram negative aerobic bacteria	*Enterobacter aerogenes*	12	12	22	
	Pseudomonas aeruginosa	14	10	25	
Gram negative anaerobic bacteria	*Porphyromonas gingivalis*	16	No effect	46	Metronidazole (100 mg/ml)

IUP: instantly used powder, AE: aqueous extract, St: standard drug

Table 5: Determination of MIC and MBC of *S. persica* IUP and AE on tested bacterial strains

Bacteria	Mean (OD ± S.E)	MIC (mg/ml)	MBC (mg/ml)
P. gingivalis	1.44 ± 0.01		
IUP	0.09 ± 0.01	100	>100
B. subtilis	1.39 ± 0.03		
IUP	0.06 ± 0.01	12.5	50
AE	0.92 ± 0.02	No inhibition	--------
S. aureus	1.43 ± 0.01		
IUP	0.96 ± 0.13	No inhibition	------
AE	0.89 ± 0.04	No inhibition	------
E. aerogenes	1.36 ± 0.01		
IUP	1.28 ± 0.03	No inhibition	-------
AE	1.31 ± 0.01	No inhibition	------
P. aeruginosa	1.43 ± 0.01		
IUP	0.05 ± 0.02	6.25	25
AE	1.27 ± 0.03	No inhibition	-------

O.D: optical density, S.E: standard error

Table 6

Time (min)	Solvent A (0.1% Formic acid in water)	Solvent B (0.1% Formic acid in acetonitrile)
0-14.9	95	5
15-29.9	85	15
30-44.9	75	25
45-60	70	30

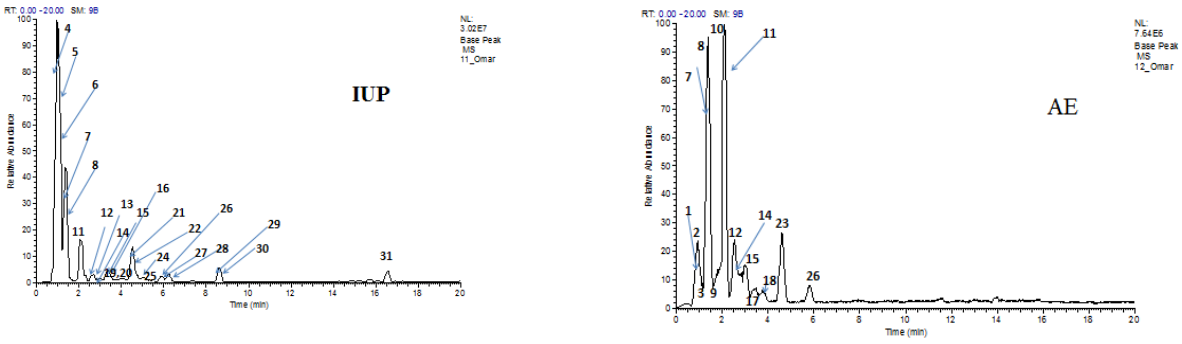


Figure 1: HPLC chromatograms of *S. persica* IUP and AE

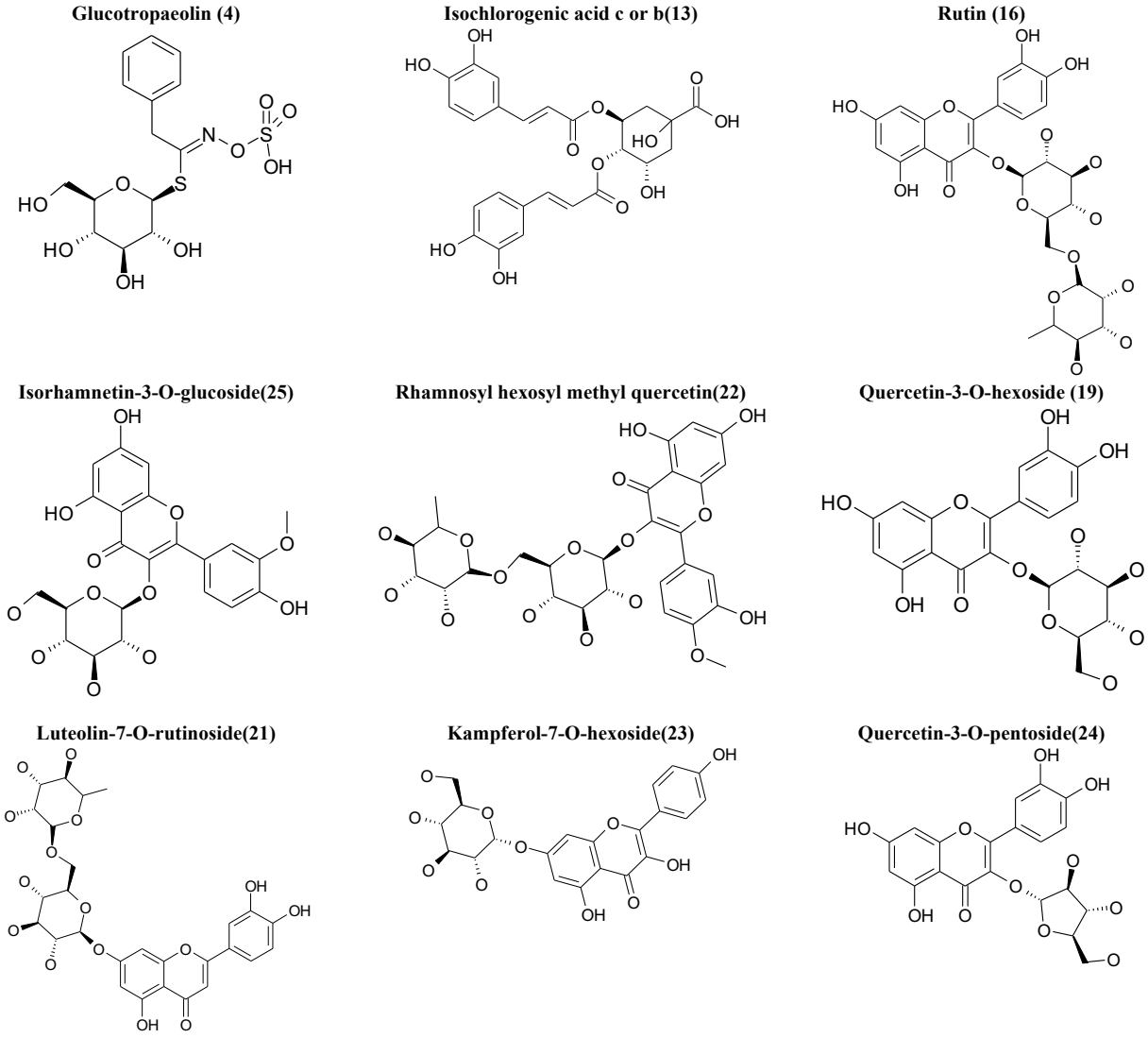


Figure 2: Structures of certain tentatively identified metabolites in *S. persica* IUP by HPLC-PDA-ESI-MS/MS.

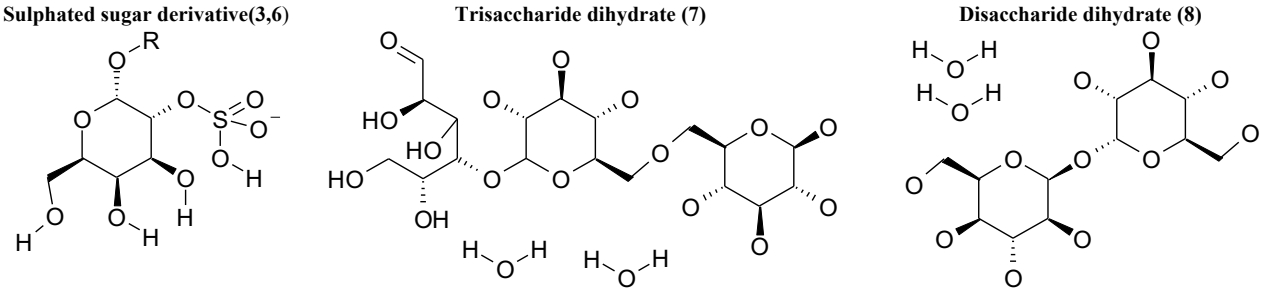


Figure 3: Structures of certain tentatively identified metabolites in *S. persica* AE by HPLC-PDA-ESI-MS/MS

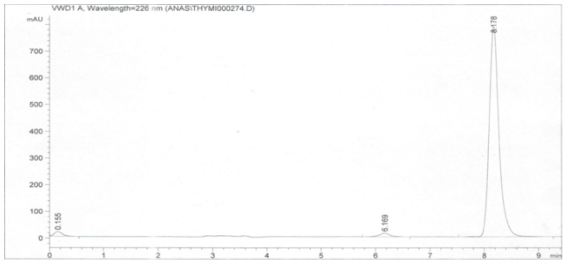


Figure 4: HPLC chromatogram of BITC

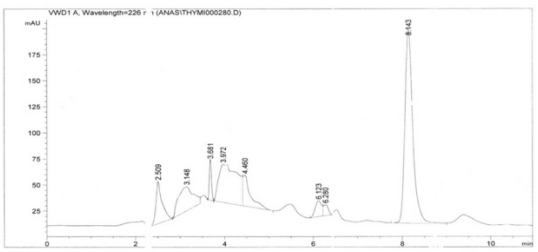


Figure 5: HPLC chromatogram of MDE

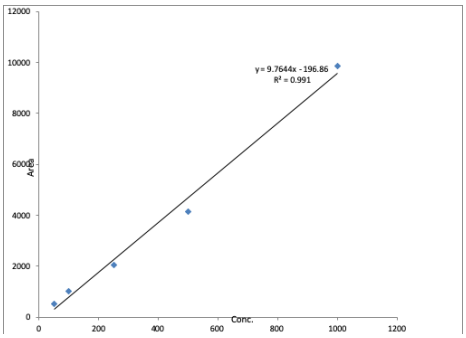


Figure 6: calibration curve of BITC

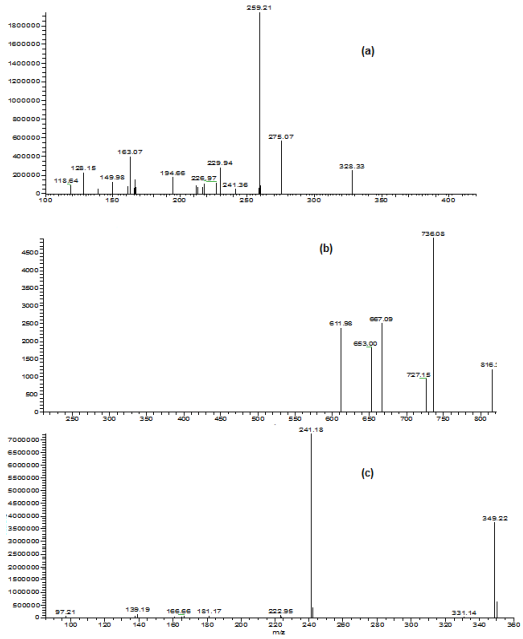


Figure S1: (a) MS/MS fragmentation of glucotropaeolin at [M - H]$^-$ (*m*/*z*) at 408.12; (b) MS/MS fragmentation of glucotropaeolin dimer at [M - H]$^-$ (*m*/*z*) at 816.64; (c) MS/MS fragmentation of sulphated sugar derivative at [M - H]$^-$ (*m*/*z*) at 349.3

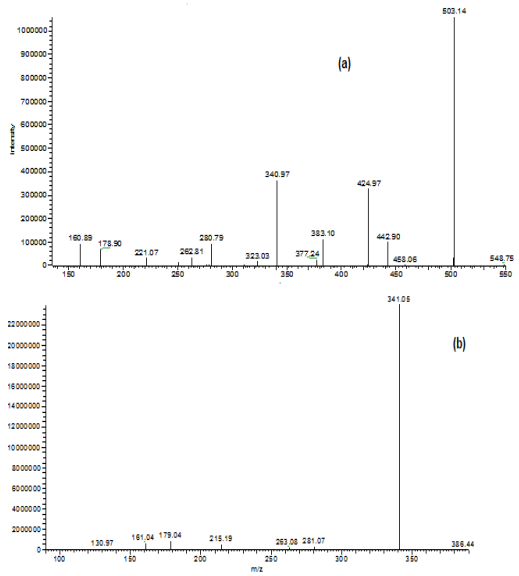


Figure S2: Negative ion ESI-MS/MS spectra of trisaccharide dihydrate at (a) MS2 of [M - H]$^-$ (*m*/*z*) at 539.19; (b) MS3 fragmentation of main daughter ion disaccharide dihydrate at [M - H]$^-$ (*m*/*z*) at 377.09

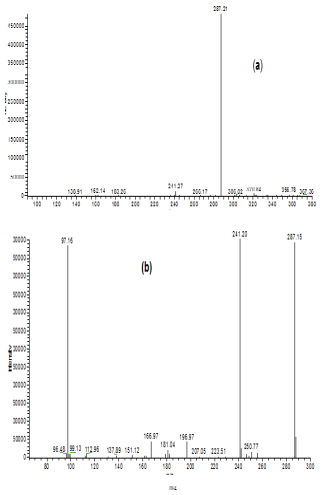


Figure S3: Negative ion ESI-MS/MS spectra of sulphated sugar derivatives (a) MS2 of [M - H$^-$ m/z 366.86; (b) MS3 of main daughter ion at m/z 287.27

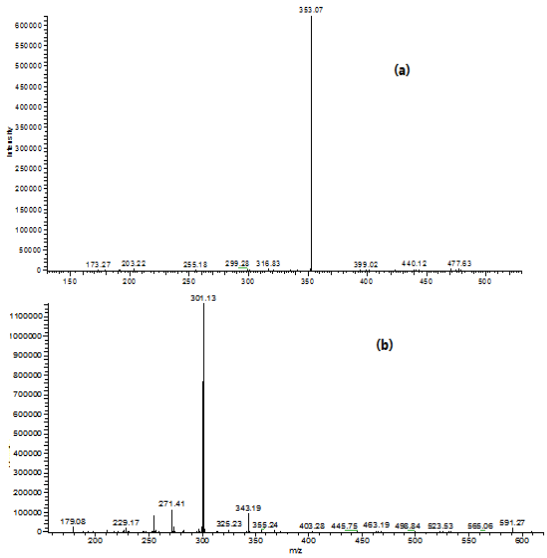
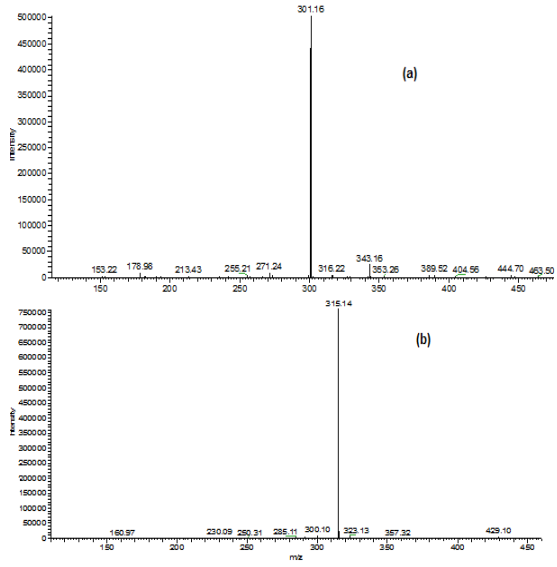


Figure S_4: MS/MS fragmentation of Isochlorogenic acid b or c [M - H]$^-$ (*m/z*) at 515.09; (b) MS2of Rutin at [M - H]$^-$ (*m/z*) at 609.26



FigureS_5: (a) MS/MS fragmentation of quercetin-o-hexoside at [M − H]$^-$ (m/z) at 463.26; (b) MS/MS fragmentation of kampferol-o-hexoside at [M − H]$^-$ (m/z) at 447.24

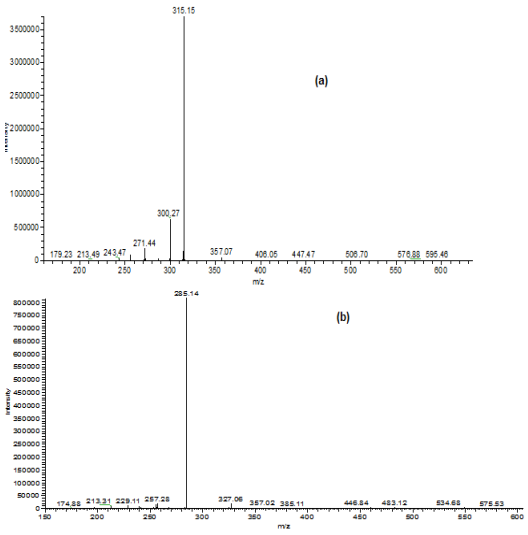


Figure S_6: Negative ion ESI-MS/MS spectra of flavonoid glycosides (a) MS2 of [M - H]$^-$ m/z 623.16 (Rhamnosylhexosyl methyl quercetin) (b) MS2 of [M - H]$^-$ m/z 593.27(Luteolin-7-O-rutinoside)

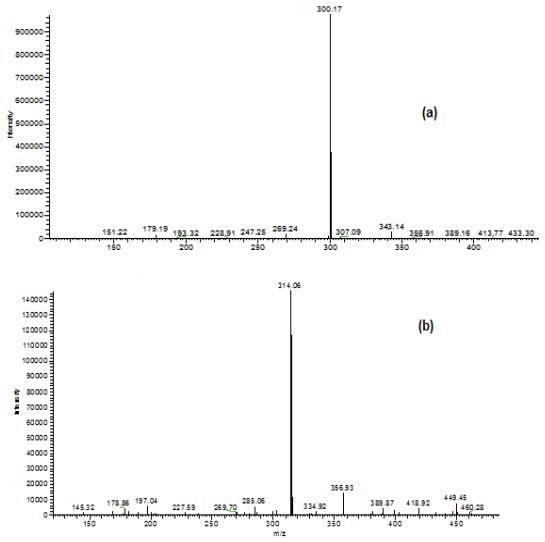


Figure S_7: Negative ion ESI-MS/MS spectra of flavonoid glycosides (a) MS2 of [M - H]$^-$ m/z 433.2 (Quercetin 3-alpha-L-arabinofuranoside) (b) MS2 of [M - H]$^-$ m/z 447.26 (Isorhamnetin-3-O-glucoside)

Peaks (13 and 16) in IUP displayed molecular ion peaks at [M - H]$^-$ (m/z) 515.09 and 609.26 respectively. In MS2, they produced fragment ions as (M-H-162) (m/z) 353, 399 and 299 for peak 13 (Figure S_4a) that was assigned to isochlorogenic acid C or b and (M-H-162 (hexose moiety) (m/z) 301, 343 and 271 for peak 16that was characterized as rutin (Figure S_4b).

In IUP peak (19) showed MS2 fragmentation of a molecular ion peak [M - H]$^-$ (m/z) 463.26 and daughter ions at [M-H-162(hexose)](m/z) 301,343 and 300 which was assigned as quercetin -O-hexoside (Figure S_5a). Peak 20 displayed MS2 fragmentation of a molecular ion peak [M - H]$^-$(m/z) 447, produced fragment ions [(M-H-162 (hexose)] (m/z) 315, 316 and 300 that was identified as Kaempferol-7-O-hexoside (Figure S_5b).

Peaks (21 and 22) in IUP showed molecular ion peaks at [M - H]- (m/z) 593.27 and 623.16 respectively. In MS_2 of peak (21) a base peak at (m/z) 285.14 [M-H-308 (rutinoside)] characterized for Luteolin-7-O-rutinoside was observed (Figure S_6b). MS2 fragmentation of peak (22) produced major fragment ion at (m/z) 315.15 [(M-H-308 (162 hexose-146 rhamnose] that was tentatively identified as rhamnosyl hexosyl methyl quercetin (Figure S6a).

The last two peaks (24 and 25) in IUP exhibited molecular ion peaks at [M - H]$^-$ (m/z) 433.2 and 477.26 respectively. MS/MS

fragmentation of peak (24) showed base peak at (m/z) 300.1 [M-H-133(arabinofuranoside)] which was assigned to quercetin 3-alpha-L-arabinofuranoside (Figure S_7a). MS_2 fragmentation of peak (25) produced major fragment ion at (m/z) 314.15 (M-H-162 hexose) that was identified as Isorhamnetin-3-O-glucoside (Figure S_7b).

Determination of BITC in MDE by HPLC

The HPLC method showed sharp and high resolved peak of BITC for both standard and MDE (Figures 4 and 5). Validation of HPLC method was achieved by the external standard method applying a sequence of serial dilution of BITC standard and calibration curve was constructed showing linearity ($r^2 = 0.991$) (Figure 6). It was found that one gram Miswak powder contains 95 µg/ml BICT. The linear regression equation, linear ranges, calibration equation, determination coefficients (r^2), LOD and LOQ were recorded in Table (2).

Antiviral activity

Determination of MNTC of each sample was evaluated by viability % and CC_{50}. The antiviral activity for both Miswak IUP and AE against *Herpes simplex* virus type-I (HSV-I) were represented by percentage inhibition in Table 3. IUP exhibited more potent antiviral activity (34.79 %) than AE (25.23 %).

Antibacterial activity

Screening of the antibacterial activity of IUP and AE against different strains of aerobic and anaerobic bacteria was performed and the results were recorded in Table 4. It was found that IUP caused wider growth inhibition zones than AE against aerobic bacteria. IUP was effective against anaerobic bacteria; however AE had no effect on anaerobic bacteria. The MIC and MBC are presented in Table 5. IUP showed antibacterial activity against aerobic (*Pseudomonas aeruginosa* MIC of 6.25 mg/ml, MBC of 25 mg/ml and *Bacillus sphaericus* MIC 12.5 mg/ml, MBC of 50 mg/ml) and anaerobic (*Porphyromonas gingivalis* MIC of 100 mg/ml) strains. No significant antibacterial activity was traced for AE against tested strains. The presence of glucotropaeolin in IUP only is suggested to be responsible for its potent antibacterial activity.

CONCLUSION

The instantly used powder(IUP) of Miswak exhibited more potent antiviral activity against *Herpes simplex* virus type 1 (HSV-I) than AE. It also demonstrated antibacterial activity against aerobic and anaerobic strains. The presence of glucotropaeolin in IUP only and its absence in AE explains its potent antimicrobial activity.

The use of *S. persica* root (Miswak) in its original form (tooth stick) as prophet Mohammed (Peace And Pray Be Upon Him) recommended[17] or as instantly used powder (IUP) is more effective than to use its aqueous extract(AE) to be incorporated in any dosage form, as the active metabolites are damaged during processing.

A simple and validated method for quantification of BITC in Miswak dichloromethane extract MDE was performed and 95 µg BITC was found to be in one gram of Miswak powder.

Further analysis using metabolomics tools are highly recommended for identification of other metabolites of *S. persica* root.

ACKNOWLEDGMENT

The authors are grateful to Prof. Dr. Mansour Sobeh, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University for his help in HPLC-PDA-MS/MS analysis.

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Cite this article as:

Elsayed A. Aboutabl *et al*. HPLC-PDA-MS/MS profile, antimicrobial activity of instantly used miswak (*Salvadora persica* L.) Root versus its aqueous extract and benzyl isothiocyanate quantification. Int. Res. J. Pharm. 2019; 10(5):40-48 http://dx.doi.org/10.7897/2230-8407.1005160

Source of support: Nil, Conflict of interest: None Declared