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ANTIMICROBIAL ISOTHIOCYANATE DERIVATIVES FROM *SALVADORA PERSICA* ROOT "SIWAK" EXTRACT

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Abstract:

Phytochemical study directed by antimicrobial activity testing of the root extract of Salvadora persica known as "Siwak" was conducted to identify the antimicrobial components. Fresh roots were extracted with ethanol and the total extract was fractionated with $CHCl_3$ and EtOAc. The antimicrobial activity was trapped to the $CHCl_3$ soluble fraction. Column chromatography followed by CPTLC resulted in the isolation of three active compounds. In addition to the major compound benzyl isothiocyanate two new minor derivatives 3-methoxy benzyl isothiocyanate (1) and 3-hydroxy benzyl isothiocyanate (2) were identified. Structures were elucidated using different spectroscopic tools including UV, MS, 1D- and 2D-NMR. The isolated compounds were active against gram positive, gram negative bacteria and fungi.

Key words: *Salvadora persica; antimicrobial; MIC, benzyl isothocyanate derivatives.*

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INTRODUTION:

Historically, the chewing stick *Salvadora persica*, family Salvadoraceae known as Siwak is the first known oral hygiene. The use of Siwak is widespread especially in Islamic countries as a tradition inherited from Prophetic medicine [1, 2]. The antimicrobial effect of *S. persica* roots was referred to the major component benzyl isothiocyanate [3]. In addition to benzyl isothiocyanate seventeen compounds were detected from root oil by GC-MS analysis including limonene and α-pinene [4]. Siwak was also reported to contain *β*-sitosterol, *m*-anisic acid, urea derivative Salvadourea, oleic, linolic and stearic acids [5, 6]. Both Egyptian and Saudi collections of Siwak reported to contain the glucosinolates; glucotropaelin and sinigrin [7]. The flavonoids Kaempferol, quercetin, quercetrin, rutin and quercetin glucoside were also isolated from Siwak [8].

In this work a phytochemical study directed by antimicrobial testing was conducted to indentify all the components responsible for Siwak activity. Isolated active compounds were identified by various spectroscopic methods.

MATERIALS AND METHODS:

General

Ultraviolet absorption spectra were obtained in methanol on a Unicum Heyios a UV-Visible spectrophotometer. ^{1}H- and ^{13}C-NMR spectra as well as 2D-NMR experiments (COSY, HSQC and HMBC) were obtained using standard Bruker program on a UltraShield Plus 500 MHz (Bruker) (NMR Unite at the College of Pharmacy, Prince Sattam Bin Abdulaziz University) spectrometer operating at 500 MHz for proton and 125 MHz for carbon, respectively. The chemical shift values are reported in δ (ppm) relative to the residual solvent peak, and the coupling constants (*J*) are reported in Hertz (Hz). EIMS were obtained using SHIMAZU-GC/MS. The GC model 2010 plus connected to Mass Spectrometer model MS-2010-ultra equipped with electron multiplier detector and quadruple system analyzer. Silica gel 60/230-400 mesh (EM Science) was used for column chromatography and silica gel 60 F254 (Merck) was used for TLC. Centrifugal preparative TLC (CPTLC) using Chromatotron (Harrison Research Inc. model 7924) and 2 mm silica gel P254 disc was applied. Aqueous extract was dried using Tray and Manifold Millrock Freeze Dryer LD85 (Millrock Technology).

Plant material

The roots *Salvadora persica*, family Salvadoraceae were purchased from the local market at Al-Kharj city in March 2016. The plant material was identified by Dr. Mohammad Atiqur Rahman, Taxonomist of the Medicinal, Aromatic and Poisonous Plants Research Center (MAPPRC), College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Voucher specimen (# 9011) was deposited at the herbarium of this center.

Extraction and isolation

Fresh roots of *S. persica* "Siwak" (2 kg) were cut into small pieces and extracted with 95% ethanol at room temperature until exhaustion. The combined ethanol extract was concentrated under reduced pressure using rotary vacuum evaporator. The concentrated aqueous alcohol extract was then partitioned in separating funnel by liquid-liquid extraction starting with $CHCl_3$ (3 X 500 mL, 9.2 gm) followed by EtOAc (3 X 300 mL, 10.6 gm). The left aqueous alcohol fraction was lyophilized to give 107.4 gm of dried extract. All fractions were subjected to antimicrobial testing.

Part of the active $CHCl_3$ fraction (8 gm) was chromatographed over silica gel column (300g, 5 cm i.d.) using a gradient of pet. Ether/EtOAc. Fractions 200 ml each were collected and screened by TLC and similar fractions were pooled and subjected to antimicrobial testing. Three collections showed antimicrobial activity.

Fractions 10-22 eluted with 5% EtOAc in pet. ether afforded 2.4 g of benzyl isothiocyanate. Fractions 23-25 eluted with 5% EtOAc in pet. ether (0.2 g) were further purified by CPTLC (2 mm silica gel GF_{254} disk, solvent: pet. ether/EtOAc; 97:3) to give 45 mg of benzyl isothiocyanate and 11 mg of **1.**

Fractions 44-47 (88 mg) eluted with 25 % EtOAc in pet. ether afforded 9 mg of **2** after further purification by CPTLC (2 mm silica gel GF_{254} disk, solvent: pet. ether/EtOAc; 93:7)

Antimicrobial testing

Antimicrobial activities of the crude fractions and pure compounds were conducted against all of *Staphylococcus aureus* ATCC35501; *S. aureus* ATCC29737; *Bacillus subtilis* ATCC10400; *Escherichia coli* ATCC25992; *Enterobacter aerogenes* ATCC10102; *Salmonella Typhimurium* ATTC14028; *Pseudomonas aeruginosa* ATTC10145; *Klebsiella pneumonia* ATCC138222; *Candida albicans* ATCC14053; *Candida albicans* ATCC2091; *Aspergillus niger* ATTC16404 and four other clinical isolates of *E. Coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Staphylococcus epidermidis*. All investigations were conducted by the Cork-borer method [9]. In this method, cultures were inoculated in the form of a loopful of each test organism into about 20 ml of nutrient agar medium for bacteria and Sabaroud agar medium for fungi. The sensitivity of the tested organisms was assayed against crude fractions of concentration ranged from 0.625 to 30 mg/mL and 4- 0.03125 mg/mL for pure isolates using inhibition zone diameter in mm as criterion for the antimicrobial activity. The plates were kept at room

temperature to solidify, wells were made by cork-borer and materials under test in DMSO/ethanol (1:1) was applied into these wells. Wells loaded with the used solvent mixture served as a negative control. The plates were kept in a refrigerator for one hour to permit homogenous diffusion of tested substances before growth of the test organism. Then, plates were incubated at 37°C for 24 h in case of bacteria and at 28°C for 48 h in case of fungi.

Minimum inhibitory concentration (MIC).

The minimum inhibitory concentration was quantified by incorporating known concentrations of purified compounds into solid growth medium using a conventional agar dilution method [10]. Inocula of tested bacteria, fungi and yeasts were inoculated onto Mueller Hinton medium for bacteria and Sabouraud medium for fungi and yeasts, containing different concentrations (0.03125; 0.0625; 0.125; 0.25; 0.5, 1, 2, 4 mg/mL). After a growth period of 24 h at 37°C for bacteria and 48 h at 28°C for fungi, the plates were examined for growth and the lowest compounds concentration that inhibited the growth of each organism was determined. Mueller Hinton and Sabouraud plates, without active products and inoculated with target organisms, was used as a control.

RESULTS:

The different fractions were tested against gram positive, gram negative bacteria and fungi. The $CHCl_3$ fraction was the only active fraction at the tested concentrations. Both EtOAc and aqueous alcohol fractions were inactive at 30 mg/mL. Results are presented in Table 1.

Table 1: Antimicrobial activity of *S. persica* roots "Siwak" $CHCl_3$ soluble fraction.

Organism	Conc. (mg/mL)			
	0.625	1.25	2.5	5
***E. coli* ATCC25922**	+	-	-	-
E. Coli clinical isolate	+	+	-	-
***S. aureus* ATCC 29737**	+	-	-	-
***S. aureus* clinical isolate**	+	-	-	-
***K. Pneumonia* ATCC 13882**	+	+	-	-
***B. subtilis* ATCC 10400**	-	-	-	-
***P. vulgaris* clinical isolate**	+	-	-	-
***S. epidemidis* clinical isolate**	-	-	-	-
***P. aeruginosa* clinical isolate**	+	+	-	-
***Candida albicans* ATCC 2091**	+	-	-	-
***Candida albicans* ATCC 14053**	+	+	-	-
***A. niger* ATCC16404**	+	+	+	-

\- Inhibition of growth + Growth

Table 2. ^{1}H and ^{13}C NMR data of 1 and 2 in C_6D_6 (δ values, *J* in parenthesis in Hz)[a].

Position	1		2	
	^{1}H	^{13}C	^{1}H	^{13}C
1	-	129.94	-	129.90
2	6.49 (bs)	112.38	6.27 (bs)	113.62
3	-	160.24	-	156.21
4	6.47 (d, 7.7)	118.89	6.35 (d, 7.6)	118.68
5	6.94 (t, 7.9)	129.94	6.85 (t, 7.8)	129.90
6	6.59 (dd, 8.25, 2.3)	113.82	6.48 (dd, 8, 2)	115.19
CH_2	3.71	48.00	3.65	47.51
N=C=S	-	136.04	-	135.90
OCH_3	3.25	54.65	-	-

[a] Assignments made by combination of COSY, DEPT, HMQC, HMBC data, and comparison with the literature.

Table 3: Minimum inhibitory concentration of benzyl isothiocyanate, 1 and 2.

Organism	Benzyl isothiocyanate	1	2
***S. aureus,* ATCC35501**	0.5	0.5	1
***B.subtilis* ATCC10400**	1	0.25	2
***E. coli* ATCC25992**	0.5	0.125	1
***E. aerogenes* ATCC 10102**	1	0.5	2
***S. typhimurium* ATTC 14028**	-	-	-
***P. aeruginosa* ATTC 10145**	-	-	-
***P. aeruginosa* clinical isolate**	2	0.5	2
***K. pneumonia* ATCC 138222**	0.5	0.25	1
***C. albicans* ATCC 14053**	1	0.25	2
***C. albicans* ATCC2091**	-	0.25	-
***A. niger* ATTC16404**	0.25	0.25	0.5

Chromatographic purification of the active fraction directed by antimicrobial testing against *S. aureus*; *B. subtilis*; *E. coli*; *P. aeruginosa* and *C. albicans* utilizing column chromatography and CPTLC resulted in the isolation of benzyl isothicyanate, compounds **1** and **2** (Fig. 1A).

3-Methoxy benzyl isothiocyanate (1): C_9H_9NOS, Obtained as colourless liquid; UV (MeOH), λ_{max} 259, 195; ^{1}H-NMR and ^{13}C-NMR (C_6D_6, 500 and 125 MHz, respectively), Table 2; EIMS *m/z* 179 [M^+].

3-Hydroxy benzyl isothiocyanate (2):
C_8H_7NOS, Obtained as colourless liquid; UV (MeOH), λ_{max} 261, 196; ^{1}H-NMR and ^{13}C-NMR (C_6D_6, 500 and 125 MHz, respectively), Table 2; EIMS *m/z* 165 [M^+].

The MIC for the three active compounds was determined using the conventional agar dilution method. The results are presented in Table 3.

DISCUSSION:
The major antimicrobial agent in the $CHCl_3$ fraction was identified as benzyl isothiocyanate by comparing the UV, NMR and MS data with that reported in the literature [11, 12]. Both compounds **1** and **2** showed ^{1}H-NMR and ^{13}C-NMR signals for CH_2-NCS (Table 2). The ^{13}C-NMR signals at δ_C160.24 and 156.21 ppm in **1** and **2** indicated the presence of one oxygenated aromatic carbon in each compound. EIMS of **1** showed an M^+ at m/z 179 diagnostic for an OCH_3 substitution over the structure of benzyl isothiocyanate. The presence of OCH_3 was supported by the NMR signals at δ_H3.25 and δ_C 54.65 ppm. One the other hand the EIMS data of **2** indicated a hydroxyl derivative of benzyl isothiocyanate. The splitting pattern of the four aromatic protons as well as the chemical shifts of the aromatic carbons were all in support of a *meta* di-substitution in **1** and **2**. The broad singlet in the ^{1}H-NMR of **1** and **2** at δ_H 6.49 and 6.27ppm respectively, assigned to H-2 based on the analysis of COSY experiment results (Fig. 1B) provided the strongest evidence for *meta* di-substitution as they are free from *ortho* splitting *J* value. Confirmation of the *meta* substitution was achieved by comparison of the NMR data of **1** and **2** with *o*-cresol [13] and *m*-cresol [14]. Consequently, **1** was identified as 3-methoxy benzyl isothiocyanate and **2** as 3-hydroxy benzyl isothiocyanate. The two derivatives are reported for the first time from natural source.

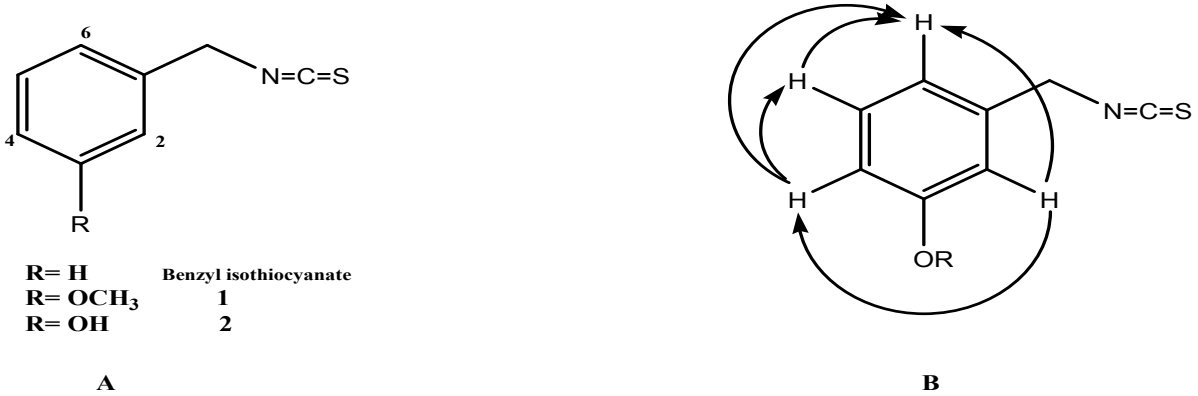

Fig. 1 A: Structures of isolated compounds. B: COSY correlations of 1 and 2.

The MIC for the three isothiocyanate derivatives was determined against panel representing gram positive, gram negative bacteria and fungi (Table 3). Compound **2** with the medium polarity among the three isolates showed the highest activity against all the tested organisms. *Escherichia coli* ATCC25992 was the most susceptible organisms with MIC at 0.125 mg/mL. The compound also showed promising activity against *C. albicans* ATCC14053, *C. albicans* ATCC2091 and *A. niger* ATTC16404. Benzyl isothiocyanate with the least polarity was less active than **1** in all tested organisms. In addition, *C. albicans* ATCC2091 was resistant at 2 mg/mL. The most polar derivative **2** was the least active and *C. albicans* ATCC2091 was also resistant at 2 mg/mL. The strongest effect of **2** was expressed against *A. niger* ATTC16404 at 0.5 mg/mL.

CONCLUSION:

Benzyl isothiocyanate is the major metabolites responsible for the antibacterial and antifungal activity of Siwak. Benzyl isothiocyanate represents about 30% of the active $CHCl_3$ fraction. Two minor derivative are also active. The 3-methoxy benzyl isothiocyanate (**1**) showed stronger activity than benzyl isothiocyanate in all the tested organism. The second compound 3-hydroxy benzyl isothiocyanate (**2**) found to be less active than the major compound. These two derivatives are reported for the first time from natural source. The study also revealed that other compounds reported from Siwak have no antimicrobial activity.

Disclosure statement

No potential conflict of interest was reported by the authors.

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