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# Benzylamides from *Salvadora persica*

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A phytochemical investigation of stems from *Salvadora persica* resulted in the first isolation of four benzylamides from a natural source. The isolated compounds were identified as butanediamide, $N^1$,$N^4$-bis(phenylmethyl)-2(*S*)-hydroxy-butanediamide (**1**), *N*-benzyl-2-phenylacetamide (**2**), *N*-benzylbenzamide (**3**) and benzylurea (**4**). The structure elucidation was accomplished using spectroscopic methods, especially 2D NMR and HREIMS. Compound **2** revealed a significant inhibitory effect on human collagen-induced platelet aggregation, and a moderate antibacterial activity against *Escherichia coli*.

**Key words:** *Salvadora persica*, Benzylamides, Human antiplatelet aggregation effect, Antibacterial activity

## INTRODUCTION

*Salvadora persica* L. (Salvadoraceae) is a subtropical tree, of medicinal interest, native to the Arabian Peninsula, Egypt, and India (Tackholm 1974, Nadkarni 1976). In Eastern Africa, the roots of this tree are used as a remedy for ancylostomiasis, rheumatic pain, gonorrhoea, gastritis, and as an ascarifuge, the leaf is also reputed to be diuretic (Watt and Breyer-Brandwijk 1962). The stems and roots ("Arak" or "Meswak") are widely used in the Middle East, especially among Muslims, as a tooth brush and to detoxify and strengthen the weakened gums; it is considered an efficient and inexpensive tool for oral hygiene (Al-Bagieh and Weinberg, 1988). The extract of the stems is reported to possess antimicrobial (Al-Bagieh and Weinberg, 1988, Al-Bagieh *et al.*, 1994, Almas and Al-Zeid, 2004), anti-inflammatory (Ezmirly *et al.*, 1979), hypoglycemic (Trovato *et al.*, 1998), and hypolipidemic (Galati *et al.*, 1999) activities. Three lignan glycosides were isolated from the stems of this species (kamel *et al.*, 1992) whereas an indole alkaloid was reported in the leaves (Malik *et al.*, 1987). The flavonoids rutin and quercetin were detected in the stems (Abdel-Wahab *et al.*, 1990). Salvadourea, (*m*-$MeOC_6H_4CH_2NH)_2CO$, has been reported in the roots. (Ray *et al.*, 1975). Benzylisothiocyanate was also isolated from the roots and is claimed to be responsible for antiviral activity against HSV-1 (Al-Bagieh *et al.*, 1992) as well as controlling dental caries (Al-Bagieh and Weinberg, 1988). The need for more scientific evidence to validate the wide traditional uses of *S. persica* has provoked the present study. Chromatographic fractionation of the methanol extract from the stems of *S. persica* has resulted in the isolation of four benzylamides. The isolated compounds were identified as $N^1$,$N^4$-bis(phenylmethyl)-2(*S*)-hydroxy-butanediamide (**1**), *N*-benzyl-2-phenylacetamide (**2**), *N*-benzylbenzamide (**3**), and benzylurea (**4**). Although these amides were previously synthesized (Cousins *et al.*, 1995; Yadav *et al.*, 2003; Salehi *et al.*, 2001), this is the first time to be isolated from a natural source. The structure elucidation was achieved using spectroscopic methods, especially 2D NMR and HREIMS. We investigated the human anti-platelet aggregation and antibacterial activities of the major compounds (**1** and **2**).

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## MATERIALS AND METHODS

### Instruments and reagents

Melting points were determined using a Laboratory Devices Mel-Temp II and were uncorrected. Optical rotations were run on a JASCO DIP-370 digital polarimeter. IR spectra were measured using a Hitachi 260-30 spectrophotometer. The $^1$H-, $^{13}$C-NMR, COSY, HMQC, HMBC, and NOESY spectra were recorded on a Varian Unity INOVA 500 FT-NMR spectrometers at 500 MHz for $^1$H and 125 MHz for $^{13}$C respectively, using TMS as the internal standard. The chemical shifts are given in $\delta$ value (ppm)

and coupling constants in Hz. Low-resolution EIMS and FABMS were recorded on a VG Quattro 5022 mass spectrometer, while HREIMS were measured on a JEOL JMS-HX 110 mass spectrometer. Silica gel for the column chromatography was obtained from E. Merck (Germany) and agar from Sigma Co. (U.S.A.). All microorganisms were obtained from the Dept. of Microbiology, Faculty of Pharmacy, Mansoura University.

## Plant material

The stems of *Salvadora persica* L. were purchased from a local drug store and identified by Professor I. Mashaly, Faculty of Science, Mansoura University. A voucher specimen (Salp05) was archived in the Dept. of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Egypt.

## Extraction and isolation

The stems (500 g) were chopped into small pieces and extracted thrice with MeOH. The extract was filtered, concentrated under vacuum to 100 mL and diluted with an equal volume of distilled water. The hydroalcoholic extract was defatted by shaking with *n*-hexane followed by extraction with $CH_2Cl_2$. The $CH_2Cl_2$ extract was concentrated using a vacuum to yield a crude residue (750 mg). Column chromatography of the residue on silica gel, using a gradient of *n*-hexane/EtOAc for elution and hydroxylamine/$FeCl_3$ reagent (Stahl, 1969) for the visualization of TLC, afforded ten fractions ($F_1$-$F_{10}$). Fractions $F_2$, $F_3$ and $F_4$ containing compounds with benzyl radicals (as monitored by $^1$H-NMR) were separately re-chromatographed on silica gel columns using a gradient of $CH_2Cl_2$/MeOH. Fraction $F_2$ yielded **1** (74 mg) and **2** (50 mg) while $F_3$ presented **3** (14 mg) and $F_4$ afforded **4** (7 mg).

### *N*$^1$,*N*$^4$-Bis(phenylmethyl)-2(*S*)-hydroxy-butanediamide (1)

Mp 153-155°C; $[\alpha]_D^{25}$ -24.2° (*c* 0.05, MeOH); IR ($CH_2Cl_2$) $\nu_{max}$ cm$^{-1}$: 3281 br (O-H st, N-H st), 3028 (C-H ar), 2925 (C-H aliph), 1650, 1647 (amide C=O), 1601, 1543, 1494 (ar C=C), 1028 (C-O), 696 (monosub. benzene); EIMS (rel. int.) *m/z*: 312 $[M]^+$ (2), 206 (6), 178 (42), 106 (70), 91 (100), 77 (15); HREIMS *m/z*: 312.1474 ($C_{18}H_{20}N_2O_3$, calcd 312.1468); $^1$H-NMR (500 MHz, pyridine-$d_5$) $\delta_H$: δ 5.34 (ddd, *J* = 9.0, 6.0, 3.5 Hz, H-2), 3.16 (dd, *J* = 14.0, 9 Hz, H-3a), 3.50 (dd, *J* = 14.0, 3.5 Hz, H-3b), 4.71 (2H, dd, *J* = 15.0, 6.0 Hz, H-1'), 4.74 (2H, dd, *J* = 15.0, 6.0 Hz, H-1"), 7.46 (4H, m, H-3',7',3",7"), 7.29 (4H, m, H-4',6',4",6"), 7.25 (2H, m, H-5',5"), 9.38 (t, *J* = 6.0 Hz, NH-a), 9.20 (t, *J* = 6.0 Hz, NH-b), 8.04 (d, *J* = 6.0 Hz, OH); $^{13}$C-NMR (125 MHz, pyridine-$d_5$): Table I.

### *N*-Benzyl-2-phenylacetamide (2)

IR ($CH_2Cl_2$) $\nu_{max}$ cm$^{-1}$: 3307 (N-H), 3028 (C-H ar), 2927 (CH aliph), 1648, (amide C=O), 1621, 1541, 1491 (ar C=C), 697 (monosub. benzene); EIMS *m/z* (rel. int.): 255 $[M]^+$ (1), 227 $[M\text{-}CO]^+$, 154 (31), 136 (30), 91 (100), 89 (23), 77 (28), $C_{15}H_{15}NO$; HREIMS *m/z*: 226.1233 $[M+H]^+$ ($C_{15}H_{15}NO$, calcd 226.1232); $^1$H-NMR (500 MHz, pyridine-$d_5$) $\delta_H$: 3.56 (2H, s, $H_2$-2), 7.34 (2H, d, *J* = 7.0 Hz, H-4,8), 7.29 (2H, brd, *J* = 7.0 Hz, *m*-H-5,7), 7.23 (H, m, H-6), 4.37 (2H, d, *J* = 6.0 Hz, C<u>H</u>$_2$-1'), 7.25 (2H, m, H-3',7'), 7.24 (2H, m, H-4',6'), 7.22 (H, m, H-6'), 7.61 (br t, *J* = 6.0 Hz, NH); $^{13}$C-NMR (125 MHz, pyridine-$d_5$): Table I.

### *N*-Benzylbenzamide (3)

IR ($CH_2Cl_2$) $\nu_{max}$ cm$^{-1}$: 3307 (N-H), 3028 (C-H ar), 2927 (CH aliph), 1648, (amide C=O), 1621, 1541, 1491 (ar C=C), 697 (monosub. benzene); EIMS *m/z* (rel. int.): 212 $[M+1]^+$ (21), 105 (36), 91 (100), 77 (27); HREIMS *m/z* : 212.1076 $[M+1]^+$ ($C_{14}H_{13}NO$, calcd 212.1075); $^1$H-NMR (500 MHz, pyridine-$d_5$) $\delta_H$: 4.60 (2H, d, *J* = 6.0 Hz, $CH_2$-1'), 7.94 (2H, d, *J* = 7.5 Hz, H-3,7), 7.46 (2H, t, *J* = 7.5 Hz, H-4,6), 7.52 (H, t, *J* = 7.5 Hz, H-5), 7.37 (2H, d, *J* = 7.5 Hz, H-3',7'), 7.31 (2H, t, *J* = 7.5 Hz,, H-4',6'), 7.23 (H, t, *J* = 7.5 Hz, H-5'), 8.25 (br s, NH); $^{13}$C-NMR (125 MHz, pyridine-$d_5$): Table I.

### Benzyl urea (4)

IR ($CH_2Cl_2$) $\nu_{max}$ cm$^{-1}$: 3434, 3332 ($NH_2$ st), 3006 (C-H ar), 2914, 2870 (C-H aliph), 1663, (<u>CO</u>-NH), 1603 (ar C=C), 710 (monosub. benzene); EIMS *m/z*: 150 ($C_8H_{10}NO$), FABMS *m/z*: 151 $[M+1]^+$; $^1$H-NMR (500 MHz, pyridine-$d_5$) $\delta_H$: 4.77 (2H, d, *J* = 6.5 Hz, $H_2$-1'), 7.48 (2H, d, *J* = 7.0 Hz, H-3',7'), 7.30 (2H, dd, *J* = 7.5, 7.0 Hz, H-4',6'), 7.24 (H, d, *J* = 7.5 Hz, H-5'), 10.33 (H, t, *J* = 6.5 Hz, NH), 4.97 (2H, s, $NH_2$); $^{13}$C-NMR (125 MHz, pyridine-$d_5$): Table I.

## Antiplatlet aggregation effect

Human blood anticoagulated with acid citrate dextrose was obtained from healthy human volunteers, who had not taken any drugs within the last two weeks. Platelet aggregation was measured using the turbidimetric method with a light-transmission aggregometer (Chronolog Co., U.S.A.) (Wu *et al.*, 2003). The extent of platelet aggregation was measured as the maximal increase of light transmission within 5 min after the addition of the stimulators [human a-thrombin and collagen (type 1, bovine Achilles tendons), Sigma. Chem. Co. U.S.A.]. The $IC_{50}$ value was defined as the final concentration that caused 50% inhibition (Zeng *et al.*, 1997).

## Investigation of the antimicrobial activity

Compounds **1** and **2** were screened for their *in vitro* antimicrobial activity against gram positive (*staphylococcus aureus*) bacteria, gram negative (*Pseudomonas aeruginosa*,

*Escherichia coli*) bacteria, and fungi (*Candida albicans*) using the agar diffusion method (Lorian, 1980; Blair *et al.*, 1970). Ampicillin and gentamicin (each 20 μg/mL) were employed as positive controls. The resulting inhibition zones were measured with a verniar caliper, after 24 h of incubation at 37°C.

## RESULTS AND DISCUSSION

Compound **1** was obtained in the form of colorless needle-shaped crystals with a molecular formula of $C_{18}H_{20}N_2O_3$ as deduced from its HREIMS, consistent with 10 degrees of unsaturation. The IR spectrum showed bands diagnostic of bonded hydroxyl (3281 $cm^{-1}$), amide carbonyl (1650, 1642 $cm^{-1}$) and monosubstituted benzene (696 $cm^{-1}$). The $^{13}$C-NMR spectrum (Table I) revealed two carbonyls ($\delta_C$ 172.1 and 175.1), three methylenes ($\delta_C$ 42.6, 43.4 and 43.9), two quaternaries ($\delta_C$ 140.5 and 140.7), one oxymethine ($\delta_C$ 70.7), along with two sets of five aromatic methines ($\delta_C$ 127.7 to 129.3). The $^1$H-NMR spectrum (experimental section) displayed ten aromatic protons at $\delta_H$ 7.25 (2H), 7.29 (4H) and 7.46 (4H), two benzyl methylenes at $\delta_H$ 4.71, 4.74 (each 2H, dd, $J$ = 15.0, 6.0 Hz, H-1' and H-1" respectively). The presence of benzyl moiety was proved by the EIMS base peak at *m/z* 91 (100%). The 2D techniques (COSY, NOESY, HMQC, HMBC) were invaluable for the complete assignment of all the NMR signals, especially the NH and OH signals. The two downfield triplets at $\delta_H$ 9.38 and 9.20 showed NOESY correlations with two benzyl $CH_2$ at $\delta_H$ 4.71 and 4.74 respectively, implying the presence of a pair of Ph-$CH_2$-NH groups (Fig. 1). In addition, the two *gem*-coupled protons at $\delta_H$ 3.50 (dd, $J$ = 14.0, 3.5 Hz, H-3a) and $\delta_H$ 3.16 (dd, $J$ = 14.0, 9.0 Hz, H-3b) were directly attached to a $\underline{C}H_2$ (C-3) at $\delta_C$ 42.6 (HMQC spectrum) and $^2J$-correlated to a carbonyl at $\delta_C$ 175.1 (C-4) in the HMBC spectrum. The oxymethine proton resonating at $\delta_H$ 5.34 (ddd, $J$ = 9.0, 6.0, 3.5 Hz) exhibited a strong NOESY correlation to one $C\underline{H}_2$ signal at $\delta_H$ 3.50 (H-3a), COSY correlations to $CH_2$ at $\delta_H$ 3.50, 3.16 (H-3a and H-3b), and HMBC correlations to the carbonyl at $\delta_C$ 175.1 (C-4) and $\underline{C}H_2$ at $\delta_C$ 42.6 (C-3). This suggested the presence of a partial amide structure Ph-$CH_2$-NH-CO-$CH_2$-CHOH-, and was further supported by the EIMS fragment at *m/z* 178 (Fig. 2). The NOESY spectrum revealed a correlation between the proton at $\delta_H$ 3.16 (H-3b) and NH-b indicating their close proximity. The COSY correlations between H-1'/NH-a; H-2/OH, H-3a, H-3b; H-1"/NH-b together with MS fragments (Fig. 1) verified the presence of two benzylamide units connected through -$CH_2$-CHOH- moiety. The α-configuration of the OH was determined by comparing the sign and magnitude of the optical rotation in MeOH (-24.2, $c$ = 0.05) with that of a similar (*S*)-synthetic compound (-32.3, $c$ = 0.26) (cousins *et al.*, 1995). As a consequence, the structure of compound **1** was established as *N*$^1$,*N*$^4$-bis(phenylmethyl)-2(*S*)-hydroxy-butanediamide or (*S*) *N*$^1$,*N*$^4$-

**Table I.** $^{13}$C-NMR (125 MHz, pyridine-$d_5$) data of compounds **1** and **2**

| C-atom | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | 172.1 s | 171.1 s | 167.3 s | |
| 2 | 70.7 d | 43.9 t | 135.9 s | 161.9 s |
| 3 | 42.6 t | 137.4 s | 128.1 d | |
| 4 | 175.1 s | 130.1 d | 129.2 d | |
| 5 | | 129.2 d | 132.0 d | |
| 6 | | 127.4 d | 129.2 d | |
| 7 | | 129.2 d | 128.1 d | |
| 8 | | 130.1 d | | |
| 1' | 43.9 t | 43.6 t | 43.9 t | 43.9 t |
| 2' | 140.5 s | 140.6 s | 140.8 s | 139.8 s |
| 3' | 128.2 d | 128.3 d | 128.5 d | 128.4 d |
| 4' | 129.2 d | 129.2 d | 129.2 d | 129.3 d |
| 5' | 127.7 d | 127.7 d | 127.7 d | 127.9 d |
| 6' | 129.2 d | 129.2 d | 129.2 d | 129.3 d |
| 7' | 128.2 d | 128.3 d | 128.5 d | 128.4 d |
| 1" | 43.4 d | | | |
| 2" | 140.7 s | | | |
| 3" | 128.4 d | | | |
| 4" | 129.3 d | | | |
| 5" | 127.8 d | | | |
| 6" | 129.3 d | | | |
| 7" | 128.4 d | | | |

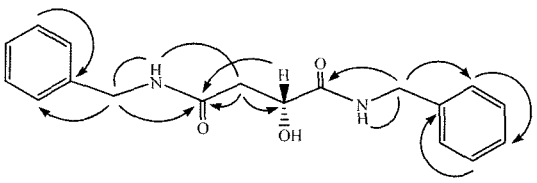

**Fig. 1.** Key HMBC (arrow) and NOESY (curved line) correlations of compound **1**.

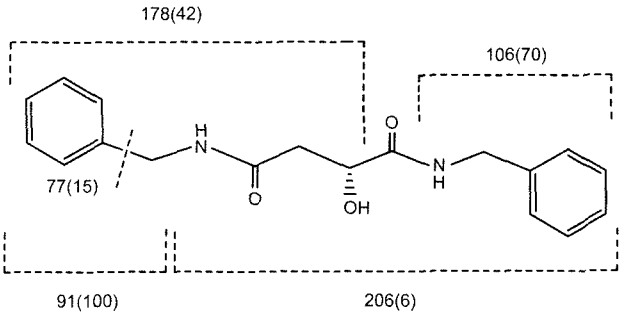


**Fig. 2.** Selected EIMS fragments from compound **1**

dibenzyl-2-hydroxy-succinamide.

The IR spectrum of compound **2** revealed an absorption band for carbonyl amide (1648 $cm^{-1}$) and an EIMS fragment at *m/z* 91 $[C_7H_7]^+$ indicated a benzyl moiety. The $^{13}$C-NMR spectrum exhibited one carbonyl ($\delta_C$ 171.1), two methylenes ($\delta_C$ 43.6 and 43.9), along with two aromatic rings ($\delta_C$ 127.4-140.6). The $^1$H-NMR of compound **3** showed ten aromatic protons and a benzylic methylene at $\delta_H$ 4.37 (2H, d, *J* = 6 Hz, C<u>H</u>$_2$NH) having HMBC correlations with the aromatic <u>C</u>H carbon at $\delta_C$ 128.3 and the quaternary aromatic carbon at $\delta_C$ 140.6. Another methylene singlet was detected at $\delta_H$ 3.56 (C<u>H</u>$_2$-CO, corresponding to $\delta_C$ 43.9) and revealed HMBC correlations with aromatic <u>C</u>H at $\delta_C$ 130.1 and a carbonyl carbon signal at $\delta_C$ 171.1. The NOESY spectrum displayed correlations between the benzyl methylenes at $\delta_H$ 3.56, 4.37 and the aromatic *o*-protons at $\delta_H$ 7.34 and 7.25, respectively. The fragment ions at *m/z* 120 $[Ph\text{-}CH_2\text{-}CHO]^+$, 107 $[Ph\text{-}CH_2\text{-}NH_2]^+$, and 134 $[Ph\text{-}CH_2\text{-}CO\text{-}NH]^+$ supported a proposed structure of Ph-$CH_2$-CO-NH-$CH_2$-Ph. Thus the structure of compound **2** was deduced as *N*-benzyl-2-phenylacetamide, and was further confirmed by the HREIMS molecular ion at *m/z* 226.1233 $[M+H]^+$consistent with molecular formula $C_{15}H_{15}NO$.

The $^{13}$C-NMR spectra of compound **3** revealed signals for two aromatic rings ($\delta_C$ 127-140.8) and one amide carbonyl $\delta_C$ 167.3 (IR band at 1651 $cm^{-1}$). The NMR signals at $\delta_H$ 7.94 (2H, dd, *J* = 7.5, 0.9 Hz, H-3,7), 7.46 (2H, t, *J* = 7.5 Hz, H-4,6), 7.52 (H, t, *J* = 7.5 Hz, H-5) and carbonyl at $\delta_C$ 167.3 were characteristic of a benzoyl moiety. The second aromatic ring formed a *N*-benzyl moiety as indicated by the benzyl C<u>H</u>$_2$ signal at $\delta_H$ 4.60 (2H, d, *J* = 6.0) that revealed NOESY correlations with N<u>H</u> ($\delta_H$ 8.25) and *o*-aromatic protons ($\delta_H$ 7.37). The benzyl protons were $^3$*J*-correlated with the benzoyl carbonyl at $\delta_C$ 167.3 and aromatic <u>C</u>H at $\delta_C$ 128.5, suggesting the attachment of *N*-benzyl to a benzoyl group (Fig. 3). This was proved by the EIMS fragments at *m/z* 105 $[Ph\text{-}CO]^+$, 121 $[Ph\text{-}CO\text{-}NH_2]^+$, and 91 $[Ph\text{-}CH_2]^+$, and the NOESY correlation between the NH signal at $\delta_H$ 8.25 (br s) and *o*-aromatic protons at $\delta_H$ 7.94. A final confirmation was obtained from the HREIMS data revealing a molecular ion peak at *m/z* 212.1076 $[M+1]^+$ corresponding to a molecular formula of $C_{14}H_{13}NO$. The structure of **3** was unambiguously elucidated as *N*-benzylbenzamide.

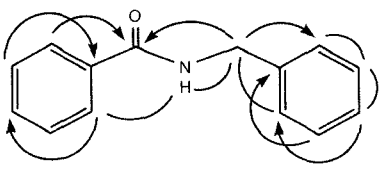


**Fig. 3.** Key HMBC (arrow) and NOESY (curved line) correlations of compound **3**

The IR spectrum of compound **4** revealed the presence of absorption bands diagnostic of a primary amine group (3434, 3332 $cm^{-1}$) and an amide carbonyl (1663 $cm^{-1}$). The $^1$H-NMR spectrum displayed 5 aromatic protons [7.48 (2H, d, *J* = 7.0 Hz, H-3',7'), 7.30 (2H, dd, *J* = 7.5, 7.0 Hz, H-4',6'), 7.24 (H, d, *J* = 7.5 Hz, H-5')] and benzylic C<u>H</u>$_2$ at $\delta$ 4.77 (d, *J* = 6.5 Hz). The latter methylene was vicinally coupled with N<u>H</u> at $\delta_H$ 10.33 (H, t, *J* = 6.5 Hz) in the COSY spectrum, and possessed HMBC correlations with a carbonyl carbon signal at $\delta_C$ 161.9 and aromatic <u>C</u>H at $\delta_C$ 128.4. Furthermore, NOESY correlations were detected between methylene protons ($\delta$ 4.77), NH signal ($\delta_H$ 10.33), and the *o*-aromatic CH ($\delta_H$ 7.48). The EIMS fragment ion at *m/z* 91 $[C_7H_7]^+$ and FABMS molecular ion at *m/z* 151 $[M+1]^+$ ($C_8H_{10}NO$) verified that compound **4** was benzylurea.

## Antiplatelet aggregation effect

We evaluated the *in vitro* inhibitory effects of compounds **1** and **2** against human platelet aggregation induced by thrombin (0.1 U/mL) and collagen (10 μg/mL). The results showed that compound **2** exhibited a significant inhibitory effect on platelet aggregation induced by collagen (Table II). Although the exact mechanism is unknown, it is probably ascribed to suppression of cyclooxygenase (COX) or thromboxane synthetase (TXS) (Kuo *et al.*, 1995; Murray *et al.*, 1993). This suggests that compound **2** may contribute to the anti-inflammatory action of the crude remedy (Glitz *et al.*, 1997) and provide a rationale for its traditional use.

## Antimicrobial activity

Compound **1** was inactive against all tested organisms while compound **2** was only moderately active against *Escherichia coli* at a concentration of 87 μg/mL (equivalent to 20 μg/mL of gentamicin). Together with other bacteria, *Escherichia coli* forms a dental biofilm harboring oral bacteria strongly correlated with the progression of dental disease (Steinberg *et al.*, 2005). Compound **2**, together with other constituents of *S. persica* stems such as lignans and flavonoids, with reported antimicrobial activity (Saleem *et al.*, 2005), were deemed accountable for the oral hygienic effects caused by the stems.

**Table II.** *Antiplatelet aggregation effects of compounds* **1** *and* **2**

| Compound | $IC_{50}$ (M) | |
|---|---|---|
| | Thrombin (0.1 U/mL) | Collagen (10 μg/mL) |
| **1** | >100 | 2.62 ± 0.07 |
| **2** | >100 | 1.21 ± 0.02 |

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