Bioactive Secondary Metabolites from *Salix tetrasperma* Roxb.

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Column chromatography of the light petroleum fraction from the methanolic extract of the stem bark of *Salix tetrasperma* Roxb. (Salicaceae) resulted in the isolation of *β*-sitosterol acetate, friedelin, 3*β*-friedelinol, *β*-amyrin, *β*-sitosterol, *β*-sitosterol-*O*-glucoside in addition to palmitic acid. From the dichloromethane fraction of the leaves, catechol and tremulacin were isolated. Salicin and its derivatives tremuloidin and 2'-*O*-*p*-(*E*)-coumaroyl salicin were isolated from the ethyl acetate fraction of the leaves. The isolated compounds were identified by MS, and 1D NMR (^{1}H and ^{13}C) and 2D NMR (H-H COSY, HSQC, and HMBC) spectral analyses. The total methanolic extract exhibited significant anti-inflammatory activity (rat hind paw oedema). The extract with a content of 120 mg/kg body weight produced 52% inhibition equivalent to the standard diclofenac sodium (54% inhibition). The antioxidant (DPPH free radical scavenging) and analgesic activities, respectively, were also evaluated.

Key words: Salix tetrasperma, Phenolic Glycosides, Biological Activity

Introduction

The genus *Salix* (willow) comprises about 500 species that are mainly distributed in the temperate region worldwide and also in higher altitudes of the tropics (Boulos, 1999; Evans, 2009). The species are rich in phenolic constituents such as salicylates, flavonoids, and tannins, that have many important pharmacological activities and medicinal uses (Malterud *et al*., 1985; El-Shemy *et al*., 2003; Pohjamo *et al*., 2003; Alam *et al*., 2006; Barnes *et al*., 2007; Khare, 2007; Li *et al*., 2008; Enayat and Banerjee, 2009; Fernandes *et al*., 2009; Sonboli *et al*., 2010; Mondal *et al*., 2010; Chhetree *et al*., 2010; Hussain *et al*., 2011).

Only two phytochemial investigations of *Salix tetrasperma* Roxb. growing in Egypt have been reported previously based only on thin-layer chromatography (TLC) screening (Fahmy and Abdel-Latif, 1948; Sahsah, 1978). Recently, anti-inflammatory activity of the aqueous methanolic leaf extract of this plant was studied (Karawya *et al*., 2010). The aim of the present study was to characterize the chemical profile of the leaves and stem bark of the plant and to evaluate the anti-inflammatory (rat hind paw oedema), antioxidant (DPPH free radical scavenging), and analgesic activities, respectively.

Material and Methods

Plant material

Leaves and stem bark of *Salix tetrasperma* Roxb. (Salicaceae) were collected in March 2007 in the vicinity of Zagazig City, Sharkia Province, Egypt. The identification was verified by Dr. H. Abdel Baset, Professor of Botany, Faculty of Science, Zagazig University, Zagazig, Egypt. A voucher specimen has been deposited with the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazing, Egypt.

Extraction and fractionation

The dried stem bark of *S. tetrasperma* (4 kg) was extracted with 80% aqueous methanol (3 x 10 L). The solvent was removed under reduced pressure to yield 200 g of a viscous brown extract. The concentrated extract was suspended in 500 mL water/methanol mixture (9:1 v/v) and fractionated by extraction with light petroleum (60–80 °C), dichloromethane, and ethyl acetate. The organic extracts were concentrated to yield 17, 3.4, and 51.3 g, respectively.

The dried leaves (5 kg) were extracted and fractionated in the same manner as with the stem bark. The total methanolic extract gave 700 g of a dark green viscous residue. The yield of the light

petroleum, dichloromethane, and ethyl acetate fractions were 32, 74, and 73 g, respectively.

Isolation of compounds

Stem bark

About 15 g of the light petroleum fraction from the stem bark were chromatographed over a silica gel column (80 x 3 cm, 250 g), packed with light petroleum. The polarity of the eluent was increased gradually using dichloromethane followed by methanol. Fractions of 250 mL each were collected, concentrated under vacuum, and monitored by TLC using pre-coated silica gel GF_{254} plates (Merck, Darmstadt, Germany) and a mixture of light petroleum/chloroform/methanol (15:15:2 v/v/v) as solvent for development. Spots were visualized by spraying with 10% (v/v) aqueous H_2SO_4 followed by heating at 105 °C. By repeated crystallization and/or preparative layer chromatography of the column fractions, compounds **1**–**7** were isolated.

Leaves

The dried dichloromethane fraction (30 g) was chromtographed over a silica gel column (150 x 4 cm, 500 g), packed with light petroleum. The column was eluted with a gradient using dichloromethane followed by methanol. Fractions were collected and monitored by TLC using chloroform/methanol (9:1 v/v). Compounds were detected under UV light and spraying with 10% (v/v) aqueous H_2SO_4 followed by heating at 105 °C. Repeated crystallization of the column fractions resulted in the isolation of two compounds **8** and **9**.

The dried ethyl acetate fraction (30 g) was applied as dry mixed initial zone on the top of a silica gel column (150 x 4 cm, 500 g), packed with dichloromethane. The column was eluted with dichloromethane, and the polarity of the eluent was increased gradually using ethyl acetate followed by methanol. Fractions (250 mL each) were collected, concentrated, and monitored by TLC using ethyl acetate/acetic acid/formic acid/water (100:11:11:27 v/v/v/v) as system for development. UV light and spraying with 10% (v/v) aqueous H_2SO_4 were used for visualization. Three phenolic compounds **10**–**12** were isolated by repeated crystallization.

Spectral analyses

NMR spectra (^{1}H and ^{13}C) were recorded on Varian MAT 500 (Palo Alto, CA, USA), Mercury 300 BB (Palo Alto, CA, USA), and JEOL 500 (Tokyo, Japan) instruments, at 500, 300, and 500 MHz for ^{1}H measurements and 125, 125, and 75 MHz for ^{13}C measurements, respectively. CD_3OD and DMSO-d_6 were used as solvents. Chemical shifts were determined in ppm with tetramethylsilane (TMS) as internal standard. APT, 2D-NMR, H-H-COSY, HSQC, and HMBC experiments were done to obtain reliable assignments. Electron impact mass spectra were recorded on a Shimadzu GC-MS-QP5050A (Kyoto, Japan) mass spectrometer at 70 eV. FAB-MS was performed on a MATT 8200 (Ringoes, NJ, USA) instrument using 3-nitrobenzyl alcohol as a matrix. Infrared spectral analyses were recorded in potassium bromide disks on a Pye Unicam SP 3000 (Philips, Cambridge, UK), a Shimadzu IR spectrophotometer, and a Jasko FT/IR-460 plus (Hachioji, Japan) instrument. A Shimadzu UV-1700 spectrophotometer was used for UV spectra.

Chemicals

Ascorbic acid, gum acacia, diclofenac sodium, dexamethasone, carrageenan, and acetic acid were purchased from El-Naser Chemical Company (Zagazig, Egypt) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical from Sigma-Aldrich (St. Louis, MO, USA). Authentic samples of *β*-sitosterol, *β*-sitosterol-*O*-glucoside, *β*-sitosterol acetate, *β*-amyrin, friedelin, 3*β*-friedelinol, and hesperidin were obtained from the Department of Pharmacognosy, Faculty of Pharmacy, University of Zagazig, Zagazig, Egypt. Methanol, dichloromethane, chloroform, light petroleum, benzene, ethyl acetate, formic acid, and other solvents used for extraction, separation, and/or detection were of analytical grade.

Antioxidant activity

The free radical scavenging activity of the crude methanolic extract of the leaves and stem bark of *S. tetrasperma* was tested according to the procedure described by Dordevic *et al.* (2007). A stock solution was prepared by dissolving 10 mg of the extract in 1 mL methanol. Serial dilutions were done from this solution, and each dilution was tested by adding 500 μL 0.2 mM DPPH. The

final volume was brought to 1 mL, the mixtures were vigorously shaken and allowed to stand in the dark for 30 min at room temperature, before absorbance was measured at 517 nm against blanks lacking DPPH (negative control). The antioxidant activity, expressed as IC_{50} (μg/mL), was compared with standard antioxidants such as ascorbic acid and hesperidin.

Anti-inflammatory activity

The anti-inflammatory activity of the crude methanolic extract of the leaves and stem bark of *S. tetrasperma* was studied using the hind paw oedema method as reported by Winter *et al.* (1962). Diclofenac sodium and dexamethasone were used as reference standards. Twenty male albino rats were divided into four groups of five rats each. The first group served as control and was given gum acacia mucilage (7%). The second group received diclofenac sodium at the dose of 4 mg/kg body weight (BW) and the third group dexamethasone at the dose of 0.5 mg/kg BW. The fourth group was given the total alcoholic extract (120 mg/kg BW) suspended in 7% gum acacia. All treatments were administered orally. Thirty min later, paw oedema was induced by subcutaneous injection of 0.1 mL carrageenan (1% suspension in saline) into the subplantar surface of the right hind paw of each animal (including control), while 0.1 mL normal saline was injected into the left hind paw. The hind paw diameter was measured, using a micrometer, just before the injection of carrageenan and 1, 2, 3, 4, 5, 6, 12, and 24 h after the injection, and the mean thickness of the oedema was calculated and compared with that of the controlled inflamed rats.

Since the time course of the effect was plotted against percentage increase in paw oedema, the area under the curve (*AUC*) was considered as the cumulative anti-inflammatory effect during the entire observation period. Because the *AUC* represents the integrated anti-inflammatory effect (variation of paw diameter) during the observation period, it includes both the maximal response and the duration of action.

The *AUC* relating variation of oedema to time was obtained using the trapezoidal rule (Tallarida and Murray, 1981). Total inhibition (*TI*, %) was calculated for each group and at each record, using the following equation:

$$TI\ (\%) = (AUC\ \text{control} - AUC\ \text{treated}) \cdot 100 / AUC\ \text{control}.$$

Analgesic activity

The analgesic activity of the crude methanolic extracts was determined using the acetic acid-induced writhing technique (Elisabetsky *et al.*, 1995). Acetic acid was used as an inducer of writhing. A sensitivity test for acetic acid was carried out 1 d before the experiment as follows: each male mouse was injected interaperitoneally with 0.1 mL/10 g BW of 0.6% acetic acid. Mice were observed for 15 min, contraction of the abdominal muscles and stretching of hind limbs was considered a positive response. Twenty four h after the sensitivity test, acetic acid-sensitive mice were divided into two groups (n = 8–10). The first group was given gum acacia mucilage (7%) interaperitoneally and served as control. The second group received the extract of *S. tetrasperma* suspended in 7% gum acacia, in an orally given dose of 120 mg/kg BW. After 1 h, acetic acid was injected and the number of writhings during the following 25-min period was counted in 25-min intervals.

Animals

Animal studies were conducted in accordance with the international acceptance principles for laboratory animal use and care. The Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt provided the experimental animals. All animals were kept under standard laboratory conditions in the animal house of the Faculty of Pharmacy, Zagazig University at 27 °C with a 12 h/12 h light-dark cycle. They were fed laboratory diet and water *ad libitum*. Adult male albino rats, weighing 200–20 g, and adult male mice (20–25 g) were used in carrageenan-induced paw oedema and acetic acid-induced writhing tests, respectively. The animals were deprived of food for 18–20 h before the beginning of the experiments with free access to tap water.

Statistical analysis

All experiments were carried out at least three times. Results are reported as means ± SE. The IC_{50} value was determined as the drug concentration which resulted in a 50% reduction in cell viability or inhibition of the biological activity.

IC_{50} values were calculated using a four-parameter logistic curve (SigmaPlot® 11.0).

Chromatographic and spectroscopic data

Catechol (**8**): Reddish-brown crystals. – Yield: 80 mg [eluted by 60% CH_2Cl_2 in light petroleum (v/v)]. – R_f = 0.53 ($CHCl_3$/MeOH 9:1 v/v). – UV (MeOH): λ_{max} = 276 nm. – EI-MS (rel. int., %): m/z = 110 $[M]^+$ (92), 92 (10), 81 (82), 62 (48), 51 (100). – ^{1}H NMR (300 MHz, CD_3OD): δ (ppm) = 6.65 (2H, m, H-3, H-6), 6.77 (2H, m, H-5, H-4). – ^{13}C NMR (75 MHz, CD_3OD): δ (ppm) = 146.1 (s, C-1, C-2), 116.4 (d, C-3, C-6), 120.4 (d, C-4, C-5) (Silverstein *et al*., 1981; Egawa *et al*., 2004).

Tremulacin (**9**): White amorphous powder. – Yield: 120 mg [eluted by 2% MeOH in CH_2Cl_2 (v/v)]. – R_f = 0.38 ($CHCl_3$/MeOH 9:1 v/v). – UV (MeOH): λ_{max} = 273, 314 nm (sh). – EI-MS (rel. int., %): m/z = 528 $[M]^+$ (0.1), 373 (0.1), 369 (12), 125 (14), 124 (10), 106 (26), 105 (100), 78 (11), 77 (21). – ^{1}H NMR (500 MHz, CD_3OD): see Table SI. – ^{13}C NMR (125 MHz, CD_3OD): see Table SII (Zapesochnaya *et al*., 2002).

Tremuloidin (**10**): White amorphous powder. – Yield: 300 mg [eluted by 50% ethyl acetate in CH_2Cl_2 (v/v)]. – R_f = 0.64 ($CHCl_3$/EtOAc/MeOH 3:1:1 v/v/v). – UV (MeOH): λ_{max} = 273, 314 nm. – EI-MS (rel. int., %): m/z = 390 $[M]^+$ (1), 373 (2), 311 (1), 269 (17), 126 (23), 125 (25), 124 (10), 106 (45), 105 (100), 77 (20). – ^{1}H NMR (500 MHz, CD_3OD): see Table SI. – ^{13}C NMR (125 MHz, CD_3OD): see Table SII (Mizuno *et al*., 1991).

2'-*O-p-(E)-Coumaroyl salicin* (**11**): White amorphous powder. – Yield: 30 mg [eluted by 75% ethyl acetate in CH_2Cl_2 (v/v)]. – R_f = 0.60 ($CHCl_3$/EtOAc/MeOH 3:1:1 v/v/v). – UV (MeOH): λ_{max} = 313 nm. – EI-MS (rel. int., %): m/z = 264 $[M]^+$ (1), 147 (2), 125 (5), 124 (1), 106 (5), 77 (36), 60 (45), 42 (100). – ^{1}H NMR (500 MHz, CD_3OD): see Table SI. – ^{13}C NMR (125 MHz, CD_3OD): see Table SII (Dagvadorj *et al*., 2010).

Salicin (**12**): White amorphous powder. – Yield: 2.5 g [eluted by 2% MeOH in ethyl acetate (v/v)]. – R_f = 0.38 ($CHCl_3$/EtOAc/MeOH 3:1:1 v/v/v). – UV (MeOH): λ_{max} = 269 nm. – EI-MS (rel. int., %): m/z = 124 [M^+ – glucose] (5), 106 (78), 91 (11), 77 (100), 59 (44). – ^{1}H NMR (500 MHz, CD_3OD): see Table SI. – ^{13}C NMR (125 MHz, DMSO-d_6): see Table SII (Mizuno *et al*., 1991; Zapesochnaya *et al*., 2002).

Results and Discussion

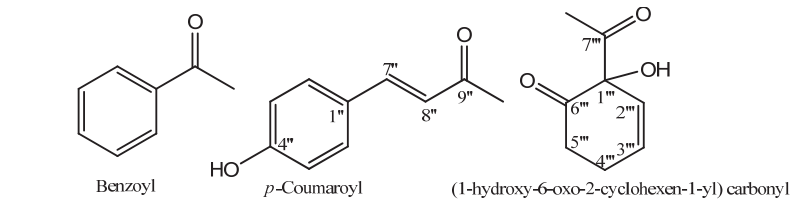
Identification of compounds

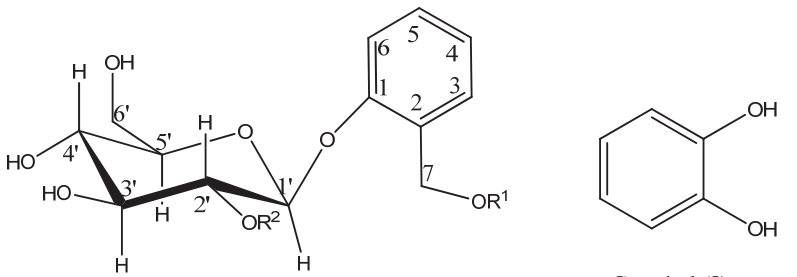
The crude methanolic extract of *S. tetrasperma* was fractionated using different solvents (*e.g.* light petroleum, dichloromethane, and ethyl acetate), and each fraction was chromatographed over a silica gel column. Column fractions were examined by TLC, and fractions of the same R_f value were pooled and the compounds purified either by crystallization or by preparative TLC. Fractionation of the extracts yielded well-known substances, however, these had not been described before as constituents of *S. tetrasperma*. The chemical structures of the isolated compounds were identified by IR, UV, MS, 1D- and 2D-NMR analyses including APT, H-H-COSY, HSQC, and HMBC as well as by comparison with reference samples and literature data.

Altogether seven compounds were isolated and identified from the light petroleum fraction of the aqueous methanolic extract of the stem bark. The isolated compounds included β-sitosterol acetate (**1**), β-amyrin (**4**), β-sitosterol (**5**) (Goad and Akihisa, 1997), friedelin (**2**), 3β-friedelinol (**3**) (Qaisar *et al*., 2008), palamitic acid (**6**), and β-sitosterol-*O*-glucoside (**7**) (Hamdan *et al*., 2011). The yields were 8, 50, 150, 50, 6, 180, and 250 mg, respectively. Catechol (**8**) and tremulacin (**9**) were isolated from the dichloromethane fraction. From the ethyl acetate fraction, tremuloidin (**10**), 2'-*O-p-(E)*-coumaroyl salicin (**11**), and salicin (**12**) were isolated (Tables SI and SII in the supplementary material; access via http://www.staff.zu.edu.eg/a_m_elshazly/userdownloads/Bioactive%20Secondary%20Metabolites%20from%20Salix%20tetrasperma%20Roxb..doc; Fig. 1). The isolated compounds are reported here for the first time as constituents of *S. tetrasperma*, except salicin which had been previously reported from this plant (Fahmy and Abdel-Latif, 1948; Sahsah, 1978).

Antioxidant activity

The DPPH assay is considered a valid and easy assay to evaluate the antioxidant activity of natural products. Log common correlations vs. absorbance were obtained for the tested sample and the positive controls (ascorbic acid and hesperidin). The concentrations giving 50% reduction in the absorbance of 0.2 mM DPPH solution (IC_{50})





Catechol (**8**)

R^1	R^2	
(1-hydroxy-6-oxo-2-cyclohexen-1-yl) carbonyl	Benzoyl	Tremulacin (**9**)
H	Benzoyl	Tremuloidin (**10**)
H	*p*-(*E*)-Coumaroyl	2'-*O*-*p*-(*E*)-Coumaroyl salicin (**11**)
H	H	Salicin (**12**)

Fig. 1. Phenolic compounds isolated from *Salix tetrasperma*.

were determined from sigmoid curves. They were 94.5 μg/mL for the methanolic extract of *S. tetrasperma*, 16.3 μg/mL for ascorbic acid and 361.5 μg/mL for hesperidin.

Anti-inflammatory activity

As shown in Table I and Fig. 2, the intradermal injection of 0.1 mL carrageenan (1%) in the rat hind paw significantly increased the paw thickness at all specified time points. On the other hand, oral pretreatment with *S. tetrasperma* extract significantly decreased rats' hind paw oedema thickness compared to the control group. In addition, *AUC* calculations showed that the potency of the anti-inflammatory activity of the extract at a dose of 120 mg/kg BW is 52% when compared with that of diclofenac sodium (54%) and dexamethasone (76%). Previous evaluation of a 50% aqueous methanolic leaf extract of this plant revealed anti-inflammatory effects after 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 h following carrageenan injection with 20, 21, 9, 30, 58, and 38% inhibition (Karawya *et al.*, 2010).

Analgesic activity

At 120 mg/kg BW, the extract reduced the total number of acetic acid-induced writhings from

Table I. Effect of *S. tetrasperma* extract (120 mg/kg BW) on hind paw thickness percentage inhibition after 1, 2, 3, 4, 5, 6, 12, and 24 h deduced from the area under the curve after carrageenan-induced oedema.

Group	*AUC*	Total inhibition (%)
Control	953.00 ± 56.31	0
Diclofenac sodium (4 mg/kg BW)	441.67 ± 20.43	54
Dexamethasone (0.5 mg/kg BW)	229.12 ± 14.48	76
S. tetrasperma extract	459.35 ± 13.25	52

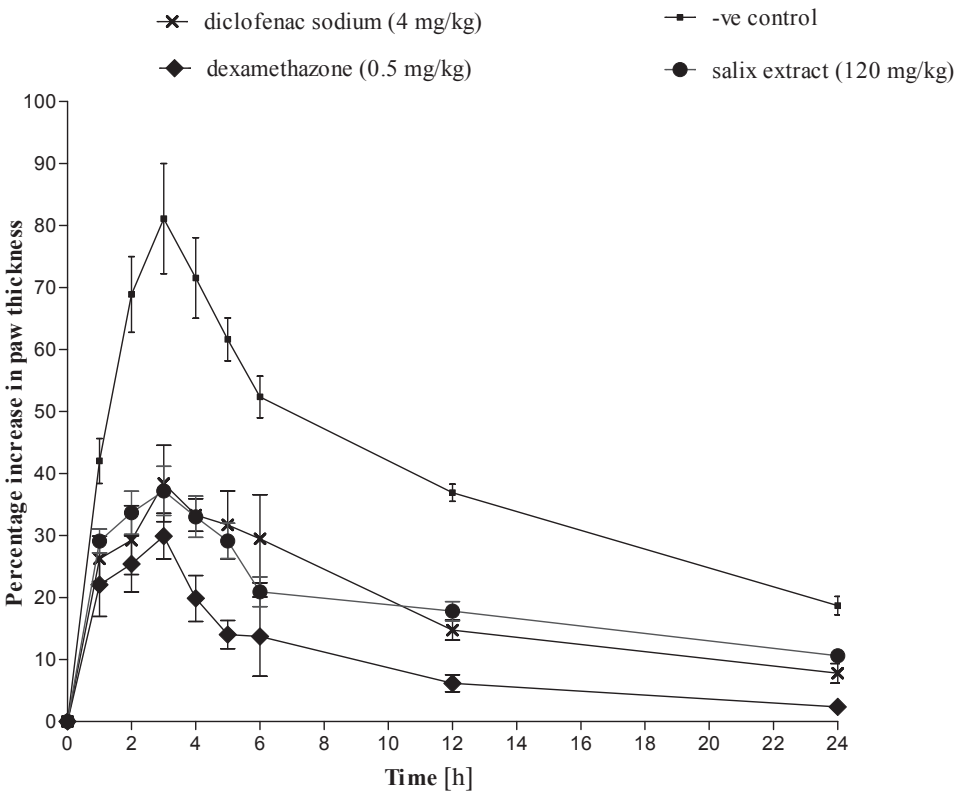


Fig. 2. Effect of oral administration of *S. tetrasperma* extract (120 mg/kg BW) on percentage increase of carrageenan-induced oedema measured after 1, 2, 3, 4, 5, 6, 12, and 24 h.

75.29 ± 4.01 to 33.71 ± 1.28, *i.e.* by 55%, and thus clearly had a pain-relieving effect.

Conclusion

The isolated compounds are reported here for the first time as constituents of *S. tetrasperma* except salicin which had been previously reported from this plant. The present biological studies established the scientific basis for the utility of this plant and other species of *Salix* as anti-inflammatory and analgesic agents and justify their tradional use as folk remedies (Chrubasik *et al.*, 2000; Khare, 2007; Barnes *et al.*, 2007; Chhetree *et al.*, 2010; Mondal *et al.*, 2010).

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