GC-MS Analysis and Anti-Microbial Activity of Acidic Fractions Obtained from *Paeonia peregrina* and *Paeonia tenuifolia* Roots

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Paeonia peregrina and *tenuifolia*, Phenolic and Aliphatic Acids

Fourteen aromatic and 24 aliphatic acids were determined by GC-MS analysis of acidic fractions obtained from *Paeonia peregrina* and *Paeonia tenuifolia* roots. Benzoic acid and its monohydroxy-, dihydroxy- and trihydroxy-derivatives are the main acid components of both *Paeonia* species. Some fractions could serve as a source of benzoic, 4-hydroxybenzoic, vanillic and gallic acids, as well as of ethyl gallate. The fractions inhibited the growth of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*.

Introduction

Paeonia Radix is one of the most important herbal drugs used in the traditional Chinese medicine. It is derived from the roots of several *Paeonia* species (Ranunculaceae) and is known as an analgesic, a sedative, and an anti-inflammatory agent and as a remedy for female genital diseases and blood stagnation (Yoshikawa *et al.,* 1992).

The roots of *Paeonia peregrina* Mill., the only *Paeonia* species widely distributed in our country, are also recommended by the Bulgarian traditional medicine for similar purposes (Stoyanov, 1973). *Paeonia tenuifolia* L. is a rare species and its medical application has not been so far documented.

Chemical studies on the roots of *Paeonia* species have shown the presence of paeonol derivatives, flavonoids and various monoterpenoids and triterpenoids (Yoshikawa *et al.,* 1992; Kamiya *et al.,* 1997; Kostova *et al.,* 1998). The occurrence of phenolic and aliphatic acids has been also reported (Asif *et al.,* 1983; Huiying *et al.,* 1984).

The studies on the acid constituents of the roots of the Bulgarian *Paeonia* species were limited to the isolation of free benzoic, *p*-hydroxybenzoic and gallic acids from *Paeonia peregrina* (Ulubelen, 1969; Todorova and Kostova, 1996). Ester bound benzoic acid was found in the same plant source (Kotova *et al.,* 1998). It was of scientific and practical interest to study the phenolic acids in the methanolic extracts of the roots of *Paeonia peregrina* and *Paeonia tenuifolia* as they possess antiseptic, antibacterial, anti-fungal, analgesic and other useful biological properties (Kowalczyk and Olechnowicz-Stepien, 1988; Kroes *et al.,* 1991) and contribute to the biological activity of the studied plant extracts. In this work we report our results on the GC-MS analysis and the anti-microbial activity of some acidic fractions obtained from the roots of *Paeonia peregrina* and *Paeonia tenuifolia* of Bulgarian origin.

Experimental

Plant material

Sampels of *Paeonia peregrina* Mill. and *Paeonia tenuifolia* L. roots collected in the regions of Konevska Mountain and the village of Ponor around Sofia, Bulgaria, respectively, were investigated. The plant materials were authenticated by Assoc. Prof. L. Koeva and voucher specimens deposited at the Department of Biology, Sofia University.

Preparation of acidic fractions

The *Paeonia peregrina* roots (100 g) were extracted with MeOH (3 × 200 ml) under reflux (2 h, 1 h, 1 h). The MeOH eluates were concentrated to a small volume (200 ml), diluted with H_2O (200 ml) and extracted with petroleum ether (3 ×

§ Deceased.

50 ml) and ethyl ether (6 × 50 ml) in succession to give the respective extracts designated as petroleum ether (PE), ethyl ether (EE) and water (WE) extracts.

The dried EE (358 mg) was dissolved in EtOAc and treated with 5% $NaHCO_3$ (50 ml). The water layer was acidified with cHCl to pH 2 and extracted with EtOAc (3 × 150 ml) to give fraction A (84 mg, free acids).

The WE (280 ml) was divided into two equal parts WE1 and WE2. WE1 was acidified with cHCl to concentration 2 M, refluxed for 2 h and worked-up (Kowalczyk and Olechnowicz-Stepien, 1988) to give the acidic fraction B (315 mg). WE2 was treated with KOH to concentration 2 M, refluxed for 2 h and after the standard work-up (Kowalczyk and Olechnowicz-Stepien, 1988) afforded the acidic fraction C (266 mg).

In a similar way, fractions A1, B1 and C1 were obtained from *Paeonia tenuifolia* roots.

Sample preparation for GC/MS

The dry fractions (3 mg) were dissolved in 25 μl of dry pyridine, 40 μl N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were added and the mixture heated at 80 °C for 20 min in a screw-cap vial.

Gas-chromatography/mass spectrometry

For the GC-MS analysis a 30 m × 0.2 mm I. D. HP-5 fused silica capillary column; 25 μm film thickens, was used in Hewlett-Packard 5890 gas chromatograph with a HP 5972 MSD detector. The samples were introduced *via* an all-glass injector working in the split mode, with He as the carrier gas, linear velocity 32 cm/s. Temperature program: 80–240 °C at 8 deg·min^{-1}, 240–300 °C at 12 deg·min^{-1} and a 20 min hold at 300 °C. The identification of components was accomplished using computer searches in commercial libraries.

Screening for antibacterial activity

For the investigation of the antibacterial activity we used a modification of bioautography developed by Kujumgiev *et al.* (1993), and *Staphylococcus aureus* 209 and *Escherichia coli* WF + as test microorganisms. The antibacterial activity was measured as a diameter of the inhibitory zones in the spot agar layer stained after 72 h incubation at 37 °C with methylene blue according to Loeffler (Gerhardt *et al.,* 1981). An inhibitory zone with diameter less than 5 mm corresponds to lack of activity (5 mm is the diameter of the spot). Control experiments with solvents showed that solvents do not have any activity. The inhibitory zones of 0.5 mg of each fraction (loaded on one spot on TLC) were measured.

Screening for anti-fungal activity

For the investigation of the anti-fungal activity the agar cup method was applied (Spooner and Sykes, 1972). As a test microorganism, *Candida albicans* 562 was used. The anti-fungal activity was measured as a diameter of the inhibitory zones. Control experiments with solvents showed that solvents do not have any activity. The inhibitory zones of 0.5 mg of each fraction were measured.

Results and Discussion

Six acidic fractions were prepared from the roots of *Paeonia peregrina* (fractions A, B, C) and *Paeonia tenuifolia* (fractions A1, B1, C1) following a modification of the procedure of Kowalczyk and Olechnowicz-Stepien (1988). The method applied allowed the isolation of the aromatic and aliphatic acids, present as free (fractions A, A1) or bound to other compounds (fractions B, B1, C and C1) by ester or glycosidic linkages.

The acidic fractions were silylated and subjected to GC-MS investigation (Table I). It is evident from this table that all fractions have a complex chemical composition. Some of the GC-MS peaks remained unidentified, because of lack of authentic samples and library data of corresponding compounds.

A general observation is that the quantity of the aromatic acids is more than that of the aliphatic acids in both *Paeonia* species. Benzoic acid (**1**) and its monohydroxy-, dihydroxy- and trihydroxy- derivatives **2–12** predominate over the cinnamic acid derivatives **13–14**.

Table I demonstrates some similarities and differences in the acid composition of the studied plants. The aromatic acids **1–6** and ethyl gallate (**10**) occur in both *Paeonia* species. However, higher content of 4-hydroxybenzoic acid (**3**), 3,4,5-trihydroxybenzoic (gallic) acid (**5**) and 3-OMe-4-

Table I. GC-MS analysis of the acidic fractions obtained from *Paeonia peregrina* and *Paeonia tenuifolia* roots.

Compounds	*Paeonia peregrina* Fractions			*Paeonia tenuifolia* Fractions		
	A	B	C	A1	B1	C1
	in% of total ion current[a]					
I. Aromatic acids and esters						
Benzoic acid (**1**)	18.4	5.4	40.4	10.8	36.4	0.4
2-Hydroxybenzoic acid (**2**)	1.5	2.0	–	–	2.0	–
4-Hydroxybenzoic acid (**3**)	0.1	4.4	0.4	1.3	4.0	28.8
3,4-Dihydroxybenzoic acid (**4**)	0.4	–	–	0.2	0.7	1.9
3,4,5-Trihydroxybenzoic acid (**5**)	3.7	–	2.7	1.3	18.8	6.0
3-OMe-4-OH-Benzoic acid (**6**)	5.2	6.8	0.3	2.0	5.9	21.6
3,4-Dimethoxybenzoic acid (**7**)	0.2	–	–	–	–	–
3,5-Dimethoxy-4-OH-benzoic acid (**8**)	–	0.3	–	–	–	–
4-OMe-3-OH-Benzoic acid, methyl ester (**9**)	0.1	0.2	–	–	–	–
3,4,5-Trihydroxybenzoic acid, ethyl ester (**10**)	–	36.2	–	–	7.6	–
3-OMe, 4-OH-Phenylpropanoic acid (**11**)	0.1	–	–	–	–	–
4-Me-2-OH-Benzoic acid or 3-Me-2-OH-Benzoic acid (**12**)	–	0.3	–	–	–	–
4-OH-Cinnamic acid (**13**)	–	–	–	0.2	–	–
4-OMe-3-OH-Cinnamic acid (**14**)	0.8	–	–	–	–	–
II. Aliphatic acids and esters						
Hexanoic acid (**15**)	0.1	–	–	–	–	–
Octanoic acid (**16**)	0.9	–	–	–	–	–
Hexadecanoic acid (**17**)	3.4	–	–	0.8	–	2.6
Heptadecanoic acid (**18**)	0.3	0.4	–	–	–	–
Octadecanoic acid (**19**)	–	–	–	0.3	–	0.7
Eicosanoic acid (**20**)	0.2	–	–	–	–	–
Docosanoic acid (**21**)	–	–	–	0.1	–	–
Tricosanoic acid (**22**)	–	–	–	0.3	–	–
2-Hydroxypropanoic acid, dimer (**23**)	0.1	–	0.3	–	–	0.5
2- Hydroxybutanoic acid (**24**)	–	–	0.5	–	–	–
2-Hydroxyheptanoic acid (**25**)	0.4	–	–	0.3	–	–
4-Hydroxypentanoic acid (**26**)	–	0.7	–	–	–	–
3-Hydroxyoctanoic acid (**27**)	0.2	–	–	0.2	–	–
2,3-Dihydroxyhexadecanoic acid (**28**)	0.3	–	–	–	–	–
Propanedioic acid (**29**)	–	–	–	–	–	1.5
Butanedioic acid (**30**)	–	0.2	–	0.5	0.8	–
Octanedioic acid (**31**)	–	–	–	0.3	–	–
Nonanedioic acid (**32**)	3.8	–	–	2.5	–	–
2-Hydroxybutanedioic acid (**33**)	–	–	–	–	1.1	1.4
3-Hydroxypentanedioic acid (**34**)	–	–	–	–	–	0.4
Butenedioic acid (**35**)	–	0.1	–	–	–	3.4
9-Octadecanoic (Z) acid (**36**)	–	–	–	0.5	–	–
9,12-Octadecadienoic (Z, Z) acid (**37**)	1.7	–	–	0.6	–	–
4-Oxo-Pentanoic acid (**38**)	–	1.5	–	–	0.8	–
III. Others						
Glycerol (**39**)	–	–	–	1.6	–	24.5
Sitosterol (**40**)	1.0	–	–	–	–	–
L-Proline-5-oxo-1-carboxylic acid (**41**)	–	–	–	–	0.4	–
Phosphoric acid (**42**)	0.5	–	–	–	–	–
α-Glycerophosphoric acid (**43**)	0.8	–	–	–	–	–
2-Furancarboxylic acid (**44**)	–	–	–	–	0.2	–
5-Hydroxymethyl-2-furoic acid (**45**)	–	0.3	–	–	–	–
5-Methoxymethyl-2-furoic acid (**46**)	–	0.3	–	–	–	–
IV. Some unknown components						
M^+ 310 (**47**)	–	4.1	–	–	0.7	–
M^+ 354 (**48**)	–	3.4	–	–	0.2	–
M^+ 475 (**49**)	3.4	–	–	5.2	–	–
M^+ 590 (**50**)	14.9	–	–	32.4	–	–

[a] The ion current generated depends on the characteristics of the compound and is not a true quantitation.

OH-benzoic (vanillic) acid (**6**) was found in *Paeonia tenuifolia*, than in *Paeonia peregrina*. Ethyl gallate (**10**), detected only in the fractions prepared by H^+ hydrolysis, is in higher quantity in *P. peregrina*, than in *P. tenuifolia*.

Table I also shows the occurrence of the phenolic acids **7**, **8**, **9**, **11**, **12** and **14** only in *P. peregrina*, and of **13** only in *P. tenuifolia*. It is interesting to note, that 3-OMe-4-OH-phenylpropanoic acid (**11**), 4-hydroxycinnamic acid (**13**) and 4-methoxy-3-hydroxycinnamic acid (**14**) were found to occur only free in the plant extracts, while 3,5-dimethoxy-4-OH-benzoic (syringic) acid (**8**), ethyl gallate (**10**) and 4-Me-2-OH-benzoic acid (**12**) exist only attached to other compounds. All fractions contain benzoic acid (**1**) and vanillic acid (**6**).

Various aliphatic acids (total number 24) were also identified in the studied fractions. They occur mainly in the fractions A and A1, containing the free acids. Nine of the aliphatic acids (**17**, **23**, **25**, **27**, **30**, **32**, **35**, **37** and **38**) are common to both species. Seven aliphatic acids (**15**, **16**, **18**, **20**, **24**, **26**, **28**) were detected only in *Paeonia peregrina* and eight (**19**, **21**, **22**, **29**, **31**, **33**, **34**, **36**) only in *Paeonia tenuifolia*. Hexadecanoic acid (**17**) and nonanedioic acid (**32**) are the major aliphatic acid components of the roots of these two *Paeonia* species.

The presence of the 2-furancarboxylic acid (**44**) and its 5-CH_2OH and 5-CH_2OMe derivatives (**45**) and (**46**) in fractions B and B1 was established.

The results from the GC-MS analysis indicate that fractions B, C, B1 and C1 could serve as a source of benzoic acid (**1**), 4-OH-benzoic acid (**3**), gallic acid (**5**), vanillic acid (**6**) and ethyl gallate (**10**).

The anti-microbial properties of the acidic fractions obtained from *Paeonia peregrina* and *Paeonia tenuifolia* against *S. aureus*, *E. coli* and *C. albicans* using known methods (Kujumgiev *et al.,* 1993; Spooner and Sykes, 1972) were studied (Table II). All fractions inhibited the growth of *S. aureus*. The data show, that the fractions B and B1 obtained by H^+ hydrolysis exhibited the strongest inhibition of *S. aureus*. The fractions A1, B1 and C1 showed some anti-fungal activity against *C. albicans*. Fraction B1 appeared to be promising for practical use.

Table II. Anti-microbial activity of acidic fractions obtained from *Paeonia peregrina* and *Paeonia tenuifolia* roots.

Fractions	Zone of inhibition ± S. D. [mm][a]		
	S. aureus	*E. coli*	*C. albicans*
Paeonia peregrina			
A (free acids)[b]	13.0 ± 1.0	13.0 ± 1.0	Not tested
B (H^+ hydrolysis)[b]	28.0 ± 2.0	Not tested	Not tested
C (OH^- hydrolysis)[b]	19.0 ± 1.0	0	Not tested
Paeonia tenuifolia			
A1 (free acids)[b]	17.3 ± 3.0	0	17.0 ± 1.0
B1 (H^+ hydrolysis)[b,c]	22.7 ± 2.5	14.7 ± 1.5	17.7 ± 0.6
C1 (OH^- hydrolysis)[b]	16.7 ± 2.5	0	15.0 ± 2.0
Streptomycin[d]	27.0 ± 1.0		
Nystatin[d]			32.0 ± 1.0

[a] Tests were done in triplicate, values are mean ± standard deviation (S. D.).
[b] 0.5 mg/spot.
[c] MIC [final concentrations: 500 μg/ml for *S. aureus* and 250 μg/ml for *C. albicans*].
[d] 0.1 mg/spot.

The minimal inhibiting concentration (MIC) measured by us for this fraction was 500 μg/ml (final concentration) against *S. aureus* and 250 μg/ml against *C. albicans*. Obviously, the qualitative and quantitative acid composition of the fractions determines their anti-microbial activity.

The derivatives of gallic (**5**) and 3,4-dihydroxybenzoic (protocatechuic, **4**) acids are known for their anti-inflammatory and anti-oxidative properties (Kroes *et al.,* 1991). This suggests that the fractions B, B1 and C1 containing **4**, **5** and ethyl gallate **10** in higher quantity would also exhibit similar activities.

Conclusions

GC-MS analysis of acidic fractions obtained from *Paeonia peregrina* and *Paeonia tenuifolia* roots revealed high content of benzoic acid and its monohydroxy-, dihydroxy- and trihydroxy-derivatives.

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