Wulfenia carinthiaca Jacq., Antioxidant and Pharmacological Activities

Rinaldo Cervellati[a,*], Ester Speroni[b], Paolo Govoni[d], Maria Clelia Guerra[b], Stefano Costa[a], Ulrike W. Arnold[c], and Hermann Stuppner[c]

[a] Dipartimento di Chimica 'G. Ciamician', Università di Bologna, Via Selmi 2, I-40126 Bologna, Italy. Fax: +390512099456. E-mail: rinaldo.cervellati@unibo.it
[b] Dipartimento di Farmacologia, Università di Bologna, Via Irnerio 48, I-40126 Bologna, Italy
[c] Institut für Pharmazie, Abteilung Pharmakognosie, Universität Innsbruck, Innrain 52, A-6020 Innsbruck, Austria
[d] Dipartimento di Medicina Sperimentale, Istologia, Università di Parma, Via Volturno 39, I-43100 Parma, Italy

* Author for correspondence and reprint requests

Z. Naturforsch. **59 c,** 255–262 (2004); received September 17/October 28, 2003

Relative antioxidant activities of a methanolic extract of three phenylpropanoid glycosides and three iridoid glycosides from *Wulfenia carinthiaca* were evaluated using the Briggs-Rauscher (BR) reaction method. This method is based on the inhibitory effects by antioxidants on oscillations of the BR reaction. The total extract showed a certain antioxidant activity with respect to resorcinol chosen as standard. The three phenylpropanoid glycosides showed a very high relative antioxidant activity while iridoid glycosides had practically no activity. These experimental results were confirmed by empirical calculations based on the BDE (Bond Dissociation Enthalpy) theory. The total phenolic content was also measured for the phenylpropanoid glycosides using the Folin-Ciocalteu reagent. The obtained values as gallic acid equivalents were in perfect agreement with the relative antioxidant activities. From a pharmacological point of view the results obtained demonstrate that the methanolic extract of *W. carinthiaca* have antinociceptive and antiedematogenic effects in the different models adopted. The plant extract produced a significant inhibition, dose related, of the rat paw edema induced by carrageenin. The anti-inflammatory activity is probably due to the phenylpropanoid compounds present in the plant. The histological sections of paw tissue in animals treated with *Wulfenia carinthiaca* extract confirmed the anti-inflammatory effects. The results of the antinociceptive assay indicated a significant reduction on the number of abdominal writhes of mice, induced by acetic acid.

Key words: Wulfenia carinthiaca, Phenylpropanoid Glycosides, Antioxidant Activity

Introduction

W. carinthiaca Jacq. (Scrophulariaceae) is of particular interest because it is a tertiary relic, whose distribution is limited to two small areas, at the border Austria/Italy and in the frontier area Albania/Montenegro/Serbia. *Wulfenia* preserves many relic characteristics as the unlimited growth of the main axis and the small variability of the leaves, the latter indicating genetic inflexibility (Lepper, 1970).

Very recently, Arnold *et al.* (2002) reported the isolation and structure elucidation of six compounds (three phenylpropanoid glycosides and three iridoid glycosides) from the underground parts of *Wulfenia carinthiaca* Jacq. The same compounds were also found in the aerial parts of the plant (Arnold and Stuppner, 2003). The structures of these compounds are reported in Fig. 1.

On the base of the content of these compounds it seems interesting to evaluate *in vitro* the possible antioxidant capacity of the crude extract of the plant, and contemporary to ascertain *in vivo* anti-inflammatory activity.

The aims of the present work are: (i) the evaluation of the antioxidant activity of a total methanolic extract and the isolated compounds using a method based on the inhibitory effects by antioxidants on the oscillations of the Briggs-Rauscher (BR) oscillating reaction recently reported by Cervellati *et al.* (2001). The BR reaction method works near the pH of the fluids in the stomach (pH $\approx$ 2) and was successfully tested on some natural polyphenolic compounds contained in vegetables, fruit and aromatic plants commonly present in the normal diet (Cervellati *et al.*, 2002);

(ii) to correlate the relative antioxidant activities of the substances isolated from *W. carinthiaca* with their total phenolic content;

0939–5075/2004/0300–0255 $ 06.00

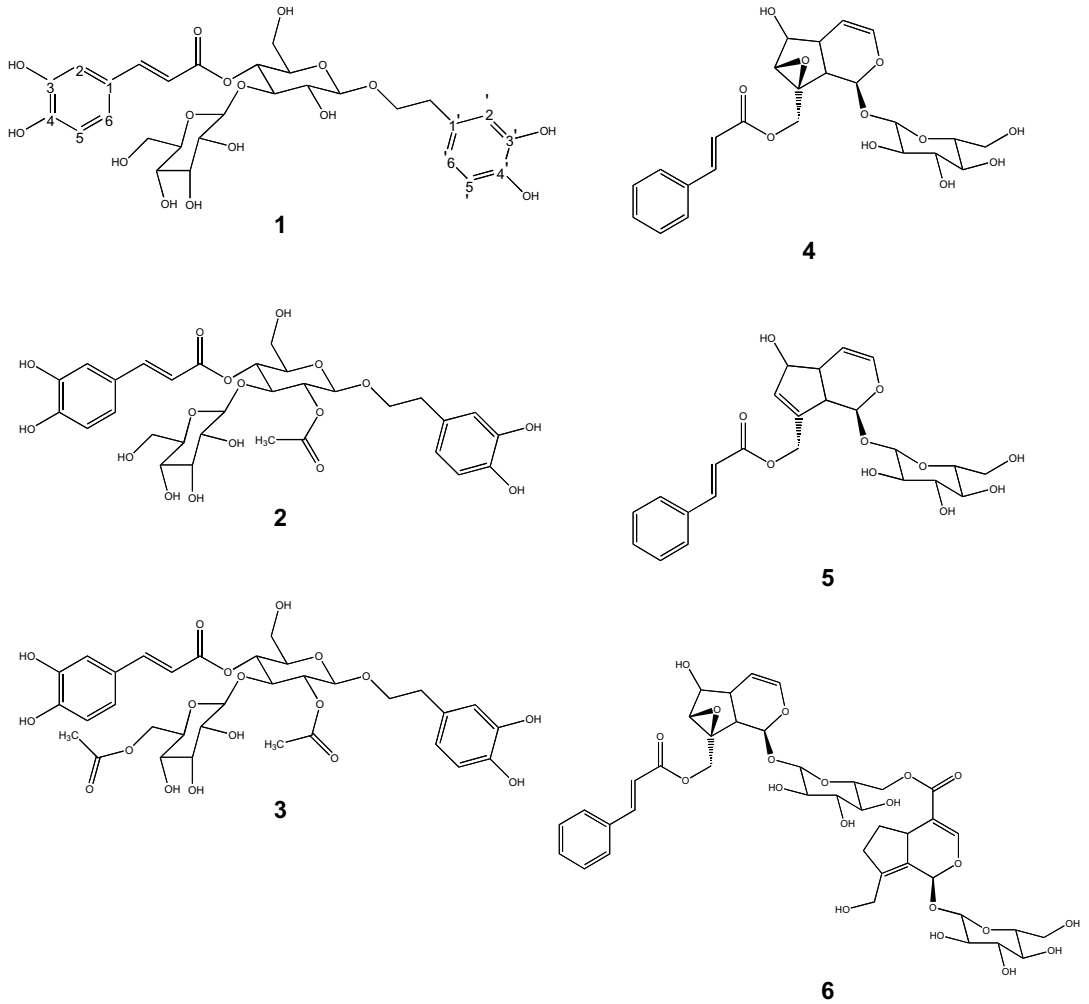

Fig. 1. Structures of phenylpropanoid glycosides [plantamajoside (**1**), 2′-*O*-acetylplantamajoside (**2**), 2′-*O*,6″-*O*-diacetylplantamajoside (**3**)] and iridoid glycosides [globularin (**4**), isoscrophularioside (**5**) and wulfenoside (**6**)] from *Wulfenia carinthiaca*.

(iii) to predict the antioxidant power of these substances using the empirical additivity rules derived from a theoretical analysis of the bond dissociation enthalpies of phenolic OH groups (Wright *et al.*, 2001);

(iv) to evaluate the anti-inflammatory and analgesic activity of a total methanolic extract in classical animal models.

Results

Relative antioxidant activity

For the total extract and for each compound, we studied the dependence of the inhibition time on the concentration. The graphs t_{inhib} *vs.* concentration for the extract and compounds **1**, **2** and **3** (phenylpropanoid glycosides) are reported in Fig. 2.

Compounds **4**, **5** and **6** (iridoid glycosides) gave very low inhibition times also at concentrations much higher than those of compounds **1**, **2** and **3**. As can be observed from Fig. 2 the experimental data are well fitted by straight lines in the explored concentration range. The R^2 values of the straight lines are: 0.999 (**1**), 0.995 (**2**), 0.989 (**3**), 0.989 (total extract), respectively.

Below a certain concentration of samples added (different for each sample) the behavior deviates

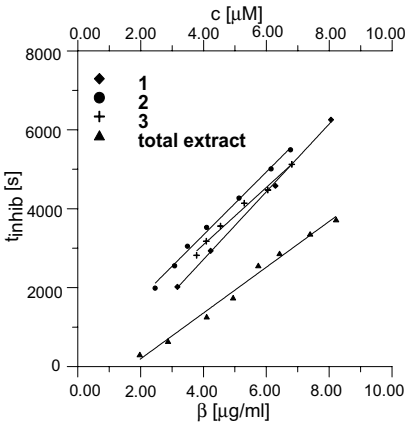

Fig. 2. Straight line of t_{inhib} *vs.* concentration [μg ml^{-1}] for the total methanolic extract (lower x axis) and straight lines of t_{inhib} *vs.* concentration [μM] for phenylpropanoid glycosides (upper x axis).

from linearity. In fact, at low concentration of sample added, the inhibition time becomes too low to be measured (Cervellati *et al.*, 2000). There is a threshold under which inhibition time cannot be detected. We believe that under these lower limits the straight line curves towards 0. At high concentration of sample the amplitude of the resumed oscillations becomes too low, until up to a given concentration (different for each sample) oscillations do not re-start. This means that the reaction reaches its end not being able to produce radicals anymore.

As shown in Fig. 2, the slopes of the straight lines are different, so the calculation of the relative antioxidant activity will depend on the substance chosen as standard and the concentration of the sample.

It was found (Cervellati *et al.*, 2001) that a way to calculate the relative antioxidant activity is to compare the concentrations of a sample and a chosen standard that give the same inhibition time, named "relative activity with respect to concentrations", r.a.c., *i.e.* the ratio:

$$\text{r.a.c.} = [\text{std}]/[\text{smp}]$$

where [smp] is the concentration of the sample added to the mixture giving a certain inhibition time and [std] is the concentration of the standard that should give the same inhibition time. Resorcinol was chosen as standard. If possible it is convenient to calculate a mean value of r.a.c. in the linear concentration range of the sample and the standard. This mean value, r.a.c.$_m$, is more significant than the r.a.c. value calculated at only one inhibition time. The obtained r.a.c. values are reported in Table I.

As can be seen from Table I the relative antioxidant activity of compounds **1**, **2** and **3** are similar and quite high. The relative antioxidant activity of the total extract is low because it is a mixture of different substances some of which are inactive, moreover the molar weight of resorcinol is lower with respect to phenylpropanoid glycosides and

Table I. r.a.c. values for the extract and the compounds studied. Straight line for resorcinol (c [μM]): t_{inhib} [s] = 465.1 [μM^{-1} s] × c [μM] – 1181 [s], R^2 = 0.994; (β [μg ml^{-1}]): t_{inhib} [s] = 4231 [μg^{-1}ml s] × β [μg ml^{-1}] – 1181 [s], R^2 = 0.994.

	β [μg/ml]	t_{inhib} [s]	r.a.c.[a]	r.a.c.$_m$ ± σ
Total extract	4.94	1742	0.14 ± 0.04	0.15 ± 0.01
	6.42	2863	0.15 ± 0.04	
	8.22	3720	0.14 ± 0.04	
	4.10	1260	0.14 ± 0.04	
	7.39	3358	0.14 ± 0.04	
	5.74	2558	0.15 ± 0.05	
	2.87	640	0.15 ± 0.04	
	1.97	301	0.18 ± 0.05	
Compound	c [μM]	t_{inhib} [s]	r.a.c.[a]	r.a.c.$_m$ ± σ
1	8.06	6256	1.98 ± 0.06	2.05 ± 0.09
	4.23	2934	2.09 ± 0.06	
	6.29	4580	1.97 ± 0.06	
	3.17	2017	2.17 ± 0.06	
2	6.15	5010	2.16 ± 0.06	2.4 ± 0.2
	3.08	2553	2.61 ± 0.08	
	5.13	4270	2.29 ± 0.07	
	2.46	1987	2.77 ± 0.08	
	4.10	3526	2.47 ± 0.07	
	3.49	3049	2.61 ± 0.08	
	6.77	5493	2.12 ± 0.06	
3	6.05	4473	2.01 ± 0.06	2.2 ± 0.1
	4.54	3559	2.24 ± 0.07	
	3.78	2819	2.27 ± 0.07	
	5.30	4139	2.16 ± 0.06	
	4.09	3177	2.29 ± 0.07	
	6.81	5124	1.99 ± 0.06	
4	505.4	421	0.0068 ± 0.0002	0.008 ± 0.001
	353.8	281	0.0089 ± 0.0003	
5	23.74	159	0.121 ± 0.004	0.14 ± 0.03
	16.62	126	0.169 ± 0.005	
6	213.1	6054	0.073 ± 0.002	0.08 ± 0.02
	106.5	2588	0.076 ± 0.002	
	42.62	955	0.108 ± 0.003	

[a] Quoted errors calculated according to the procedure suggested by Harris (1987).

iridoid glycosides. Thus the r.a.c. value of the total extract is not comparable with those of the isolated compounds.

Total phenolic content

The total phenolic content was measured only for compounds **1**, **2** and **3**. For each sample we measured the absorbance at 765 nm of the reacted mixture Folin-Ciocalteu reagent/sample at five different concentrations of sample (see Experimental section). The measured values of absorbance, A, of the samples and the standard (gallic acid) are reported in Table II.

The total phenolic content is given as gallic acid equivalents (GAE). The GAE values are obtained calculating the concentration of gallic acid that gives the same absorbance of the sample. Then this value is reported at a given concentration of the sample, for example 10 mg l^{-1}. Since the slopes of the straight lines A *vs.* c of the samples are different from that of the standard, we calculated a mean value of GAE (GAE_m) in the concentration range explored. The results are reported in Table II. The meaning of GAE_m is that for example 10 mg l^{-1} of compound **2** have the same total phenolic content of 5.4 mg l^{-1} of gallic acid.

Structure-activity relations

Recently a theoretical method to calculate the bond dissociation enthalpies (BDE) for molecules belonging to the class of phenolic antioxidants and to correlate them with their free radical scavenging activities has been reported by Wright *et al.* (2001). These authors also proposed empirical additivity rules that take into account the electronic, H-bond, conjugation and steric effects of substituents in the phenol parent molecule to evaluate the antioxidant power and the structure-activity relations. In this way a value of BDE of a phenolic compound or $\Delta BDE = (BDE_{comp} - BDE_{\phi\text{-}OH})$ can easily be calculated from the data reported by Wright *et al.* (2001). It was found that the number of phenolic OH groups is largely irrelevant and that it is the strategic placing of such groups that determine the antioxidant activity.

The three phenylpropanoid glycosides from *W. carinthiaca* have all the same free radical scavenging sites; these are the phenolic OH groups in *para a*-position with respect to the main chain as can be argued comparing our molecules with that represented in Fig. 1 (No. 24) of the Wright *et al.* article. From the data reported in Tables IV and V of this article, we found that the most active OH group is that in position 4 of the structures reported in Fig. 1 [calculated $\Delta BDE(total) = -16.2$ kcal mol^{-1}].

Anti-inflammatory and antinociceptive activity

The *W. carinthiaca* methanol extract inhibits significantly the development of rat's paw edema, as reported in Fig. 3. The higher doses (200 and 400 mg/kg) induce a reduction in the phlogistic response between 42 and 43%.

Table II. GAE calculations for the three phenylpropanoid glycosides. Straight line for gallic acid (GA): $A = 0.012$ [ml μg^{-1}] $\times \beta$ [μg ml^{-1}], $R^2 = 0.999$.

Compound	β [mg/l]	A at 765 nm	Corresponding β [mg/l] of GA	GAE referred to 10 mg/l	GAE_m (10 mg/l) $\pm \sigma$
1	50	0.20	17.2	3.45	3.7 ± 0.7
	40	0.15	13.0	3.24	
	30	0.12	10.5	3.50	
	20	0.08	6.7	3.38	
	10	0.06	5.0	5.02	
2	50	0.30	24.8	4.96	5.4 ± 0.6
	40	0.24	19.8	4.94	
	30	0.19	16.1	5.35	
	20	0.12	10.2	5.08	
	10	0.08	6.4	6.39	
3	50	0.26	22.2	4.44	4.4 ± 0.3
	40	0.21	17.6	4.40	
	30	0.16	13.8	4.61	
	20	0.11	9.6	4.82	
	10	0.05	3.9	3.94	

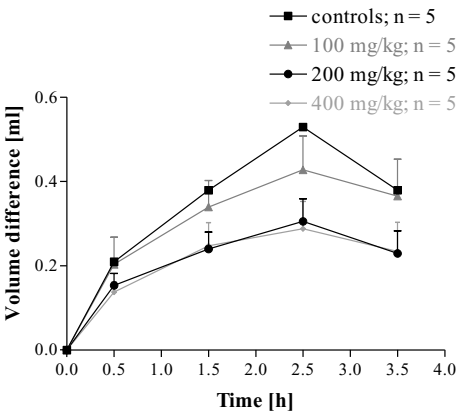

Fig. 3. Mean value and standard deviation of the measured rat's paw volumes after treating with *Wulfenia carinthiaca.*

Also the reaction to pain stimulus in the acetic acid test is in good accordance with the anti-inflammatory activity produced by the extract. The reduction of the numbers of stretches at the different time intervals is reported in Table III and represented in Fig. 4.

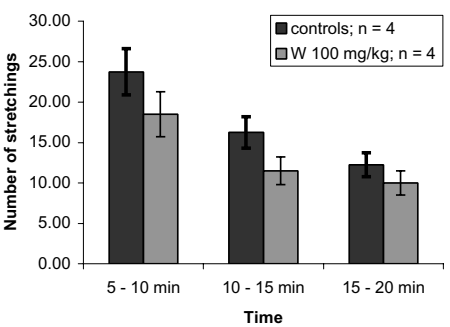

Fig. 4. Mean value and standard deviation of registered stretchings in rats treated with *Wulfenia carinthiaca* and negative controls.

Finally, a clear reduction of the phlogistic process has been observed in the histological specimens from paw of rats pretreated with *W. carinthiaca* extract. The blood vessels present a smaller caliber and there is a less infiltration of leucocytes in comparison with the samples obtained from rats injected with carrageenin.

Discussion

As previously stated the three phenylpropanoid glycosides show a high antioxidant activity at pH ≈ 2, while iridoid glycosides practically don't have free radical scavenging power. This is not surprising because it is well known that the free radical scavenging action is due to the phenolic OH group(s) contained in the molecule (Rice-Evans *et al.*, 1996). Indeed the iridoid glycosides do not contain any phenolic OH group in their molecules. The total phenolic content is sometimes used as an indication of the antioxidant activity of pure substances or in different kind of matrices (Baderschneider *et al.*, 1999; Schlesier *et al.*, 2002; Heinonen *et al.*, 1998). In our opinion, however, the GAE_m values are only a rough indication of the amount of 'phenols' compared with gallic acid. In the present work the relative antioxidant activity values well correlate with the GAE_m values, but this is not always true (Höner *et al.,* 2003).

The empirical predictions of antioxidant activity based on the Wright *et al.* (2001) empirical rules show that the calculated ΔBDEs are the same for the three phenylpropanoid glycosides. This is justified by the fact that the active sites are equal for the three molecules (see Fig. 1). Thus, it is expected that all the three phenylpropanoid glycosides have the same relative antioxidant activity. Our results reported in Table II are indeed in agreement with the predictions within the experimental errors.

Table III. Mean values (m) and standard deviations (sd) of registered stretchings in rats treated with *Wulfenia carinthiaca* (W) and negative controls.

	Extract	Dose [mg/kg]	Stretchings 5–10 min	Stretchings 10–15 min	Stretchings 15–20 min
m	W	100	18.50	11.50	10.00
sd	W	100	5.07	1.73	1.41
m	controls	–	23.75	16.25	12.25
sd	controls	–	3.59	0.96	0.96

From pharmacological point of view the extract of *W. carinthiaca* produce a significant inhibition, dose related, of the rat paw edema. The anti-inflammatory activity is probably due to the phenylpropanoid compounds, as they have been reported to exert multiple biological effects, including antithrombotic, antiischemic, anti-inflammatory effects. Phenylpropanoids have been shown to modify eicosanoid biosynthesis (antiprostanoid and anti-inflammatory response). Moreover, from a therapeutic point of view their antioxidant properties are important (Robak and Gryglewsky, 1996).

Materials and Methods

Materials

Malonic acid (MA, Merck; reagent grade, > 99%), manganese(II) sulfate monohydrate (Merck; reagent grade, > 99%), $NaIO_3$ (Merck; reagent grade, > 99.5%), anhydrous Na_2CO_3 (Merck; reagent grade, ≥ 99.9%) and gallic acid monohydrate (= 3,4,5-trihydroxybenzoic acid, Riedel-de Haën; reagent grade, ≥ 99%) were used without further purification, Folin-Ciocalteu reagent solution (Merck), $HClO_4$ (Merck; 70–72% v/v), H_2O_2 (Merck; 35% v/v), and other chemicals were of analytical grade. All stock solutions were prepared from double distilled, deionised water. Perchloric acid was analyzed by titration *vs.* standard 0.1 M NaOH (Merck). H_2O_2 was standardized daily by manganometric analysis.

W. carinthiaca plant material was collected in September 2001 from the Botanical Garden of Innsbruck, Austria. Voucher specimens (MD01-2319) have been deposited in the herbarium of the Department of Pharmacognosy, Institute of Pharmacy, University of Innsbruck, Austria. Samples of phenylpropanoid glycosides and iridoid glycosides were isolated from the roots of *W. carinthiaca*.

Extraction, fractioning, isolation and characterization of these compounds were reported by Arnold *et al.* (2002). Methanol extracts for testing the anti-inflammatory and antinociceptive activity were prepared as follows: air dried underground and aerial parts of *W. carinthiaca* (78.7 g and 150.7 g, respectively) were crushed to coarse powder and extracted with MeOH. The MeOH extracts were concentrated under vacuum yielding 24.3 g of root extract and 60.4 g extract of the aerial parts.

Antioxidant activity measurements

The method is based on the inhibitory effects on oscillations of the BR reaction (Briggs and Rauscher, 1973) by antioxidants. The BR oscillating system consists of a mixture of malonic acid, acidic iodate with hydrogen peroxide and Mn^{2+} ions as catalyst. A fundamental role in the onset of oscillations is played by the intermediate hydroperoxyl radical $HOO^{\cdot}$. When a free-radical scavenger is added to an active oscillating BR mixture there is an immediate quenching of oscillations, an inhibition time that linearly depends on the concentration of the antioxidant added, and subsequent regeneration of oscillations. Oscillations in the BR mixtures were followed potentiometrically by recording the potential of the mixture using a coupled bright platinum electrode (Hamilton, model P/N 238 945) – reference electrode (double junction Ag/AgCl, Ingold, model 373-90-WTE-ISE-S7). Electrodes were connected to a pH multimeter (WTW, model pH 540 GLP) controlled by an IBM-compatible PC. The accuracy of the multimeter was ± 1 mV. The suitable data acquisition Multi-Achat II (WTW) was used. The multimeter was equipped with a temperature sensor with an accuracy of ± 0.1 °C. All solutions and reaction mixtures were maintained at constant temperature by means of a suitable thermostating system (accuracy of ± 0.1 °C). BR mixtures were prepared by mixing the appropriate amounts of stock solutions of reagents using pipettes or burettes in a 100 ml beaker to a total volume of 30 ml. The order of addition was malonic acid, $MnSO_4$, $HClO_4$, $NaIO_3$ and H_2O_2. Oscillations start after the addition of H_2O_2. The reaction mixtures were continually well stirred with a magnetic stirrer. After the third oscillation, 1.0 ml of a suitably diluted solution of a sample was added using a micropipette.

Determination of total phenolic content

The content of total phenolics was analyzed spectrophotometrically using the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965), with gallic acid as standard. Singleton and Rossi (1965) described different procedures for the determination of the total phenolic content that substantially variate only in the amount of reagents and samples and in the volume of the reaction mixture (100 ml, 20 ml). We used in our investigation the procedure for a total volume of 20 ml. We prepared solutions of phenylpropanoid glycosides

at concentrations of 50, 40, 30, 20 and 10 mg l^{-1}, then the absorbances of the reacted mixtures were measured at 765 nm by using a Shimadzu UV-1601PC controlled by an IBM-compatible PC. The accuracy was ± 1 nm. The suitable data aquisition UVPC program was used. The cell compartment was thermostated at 24.0 ± 0.1 °C. The calibration straight line of gallic acid was constructed measuring the absorbances of reacted mixtures at the same concentrations of gallic acid as those of the samples. Absorbance values of samples were then compared with those of the standard.

Animal procedures

Sprague Dawley rats weighing 200 ± 50 g and Swiss mice weighing 20 ± 10 g (Harlan Nossan, Italy) were housed under controlled conditions (12 h light-dark cycle, 22 °C , 60% humidity). Rats were used for the antiematogenic studies, and mice for the antinociceptive studies. Eight animals were selected for each experimental group. The animals submitted to oral administration of the extract or drugs were fasted overnight before the experiment. Procedures and animal comfort were controlled by the University Veterinary Service.

Carrageenin-induced edema

A volume of 0.1 ml of 1% carrageenin (an edematogenic agent) diluted in 0.9% saline was injected into the subplantar region of the right hind paw of the rat (Winter *et al*., 1962). Paw volume was measured immediately before carrageenin injection (time 0) and at intervals of 1, 2, 3 and 4 h using a plethysmometer (model 7150, Ugo Basile, Varese, Italy). The aqueous extract at different concentrations (100, 200, 400 mg/kg) and indomethacin (10 mg/kg) were administered 1 h before carrageenin injection.

Acetic acid-induced writhing test

The response to intraperitoneal injection of 0.6% acetic acid, *i.e.* contraction of the abdominal muscle and elongation of the hind limbs, was induced by the method of Koster *et al.* (1959). Mice were pretreated orally with the extract (100 and 200 mg/kg) and morphine (2.5 mg/kg, i.p.) 30 min before intraperitoneal injection of 0.6% acetic acid (0.1 ml/10 g), and 10 min later, mouse pairs were placed in transparent cages, and the number of abdominal writhes was counted over a period of 20 min. The antinociceptive activity was evaluated in terms of percentage inhibition of writhes.

Histology

Paraffin-embedded sections, 6 μm thick, carried out perpendicularly to the skin surface, included the whole thickness of the skin. Serial sections were stained with hematoxylin-eosin to show the common morphology of the tissues, especially the cells.

Acknowledgements

The authors wish to thank Mr P. D. Schlorhaufer (Botanischer Garten, Universität Innsbruck) for raising the plant material.

Arnold U. W. and Stuppner H. (2003), Vergleich verschiedener *Wulfenia* Arten anhand ihres Inhaltsstoffspektrums. Poster presented at the "17. Wissenschaftliche Tagung der ÖPhG", Graz, Austria.

Arnold U. W., Zidorn C., Ellmerer E. P., and Stuppner H. (2002), Iridoid and phenolic glycosides from *Wulfenia carinthiaca*. Z. Naturforsch. **57c**, 969–975.

Baderschneider B., Luthria D., Waterhouse A. L., and Winterhalter P. (1999), Antioxidants in white wines (cv. Riesling): I. Comparison of different testing methods for antioxidant activity. Vitis **38**, 127–131.

Briggs T. S. and Rauscher W. C. (1973), An oscillating iodine clock. J. Chem. Educ. **50**, 496.

Cervellati R., Crespi-Perellino N., Furrow S. D., and Minghetti A. (2000), Inhibitory effects by soy antioxidants on the oscillations of the *Briggs-Rauscher* reaction. Helv. Chim. Acta **83**, 3179–3189.

Cervellati R., Höner K., Furrow S. D., Neddens C., and Costa S. (2001), The *Briggs-Rauscher* reaction as a test to measure the activity of antioxidants. Helv. Chim. Acta **84**, 3533–3547.

Cervellati R., Renzulli C., Guerra M. C., and Speroni E. (2002), Evaluation of antioxidant activity of some natural polyphenolic compounds using the Briggs-Rauscher reaction method. J. Agric. Food Chem. **50**, 7504–7509.

Harris D. C. (1987), Quantitative Chemical Analysis, 2nd ed. Freeman Publ., New York, Chapter 4.

Heinonen I. M., Lehtonen P. J., and Hopia A. (1998), Antioxidant activity of berry and fruit, wines and liquors. J. Agric. Food Chem. **46**, 25–31.

Höner K., Cervellati R., Costa S., and Sartini E. (2003), Effectiveness of determination of the total phenolic content with the Folin-Ciocalteu reagent (paper submitted).

Koster R., Anderson M., and De Beer E. J. (1959), Acetic acid for analgesic screening. Fed. Proc. Fed. Am. Soc. Exp. Biol. **18**, 412.

Lepper L. (1970), Die Evolution der Gattung *Wulfenia* Jacq., ein Beitrag zum *Wulfenia*-Problem. Wiss. Z. Friedrich-Schiller-Univ. Jena, Math. Nat. Reihe **19**, 345–361.

Rice-Evans C. A., Miller N. J., and Paganga G. (1996), Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. **20**, 933–956.

Robak J. and Gryglewsky R. J. (1996), Bioactivity of flavonoids. Pol. J. Pharmacol. **48,** 555–564.

Schlesier K., Harwat M., Böhm V., and Bitsch R. (2002), Assessment of antioxidant activity by using different *in vitro* methods. Free Rad. Res. **36,** 177–187.

Singleton V. L. and Rossi J. A. (1965), Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. **16**, 144–158.

Winter C. A., Risley E. A., and Nuss G. W. (1962), Carrageenin-induced oedema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc. Soc. Exp. Biol. and Med. **111**, 544–547.

Wright J. S., Johnson E. R., and DiLabio G. A. (2001), Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. J. Am. Chem. Soc. **123**, 1173–1183.