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Assessment of isorhamnetin 3-*O*-neohesperidoside from *Acacia salicina*: protective effects toward oxidation damage and genotoxicity induced by aflatoxin B1 and nifuroxazide

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ABSTRACT: Antioxidant activity of isorhamnetin 3-*O*-neohesperidoside, isolated from the leaves of *Acacia salicina*, was determined by the ability of this compound to inhibit xanthine oxidase activity and to scavenge the free radical 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot-}$) diammonium salt. Antigenotoxic activity was assessed using the SOS chromotest assay. This compound has the ability to scavenge the $ABTS^{\cdot+}$ radical by a hydrogen donating mechanism. We also envisaged the study of the antioxidant effect of this compound by the enzymatic xanthine/xanthine oxidase (X/XOD) assay. Results indicated that isorhamnetin 3-*O*-neohesperidoside was a potent inhibitor of xanthine oxidase and superoxide anion scavengers. Moreover, this compound induced an inhibitory activity against nifuroxazide and aflatoxine B1 (AFB1) induced genotoxicity. Taken together, these observations provide evidence that isorhamnetin 3-*O*-neohesperidoside isolated from the leaves of *A. salicina* is able to protect cells against the consequences of oxidative stress. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: isorhamnetin 3-*O*-neohesperidoside; *A. salicina*; SOS chromotest; antigenotoxicity; antioxidant capacity

INTRODUCTION

Flavonoids are a particularly attractive class of polyphenolic naturally occurring compounds that are widely distributed in the plant kingdom. They have been acknowledged as having interesting therapeutical properties. Putative therapeutic effects of many traditional medicinal drugs may be ascribed to the presence of flavonoids (Kühnau, 1976). Numerous observations on flavonoids, both *in vivo* and *in vitro*, demonstrate a wide variety of physiological and biological effects. Flavonoids are strong antioxidants (Laughton *et al*., 1989; Sichel *et al*., 1991; Ramanathan and DAS, 1992) and free radical scavengers with various degrees of activity (Robak and Gryglewski, 1988; Laughton *et al*., 1989; Sichel *et al*., 1991). Free radicals are possibly involved in cell damage and tumor promotion (Cerutti, 1985; Cerutti and Trump, 1991). Antioxidant action is a combination of several distinct chemical events such as metal chelation, quenching free radicals by hydrogen donation from phenolic groups, oxidation to a non-propagating radical, redox potential and enzyme inhibition (Williamson *et al*., 1999). When oxidation caused by free radicals and reactive oxygen species emerges in food or in biological systems, antioxidants can prevent or delay this process via one or a combination of the aforementioned mechanisms. Hence, antioxidants may help the body to protect itself from various types of oxidative damage which are linked to diseases such as diabetes, cardiovascular disorders and aging (Halliwell, 1991). Other biological functions, such as antiinflammatory, antiallergic, antiviral, anticarcinogenic and antiproliferative activities, have long been recognized (Middleton and Kandaswami, 1999). Recently, several flavonoids have been demonstrated to have an antimutagenic effect on various mutagens or carcinogens (Nagabhushan and Bhide, 1988; Edenharder *et al*., 1993; Edenharder and Tang, 1997).

In view of several drawbacks of synthetic compounds for the human organism, preparations of plant origin for this purpose have received more attention. At present, about 4000 compounds of polyphenolic structures are known. They exert a wide

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variety of biological actions including anticarcinogenic, antimutagenic and antioxidative activities (Yagi *et al*., 2002; Hayder *et al*., 2003, 2004, 2005; Kilani *et al*., 2005 a–c; Ben Ammar *et al*., 2005, 2007). Based on information gathered from traditional healers, herbalists and inhabitants from rural regions, *Acacia salicina* is frequently used in such diverse applications as the treatment of inflammatory diseases, as a 'fibrifuge' to treat cancer and to promote human fertility in southern Tunisia. In northern Tunisia, it is used for the treatment of diarrhea and rheumatism. The effects of aqueous, enriched total oligomer flavonoids (TOF), petroleum ether, chloroform, ethyl acetate and methanol extracts from leaves of *A. salicina*, on genotoxicity induced by both benzo [a]pyrene (B[a]P) and nifuroxazide, were investigated in a bacterial assay system, i.e. the SOS chromotest with *E. coli PQ 37*. These extracts exhibited an important free radical scavenging activity towards the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) free radical (Bouhlel *et al*., 2007). These results suggested deepening our study in order to purify and test molecules obtained from the most active extract detected previously (Bouhlel *et al*., 2007). Therefore, the main objective of this study was to investigate the antioxidant and the antigenotoxic activities of the major flavonoid (isorhamnetin 3-*O*-neohesperidoside), isolated from *A. salicina* leaves.

MATERIALS AND METHODS

Plant Material

Leaves of *A. salicina* were harvested in the region of Monastir in the center of Tunisia in October 2003. Botanical identification was carried out by Professor M. Chaieb (Department of Botany, Faculty of Sciences, University of Sfax, Tunisia), according to Cuénod (1954). A voucher specimen (ASm–11.03) has been deposited in the Laboratory of Pharmacognosy, Faculty of Pharmacy of Monastir, Tunisia.

Extraction Method

The leaves of *A. salicina* were dried at room temperature and reduced to coarse powder. In order to obtain an extract enriched with TOF, powder was macerated in water–acetone mixture (1:2, v/v) for 24 h with continuous stirring. The extract was filtered and the acetone was evaporated under low pressure in order to obtain an aqueous phase. The tannins were removed by precipitation with an excess of NaCl during 24 h at 5 °C, then the supernatant was recovered. This was extracted with ethyl acetate, concentrated and precipitated with an excess chloroform. The precipitate was separated and yielded TOF extract which was dissolved in water (Ghédira *et al*., 1991). Isorhamnetin 3-*O*-neohesperidoside was isolated from TOF extract. The TOF (2.4 g) was fractionated over silica gel column eluted with AcOEt–MeOH–H_2O (100:16.5:13.5) and eight fractions were collected. Fractions 3 and 4 were combined (250 mg) and were rechromatographed through a C_{18} silica gel column (1 cm i.d. × 5 cm) eluted with a gradient of H_2O–MeOH (100:0; 98:2; 94:6; 90:10; 85:15; 80:20; 75:25; 70:30; 60:40; 50:50 and 100:0) to afford 49.4 mg of isorhamnetin 3-*O*-neohesperidoside.

Elucidation of the Purified Compound

NMR spectroscopy experiments on the compound were performed on a Bruker® Avance 400 at 400 MHz (for ^{1}H NMR) and 100 MHz (for ^{13}C NMR) with methanol as solvent. FAB–MS (negative-ion mode, glycerol matrix) was recorded on an R210C (VG Instruments, Altrincham, UK) spectrometer equipped with an IPC (P2A) MSCAN WALLIS computer system. COSY, HMQC and HMBC spectra were obtained using the usual pulse sequences. We were convinced that the purity of this molecule was adequate because, when we varied the mobile phase for CCM and for HPLC, we always obtained a single spot and a single peak. We also confirmed the purity of our molecule by ^{1}H NMR spectroscopy and mass spectra. The compound was identified as isorhamnetin 3-*O*-neohesperidoside (Fig. 1) and the proposed structure was confirmed by comparison with the literature (Woo *et al*., 1983; Peng *et al*., 2005; Saleem *et al*., 2006).

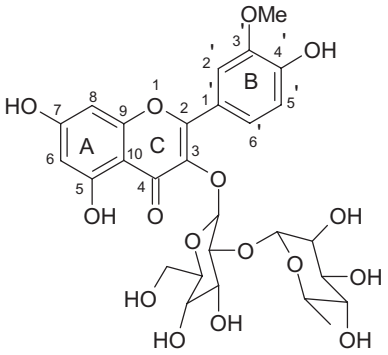

Figure 1. Isorhamnetin 3-*O*-neohesperidoside.

Radical-scavenging Activity on $ABTS^{+\cdot}$

An improved ABTS [2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] radical cation decolorization assay was used. This involves the direct production of the blue-green $ABTS^{+\cdot}$ chromophore through the reaction between ABTS and potassium persulfate. Addition of antioxidants to the pre-formed radical cation reduces it to ABTS, to an extent and on a timescale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction (Re *et al*., 1999). ABTS was dissolved in water to a 7 mM concentration. $ABTS^{+\cdot}$ was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{+\cdot}$ solution was diluted with ethanol to an absorbance of 0.7 (±0.02) at 734 nm. In order to measure the antioxidant activity of isorhamnetin 3-*O*-neohesperidoside, 10 µl of this compound at various concentrations was added to 990 µl of diluted $ABTS^{+\cdot}$ and the absorbance was recorded every 1 min. We stop the kinetic reaction when the absorbance becomes stable. Each concentration was analyzed in triplicate. The percentage decrease of absorbance at 734 nm was calculated for each point and the antioxidant capacity of the test compound was expressed as percentage inhibition (%). IC_{50} value was calculated from regression analysis. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is used as a standard in comparison for the determination of the antioxidant activity of a compound. The results are also reported as the Trolox equivalent antioxidant capacity (TEAC), which is the molar concentration of the Trolox giving the same percentage decrease of absorbance of the $ABTS^{+\cdot}$ radical cation as 1 mg ml^{-1} of the antioxidant testing compound at a specific time point (Re *et al*., 1999; Van den Berg *et al*., 2000).

Inhibition of Xanthine Oxidase Activity and Superoxide Radical Scavenging Effect

The superoxide radical ($O_2^{\cdot-}$) is a highly toxic species that is generated by numerous biological and photochemical reactions via the Haber–Weiss reaction. It can generate the hydroxyl radical, which reacts with DNA bases, amino acids, proteins and polyunsaturated fatty acids, and produces toxic effects. The toxicity of the superoxide radical could also be due to the perhydroxyl intermediates ($HO_2^{\cdot}$) that react with polyunsaturated fatty acids. Finally, superoxide may react with nitric oxide (NO) to generate peroxynitrite, which is known to be toxic towards DNA, lipids and proteins. Both inhibition of XOD activity and the superoxide anion scavenging activity, were assessed *in vitro* in one assay. The inhibition of XOD activity was measured according to the increase in UV absorbance of uric acid at 290 nm as proposed by Cimanga *et al*. (2001), while the superoxide anion scavenging activity was detected spectrophotometrically with the nitrite method described by Oyangagui (1984) with some modifications introduced by Hu *et al*. (1995) and Russo *et al*. (2005). Briefly, the assay mixture consisted of 100 µl of the tested compound solution, 200 µl xanthine (X; Sigma, USA; final concentration 50 µM) as the substrate, hydroxylamine (final concentration 0.2 mM), 200 µl EDTA (0.1 mM) and 300 µl distilled water. The reaction was initiated by adding 200 µl XOD (Sigma, USA; 11 mU ml^{-1}) dissolved in phosphate buffer (KH_2PO_4 20.8 mM, pH 7.5). The assay mixture was incubated at 37 °C for 30 min. Before measurement of the uric acid production at 290 nm, the reaction was stopped by adding 0.1 ml of HCl 0.5 M. The absorbance was measured spectrophotometrically against a blank solution prepared as described above but replacing XOD with buffer solution. Another control solution without the tested compound was prepared in the same manner as the assay mixture to measure the total uric acid production (100%). The uric acid production was calculated from the differential absorbance. To detect the superoxide scavenging activity, 2 ml of the colouring reagent consisting of sulfanilic acid solution (final concentration 300 µg ml^{-1}), N-(1-naphthyl) ethylenediamine dihydrochloride (final concentration 5 µg ml^{-1}) and acetic acid (16.7%, v/v) were added. This mixture was allowed to stand for 30 min at room temperature and the absorbance was measured at 550 nm on a helios α-spectrophotometer. The dose–effect curve for each test compound was linearized by regression analysis and used to derive the IC_{50} values.

Activation Mixture

The S9 microsome fraction was prepared from the livers of rats treated with Aroclor 1254, as described by Maron and Ames (1983). The composition of the activation mixture is the following per 10 ml of S9 mix: salt solution (1.65 M KCl + 0.4 M $MgCl_2{\cdot}6H_2O$) 0.2 ml; G6P (1 M) 0.05 ml; NADP (0.1 M) 0.15 ml; Tris buffer (0.4 M, pH 7.4) 2.5 ml; Luria broth medium 6.1 ml; S9 fraction 1 ml.

SOS Chromotest

Genotoxicity and anti-genotoxicity assays were performed according to Quillardet and Hofnung (1985). Exponential-phase culture of *E. coli PQ37* was grown at 37 °C in LB medium (1% bactotryptone, 0.5% yeast extract and 1% NaCl) plus 20 µg ml^{-1} ampicillin and diluted 1:9 into fresh medium; 100 µl aliquots were distributed into glass test tubes containing up to 50 µg of compound in a 0.6 ml final volume. A positive control was prepared by exposure of the bacteria to either nifuroxazide or AFB1. After 2 h of incubation at 37 °C, with shaking, 300 µl samples were used for assay of β-galactosidase (β-gal) and alkaline phosphatase (AP) activities. In this assay, the β-galactosidase synthesis (lac Z gene) is dependent on sfi A activation and is used as a measure of SOS repair system induction. The activity of the constitutive enzyme alkaline phosphatase was used as a measure of protein synthesis and toxicity. Enzyme activities were assayed in a spectrophotometer. The SOS induction factor (IF) in treated cells was obtained by comparing β-galactosidase and alkaline phosphatase activity in treated and untreated cells. The result was considered positive when the IF for β-galactosidase activity was >2.0.

For evaluation of the protective effect of isorhamnetin 3-*O*-neohesperidoside on induction of the SOS response by nifuroxazide (in the absence of the S9 activation system) and AFB1 (in the presence of the S9 activation system), 20 µl of nifuroxazide solution (0.5 µg per assay) or AFB1 solution (0.5 µg per assay) were added into tubes with 20 µl of the tested concentration of the compound. Anti-genotoxicity was expressed as percentage inhibition of genotoxicity induced by either nifuroxazide or AFB1 according to the formula:

$$\text{inhibition}\,(\%) = (1 - IF_1 - IF_0 / IF_2 - IF_0) \times 100$$

where IF_1 is the induction factor in the presence of both test compound and mutagen, IF_2 the induction factor in the absence of the test compound and in the presence of mutagen and IF_0 the induction factor of the untreated cells.

Statistical Analysis

Data were collected and expressed as mean ± standard deviation of three independent experiments, and analyzed for statistical significance using the Dunnett test (SPSS 11.5 for windows). The criterion for significance was set at $P < 0.05$.

RESULTS

ABTS Radical Scavenging Activity

Determination of the antioxidant capacity of isorhamnetin 3-*O*-neohesperidoside was the next approach in this study. The term 'antioxidant capacity' used in this study corresponds to the measure of the moles of a given free radical scavenged by a test solution, independently of the antioxidant activity of any antioxidant present in the mixture (Ghiselli *et al*., 2000). The radical scavenging activity of isorhamnetin 3-*O*-neohesperidoside is reported in Table 1. This compound was a very potent radical scavenger with a percentage decrease vs the absorbance of ABTS standard solution of 100, 100, 99, 96.8 and 93%, respectively, at 5, 3.5, 2.5, 1 and 0.5 mg ml^{-1}. The TEAC was also calculated. The TEAC values reflect the relative ability of hydrogen or electron-donating antioxidants of a sample to scavenge the $ABTS^{\cdot+}$ radical cation compared with that of Trolox. When referring to TEAC value, isorhamnetin 3-*O*-neohesperidoside showed a high antioxidant activity with TEAC value of 2.21 mM.

Xanthine Oxidase Inhibition and Superoxide Scavenging Activity

The antioxidant activity of isorhamnetin 3-*O*-neohesperidoside was evaluated by the X/XOD enzymatic system. The influence of

Table 1. Concentration dependent ABTS free radical scavenging activity and TEAC values of isorhamnetin 3-*O*-neohesperidoside and standard antioxidant Trolox

	Concentration	Percentage inhibition[a]	IC_{50} (mg ml^{-1})	TEAC
Isorhamnetin 3-*O*-neohesperidoside (mg ml^{-1})[b]	0.025	21.5 ± 0.9	0.06	2.21
	0.05	49.8* ± 1.4		
	0.1	61.4* ± 1.5		
	0.25	78.2* ± 1.9		
	0.5	93* ± 2.6		
	1	96.8* ± 1.8		
	2.5	99* ± 3.2		
	3.5	100* ± 0.6		
	5	100* ± 1.3		
Trolox[c] (mM)	0.5	22.07* ± 0.34	0.76	
	0.625	32.21* ± 0.28		
	0.833	53.84* ± 0.74		
	1.25	65* ± 0.28		
	2.5	96.85* ± 0.19		

[a]Inhibition of absorbance at 734 nm relative to that of standard ABTS solution. [b]Means of three experiments. [c]Positive control of anti-oxidant effect.
*$P < 0.05$ compared with the negative control (without the tested compound) using the Dunett test.

Table 2. Inhibition of XOD and scavenging of superoxide anions ($O_2^{\bullet-}$) by isorhamnetin 3-*O*-neohesperidoside at the indicated concentrations

Extracts	Concentrations (µg ml^{-1})[a]	Inhibition of xanthine oxidase activity (%)	IC_{50} (µg ml^{-1})[a]	Inhibition of superoxide anion (%)	IC_{50} (µg ml^{-1})[a]
Isorhamnetin	150	94.5* ± 1.2	48.75	70.1* ± 2.8	30
3-*O*-neohesperidoside	75	76.2* ± 3.2		59.3* ± 1.6	
	37	35.6 ± 2.9		52.8* ± 3.8	
	19	—		30 ± 2.5	

[a]Means of three experiments.
*$P < 0.05$ compared with the negative control (without the tested compound) using the Dunett test.

this compound on XOD activity evaluated by uric acid formation as the final product and its effect on the superoxide anions ($O_2^{\bullet-}$) enzymatically generated by this system were evaluated *in vitro*. The IC_{50} value of this molecule for the inhibition of XOD and as a scavenger of superoxide anions ($O_2^{\bullet-}$) are given in Table 2. Both inhibition of XOD and scavenging effect on superoxide anions were measured in one assay. Inhibition of XOD involves a decrease in production of uric acid and in superoxide anions which can be followed spectrophotometrically. Two IC_{50} values (50% inhibitory concentration) in the presence of isorhamnetin 3-*O*-neohesperidoside were calculated by linear regression analysis: 50% inhibition of XOD activity and 50% reduction of the superoxide level. The half-maximal inhibitory concentrations of this compound are listed in Table 2. Fifty percent inhibition of uric acid production was obtained at an IC_{50} of 48.75 µg ml^{-1}. Likewise it appears from the IC_{50} value of superoxide anions (30 µg ml^{-1}) that isorhamnetin 3-*O*-neohesperidoside is a potent superoxide scavenger.

Genotoxic Activity of Isorhamnetin 3-*O*-neohesperidoside

In a series of experiments preceding the antimutagenicity studies, it was ascertained that the isorhamnetin 3-*O*-neohesperidoside added to the indicator bacteria does not influence its viability (data not shown). The ability of isorhamnetin 3-*O*-neohesperidoside to induce an SOS response was examined. It was revealed that this compound at the concentrations used has no effect on the induction factor in the SOS chromotest with or without metabolic activation (Table 3). According to Kevekordes *et al.* (1998), compounds are classified as non-genotoxic if the induction factor (IF) remains <1.5, as marginally genotoxic if the induction factor ranges between 1.5 and 2 and as genotoxic if the IF exceeds 2. Based on this, isorhamnetin 3-*O*-neohesperidoside is evaluated as non-genotoxic.

Antigenotoxic Assay

A dose of 0.5 µg per assay of nifuroxazide (directly acting mutagen) and AFB1 (indirectly acting mutagen) was chosen for the study of antigenotoxicity. This dose gives the maximum genotoxicity for both nifuroxazide and AFB1. The inhibitory effect of the tested product on the genotoxicity induced by nifuroxazide and AFB1 using the SOS chromotest is illustrated in Tables 4 and 5. The tested compound from *A. salicina* leaves was effective in reducing the IF induced by the AFB1 indirectly acting

Table 3. Genotoxicity of isorhamnetin 3-*O*-neohesperidoside from of *A. salicina* leaves in the SOS chromotest conducted in the absence and in the presence of exogenous metabolic activation system (S9)

Compound	Doses (µg per assay)[a]	–S9			+S9		
		Ap (U)	(β-gal) (U)	IF	Ap (U)	(β-gal)	IF
Nifuroxazide[b]	0.5	20.2 ± 0.13	58.3 ± 0.09	10.7	—	—	—
AFB1[c]	0.5	—	—	—	21.3 ± 0.27	53.8 ± 0.3	11.5
NC	0	27.5 ± 0.23	7.6 ± 0.27	1	18.96 ± 0.15	4.3 ± 0.09	1
Isorhamnetin	1	17.8 ± 0.08	6.7 ± 0.16	1.4	15.6 ± 0.09	2.7 ± 0.42	0.8
3-*O*-neohesperidoside	10	16.9 ± 0.21	5.9 ± 0.41	1.3	18.4 ± 0.13	5.2 ± 0.31	1.3
	50	18.7 ± 0.09	5.5 ± 0.12	1.1	21.8 ± 0.31	5.7 ± 0.26	1.2
	100	20.5 ± 0.1	4.9 ± 0.43	0.9	18.6 ± 0.07	5.3 ± 0.11	1.3

β-gal: Units of β-galactosidase; AP: units of phosphatase alkaline; IF: induction factor; NC: negative control (non-treated cells).
[a]Positive control of genotoxicity (aflatoxin B1); [b]means of three experiments; [c]Positive control of genotoxicity (nifuroxazide).

Table 4. Antigenotoxic effect of isorhamnetin 3-*O*-neohesperidoside on genotoxicity induced by nifuroxazide (0.5 µg per assay) in *E. coli PQ37* using the SOS chromotest assay in the absence of the exogenous metabolic activation system (S9)

Extract	Dose (µg per assay)[a]	β-gal (U)	Ap (U)	IF	Inhibition of genotoxicity
Nifuroxazide[b]	0.5	33.2 ± 0.43	12.8 ± 0.09	9.3	—
NC	0	5.6 ± 0.11	19.6 ± 0.42	1	—
I3ON	1	29.72 ± 0.62	13.52 ± 0.83	7.85	17.4
	5	32.75 ± 0.41	16.73 ± 0.72	6.99	27.8
	50	35.10 ± 0.21	18.55 ± 0.65	6.75	30.7*
	100	10.15 ± 0.32	15.36 ± 0.42	2.36	83.6*

β-gal: units of β-galactosidase; AP: units of phosphatase alkaline; IF: induction factor; NC: negative control (non-treated cells).
[a]Means of three experiments; [b]positive control of genotoxicity (nifuroxazide).
*$P < 0.05$ compared with the negative control (without the tested compound) using the Dunett test.

Table 5. Antigenotoxic effect of isorhamnetin 3-*O*-neohesperidoside on genotoxicity induced by AFB1 (0.5 µg per assay) in *E. coli* PQ37 using the SOS chromotest assay in the presence of the exogenous metabolic activation system (S9)

Extract	Dose (µg per assay)[a]	β-gal (U)	Ap (U)	IF	Inhibition of genotoxicity
AFB1[b]	0.5	61.43 ± 0.35	19.5 ± 0.52	10.5	—
NC	0	6.11 ± 0.21	20.3 ± 0.41	1	—
I3ON	0.5	40.72 ± 0.32	17.67 ± 0.52	7.68	29.6
	1	23.48 ± 0.41	19.81 ± 0.31	3.95	68.9*
	5	17.12 ± 0.31	15.67 ± 0.53	3.64	72.2*
	50	17.18 ± 0.42	16.89 ± 0.62	3.39	74.8*
	100	17.16 ± 0.62	17.54 ± 0.41	3.26	76.2*

β-gal: units of β-galactosidase; AP: units of phosphatase alkaline; IF: induction factor; NC: negative control (non-treated cells).
[a]Means of three experiments; [b]positive control of genotoxicity (aflatoxin B1).
*$P < 0.05$ compared with the negative control (without the tested compound) using Dunett test.

genotoxic, as well as by the directly acting genotoxic, nifuroxazide. Isorhamnetin 3-*O*-neohesperidoside showed a higher antigenotoxic activity. In fact, this compound, at concentrations of 0.5, 1, 10, 50 and 100 µg per assay, significantly decreases the IF of AFB1 by respectively 29.6, 68.9, 72.2, 74.8 and 76.2%. At the concentrations of 1, 10, 50 and 100 µg per assay of this compound, the IF of nifuroxazide decreased by, respectively, 17.5, 27.8, 30.7 and 83.6%.

DISCUSSION

Free radicals (FR) are involved in the normal physiology of living organisms. They act as a messenger for signal transduction and also affect gene expression (Armario *et al.*, 1990). They are also involved in the pathogenesis of chronic diseases such as neurodegenerative diseases, aging and rheumatoid arthritis, and metabolic diseases like atherosclerosis, diabetes and

hypertension. There are several proteins and biomolecules in the living organism which act as free radical scavengers. Phenolics and flavonoids are ubiquitously found in many plant sources including different vegetables, fruits and medicinal plants. Recently the role of phenolic compounds from food and beverages in the prevention of free radical-mediated diseases has become more important due to the discovery of the link between lipid peroxidation of LDL and artereosclerosis. The phenolic compounds possess different antioxidant properties, which can be ascribed to a broad range of pharmacological activities. These compounds in general act by quenching free radicals, inhibiting the activation of procarcinogens, or by binding carcinogens to macromolecules (Krishnaswamy, 1996). Inhibition of free radical-induced damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of diseases (Brash and Harve, 2002).

The present study has demonstrated that the isorhamnetin 3-*O*-neohesperidoside, compound isolated from leaves of *A. salicina* exhibited radical scavenger and antioxidant activities. The characterization of antioxidant status by TEAC values as found by the elimination of $ABTS^{\cdot+}$ cation radical, is suitable for the investigation of the radical-scavenging activity of systems containing hydrogen/electron-donating compounds, especially phenolics (Sanchez-Moreno, 2002; Aruoma, 2003; Roginsky and Lissi, 2005; Klein and Lukes, 2006). Our data show that the isorhamnetin 3-*O*-neohesperidoside exhibited a potent scavenging activity towards ABTS radical cations in a concentration-dependent manner (Table 1), showing a direct role in trapping free radicals. According to Bors *et al.* (2001) and Cai *et al.* (2006), the required structural criteria for high radical scavenging and antioxidant activities of flavonoids include the *ortho*-dihydroxyl groups (catechol substructure) in the B-ring or in the A-ring, the 3-hydroxyl group in the C-ring, and the 2,3-double bond in conjugation with 4-oxo function (carbonyl group) in the C-ring; and finally the additional presence of 3-, 5- ,7-, 3′- and 4′-hydroxyl groups. The mechanism of reaction between antioxidant and $ABTS^{\cdot}$ depends on the structural conformation of the antioxidant. Our results on the efficiency of flavonoids in inhibiting $ABTS^{\cdot+}$ free radical are generally consistent with these criteria. Hence, isorhamnetin 3-*O*-neohesperidoside, showing a potent scavenging activity towards ABTS radical cations, only satisfies the requirements of 4′, 5- and 7-hydroxyl substitution, and the 2,3-double bond in conjugation with a 4-oxo function.

However, the chemical assay using ABTS is far from biological conditions (Cao *et al.*, 1999). Thus we used the enzymatic assay (X/XOD system), which is more related to physiological conditions. XOD is an enzyme with the capacity to catalyze the transformation of hypoxanthine into xanthine. Afterwards, the xanthine is transformed in uric acid. During the reoxidation of XOD, molecular oxygen acts as an electron acceptor, producing superoxide radical and hydrogen peroxide according to the following equation:

$$\text{Xanthine} + O_2 \xrightarrow{\text{XOD}} \text{uric acid} + O_2^{\cdot-} + H_2O_2$$

Consequently, XOD is considered to be an important biological source of superoxide radicals (Montoro *et al.*, 2005). Thus, in this study, we evaluate the XOD inhibitory effects of the studied flavonoid. Our data show that isorhamnetin 3-*O*-neohesperidoside exhibits a significant antioxidant ability, as determined through inhibition of XOD activity (IC_{50} is 48.75 µg ml^{-1}). The presence of a hydroxyl group in the C-7 and C-5 positions on the A ring may be correlated to the inhibitory effect of this compound towards the X/XO system (Paul and Li, 1998). Our results showed also that the $O_2^{\cdot-}$ production inhibition activity of the tested flavonoid was obtained at an IC_{50} of 30 µg ml^{-1}. This suggested that the number of hydroxyl groups as well as their disposition increased the superoxide radical scavenging activity of the tested flavonoid.

Comparing the results obtained from the inhibition of XOD and the scavenging of the superoxide anions, it is clear that the isorhamnetin 3-*O*-neohesperidoside showed activity in both tests, so that the inhibition of the XOD system is strengthened by the simultaneous action on the superoxide anions. 7-Hydroxy-flavonoids have been proposed to be potent inhibitors of XOD implicated in the generation of reactive oxygen species. From our results, it also appears that, for the inhibition of XOD activity by flavonoids, the hydroxyl groups at C-5 and C-7 and the 2,3-double bond are important. The unsaturation in ring C and the free hydroxyl group at C-7 enhanced the XOD inhibitory effect of isorhamnetin 3-*O*-neohesperidoside. It is proposed that the C-7 O H of flavonoid may take the place of the C-2 or C-6 OH of xanthine in the active site of the enzyme. In this assay, it has been found that inhibition of superoxide anion production in the X/XOD system was probably due to both scavenging activity and inhibition of the enzyme. This study provides evidence that the tested flavonoid exhibits interesting antioxidant properties expressed by the capacity either to scavenge free radicals or to inhibit XOD activity. These findings are noteworthy because such compounds may be useful in the treatment of many kinds of diseases related to free radical oxidation. Notably, such compounds would be well adapted to the pathogenesis of ischemic injury, which is characterized by an overproduction of the superoxide anion due (i) to a leak of electrons in the mitochondrial respiratory chain, and (ii) to the conversion of xanthine dehydrogenase to XOD (Werns and Lucchesi, 1990), which produces $O2^{\cdot-}$ when converting hypoxanthine successively to xanthine, then uric acid. Thus, compounds able to both inhibit XOD and to scavenge $O_2^{\cdot-}$ may be useful as protecting agents against cellular injury during reperfusion of ischemic tissues.

The role of antioxidants has attracted much interest with respect to their protective effect against free radical damage, which may be the cause of many diseases including cancer (Edenharder and Grunhage, 2003). In order to complete this study, genotoxic and antigenotoxic activities of the tested flavonoid were evaluated using the SOS chromotest assay. The SOS chromotest demonstrates that the isorhamnetin 3-*O*-neohesperidoside reduced strongly both AFB1 and nifuroxazide mutagenicity. This is in accordance with antimutagenic activity of flavonoids reported previously (Zani *et al.*, 1993; Calomme *et al.*, 1996). Antigenotoxicity of this compound could be due to the presence of a higher number of hydroxyl functions in the sugar moiety. Results of antigenotoxicity towards AFB1 and nifuroxazide suggest that the tested component may inhibit microsomal enzyme activation or that they may directly protect DNA strands from the electrophilic metabolite of the mutagen. However, the inhibition of mutagenesis is often complex, acting through multiple mechanisms. The inhibition of the P 450 mono-oxygenase system is known to operate in the antimutagenic effects of some plant extracts (Zani *et al.*, 1993) on the mutagen AFB1.

We deduce from the results of the present work that the antigenotoxic activity of the isorhamnetin 3-*O*-neohesperidoside from *A. salicina* leaves could be ascribed, at least in part, to their antioxidant properties, but we cannot exclude other additionally

mechanisms as DNA repair enzyme induction. The promising antimutagenic, antioxidant activities and absence of mutagenicity of the tested compound isolated from *A. salicina* leaves suggest that this compound is a phytopharmaceutical molecule of interest.

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