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Antioxidant and free radical-scavenging properties of three flavonoids isolated from the leaves of *Rhamnus alaternus* L. (Rhamnaceae) : A structure-activity relationship study

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ABSTRACT

Fractionation of the methanolic and total oligomer flavonoid enriched extracts from *Rhamnus alaternus* leaves resulted in the isolation of three flavonoids: kaempferol 3-*O*-isorhamninoside (**1**), rhamnocitrin-3-*O*-isorhamninoside (**2**) and rhamnetin-3-*O*-isorhamninoside (**3**), along with apigenin, kaempferol and quercetin. The structures were determined using data obtained from FAB–MS, ^{1}H and ^{13}C NMR spectra, as well as by various correlation experiments (COSY, HMQC and HMBC). The antioxidant activities of the isolated compounds were evaluated by measuring their ability to scavenge the DPPH radical and superoxide anions, to inhibit H_2O_2-induced lipid peroxidation in human K562 cells, and to inhibit xanthine oxidase activity. Compound **3** was a strong scavenger of DPPH· and superoxide anion radicals, and a potent inhibitor of H_2O_2-induced lipid peroxidation, with respective IC_{50} values of 1.5, 35 and 106 μg/ml, whereas compound **1** showed the better activity in the inhibition of xanthine oxidase activity with an IC_{50} value of 18 μg/ml, showing some structure–activity relationships.

1. Introduction

Reactive oxygen species (ROS) readily attack and induce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA. This oxidative damage is a crucial etiological factor implicated in several chronic human diseases, namely cardiovascular diseases, rheumatism, diabetes mellitus and cancer (Pong, 2003). Based on growing interest in free radical biology and the lack of effective therapies for most chronic diseases, the usefulness of antioxidants in protection against these diseases is warranted. Antioxidants are chemical substances that reduce or prevent oxidation. They have the ability to counteract the damaging effects of free radicals in tissues and thus are believed to protect against cancer, arteriosclerosis, heart disease, and several other diseases (Bandyopadhyay, Chakraborty, & Raychaudhuri, 2007).

Many studies have shown that phenolic compounds display antioxidant activity as a result of their capacity to scavenge free radicals (Seyoum, Asres, & El-Fiky, 2006). Phenolic compounds can also act as antioxidants by chelating metal ions, preventing radical formation and improving the antioxidant endogenous system (Al-Azzawie & Mohamed-Saiel, 2006). These compounds are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because they are stable radical intermediates. Probably the most important natural phenolics are flavonoids because of their broad spectrum of chemical and biological activities, including antioxidant and free radical scavenging properties (Kahkonen et al., 1999). In fact, flavonoids have been reported as antioxidants, scavengers of a wide range of reactive oxygen species and inhibitors of lipid peroxidation (Williams, Spencer, & Rice-Evans, 2004). These compounds, which are widely distributed across the plant kingdom, represent the most abundant antioxidants in the diet and they have gained tremendous interest as potential therapeutic agents against a wide variety of diseases, most of which involve oxidant damage (Ross & Kasum, 2002). The unusually wide pharmacological spectrum of flavonoids was originally thought to result from their antioxidant activity; however, recent studies suggest that various flavonoids may use other protective mechanisms as well. Flavonoids have also been shown

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to be highly effective scavengers of most types of oxidising molecules, including singlet oxygen and other various free radicals that are probably involved in several diseases. On the other hand, numerous studies have shown structure–activity relationships governing antioxidant capacities of flavonoids (Bors, Michel, & Stettmaier, 2001; Cai, Sun, Xing, Luo, & Corke, 2006; Cotelle et al., 1996).

The genus *Rhamnus* (Rhamnaceae), which is encountered both in temperate and in tropical countries, includes well-known medicinal species possessing various biological properties (Mai et al., 2001). Generally, *Rhamnus* species contain anthraquinones such as emodin (Wei, Lin, & Won, 1992) or chrysophanol (Alemayu, Abegaz, Snatzke, & Duddeck, 1993), as the reduced forms or their glycosides (Abegaz & Peter, 1995), whilst some others contain flavonoids (Coskun, Satake, Hori, & Tanker, 1990; Lin & Wei, 1994; Marzouk, El-Toumy, Merfort, & Nawwar, 1999).

Rhamnus alaternus (Rhamnaceae) is a small tree located principally in the North of Tunisia, where it is known as "Oud El-khir". It has traditionally been used as a digestive, diuretic, laxative, hypotensive and for the treatment of hepatic and dermatological complications (Boukef, 2001). Previous studies have shown potent antioxidant, free radical scavenging, antimutagenic and antigenotoxic activities of crude extracts from *R. alaternus* (Chevolleau, Debal, & Ucciani, 1992; Ben Ammar et al., 2005, 2007a, 2008a, 2008b). We have also reported others biological activities of *R. alaternus* extracts: antibacterial and antiproliferative. In human cells, extracts of *R. alaternus* leaves modulate the expression levels of genes implicated in both DNA repair and oxidative defence systems (Ben Ammar et al., 2007a, 2007b).

In the present study, three triglycoside flavonoids were isolated from the leaves of *R. alaternus* and identified according to their NMR and mass spectra as kaempferol 3-*O*-β-isorhamninoside (**1**), rhamnocitrin 3-*O*-β-isorhamninoside (**2**) and rhamnetin-3-*O*-β-isorhamninoside (**3**). Proton and carbon chemical shifts of these compounds were obtained by one- and two-dimensional NMR spectrum assignments. Beside the glycosides, the aglycones apigenin, kaempferol and quercetin were identified by comparison with authentic samples.

The antioxidant activities of the isolated flavonoids from *R. alaternus* leaves were evaluated by measuring their ability to scavenge the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the superoxide anion, to inhibit XOD activity, and to reduce lipid peroxidation in human leukaemia K562 cell line, showing some structure–activity relationships.

2. Material and methods

2.1. Plant material

R. alaternus leaves were collected from Guelta Safra, Tabarka (Tunisia) in November 2004. After the authenticity and the botanical identification of the species was confirmed, according to the "*Flore de la Tunisie*" (Pottier-Alapetite, 1978), by Dr. Ben Tiba, a taxonomic botanist from the "Institut Supérieur d'Agronomie de Chott-Mariam, Tunisia", a voucher specimen (Ra-12-004) was placed in the Laboratory of Pharmacognosy, Faculty of Pharmacy of Monastir, Tunisia, for future reference.

2.2. Chemicals

2,2-Diphenyl-1-picrylhdrazyl (DPPH), α-tocopherol, xanthine (X), XOD, SDS and allopurinol were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO); dimethyl sulfoxide (DMSO) from Sigma–Aldrich (Seelze, Germany). RPMI-1640, foetal bovine serum, gentamycin and L-glutamine were purchased from Gibco BRL Life Technologies (Grand Island, NY); N-(1-naphthyl) ethylenediamine dihydrochloride was purchased from Sigma–Aldrich (Steinheim, Germany) and KCl was purchased from Acros Organics (Fairlawn, NJ). Silica gel (40–63 μm) and reversed-phase silica gel C_{18} (25–40 μm) were purchased from Merck (Darmstadt, Germany). All other chemicals were reagent grade. The elucidation and purity of the flavonoids were determined by TLC, and ^{1}H and ^{13}C NMR spectroscopy.

2.3. Extraction method

Dried and powdered leaves (100 g) of *R. alaternus* were first defatted with petroleum ether (1 l), and then extracted with chloroform (1 l), ethyl acetate (1 l), and methanol (1 l) using a Soxhlet apparatus (6 h). Four different extracts were obtained. They were concentrated to dryness and kept at 4 °C in the absence of light. Amongst these extracts, only the Soxhlet methanolic extract was fractioned and purified in this study.

Additionally, in order to obtain total oligomer flavonoid (TOF) enriched extract, the powdered leaves were macerated in water: acetone mixture (1:2) for 24 h, under continuous stirring. The extract was filtered and the acetone was evaporated under low pressure, to obtain an aqueous phase. The phlobaphenes were removed by precipitation with an excess of NaCl at 5 °C for 24 h. The supernatant was extracted with ethyl acetate, concentrated and precipitated in an excess of chloroform. The precipitate was then separated and TOF extract yielded.

2.4. Fractionation and isolation methods

Compound **1** (210 mg) was directly obtained by fractionation of the TOF extract (732 mg) on a silica gel column (350 × 15 mm i.d.) with EtOAc:MeOH:H_2O (100:15:13) solvent system as eluent.

The methanolic extract (6 g) was fractionated by vacuum liquid chromatography (VLC) on a silica gel column (100 × 40 mm i.d.) eluted with CH_2Cl_2:MeOH with gradual increasing of the MeOH content (100:0 → 90:10 → 80:20 → 70:30 → 60:40 → 50:50 → 40:60 → 20:80 → 0:100) and eight fractions (**A–H**) were collected. Fractions **E**, **F** and **G** were regrouped together and rechromatographed over a silica gel column (350 × 15 mm i.d.) using an EtOAc:MeOH:H_2O (100:15:13) solvent system to give seven subfractions (**4A–4G**). The **4E** subfraction (631 mg) was rechromatographed on a C_{18} gel column (250 × 10 mm i.d.) using an H_2O:MeOH (70:30 to 0:100) gradient solvent system to afford compound **2** (99.8 mg) as well as others subfractions (**5A–5J**). Finally, the subfraction **5D** (80.5 mg) was rechromatographed on a C_{18} gel column (250 × 10 mm i.d.), using an H_2O:MeOH (70:30 to 0:100) gradient solvent system, to yield compound **3** (31.6 mg).

2.5. Nuclear magnetic resonance (NMR)

NMR spectroscopy experiments on the compounds were performed on a Bruker® Avance 400 at 400 MHz (for ^{1}H NMR) and 100 MHz (for ^{13}C NMR) with CD_3OD 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.

2.6. DPPH free radical-scavenging activity

The DPPH free radical-scavenging assay was carried out, as previously reported by Cheel, Theoduloz, Rodriguez, Caligari, and Schmeda-Hirschmann (2007) with some modifications. The pure compounds separated from *R. alaternus* leaves at various concentrations (1, 3, 10, 30 and 100 μg/ml) were added to a 0.06 mM

DPPH· solution in ethanol and the reaction mixture was shaken vigorously. After incubation for 30 min at room temperature, the absorbance at 517 nm was recorded spectrophotometrically. Compounds which displayed promising activity ($\geqslant$50% decolorisation at 100 μg/ml) were retested at lower concentrations using serial dilutions. Vitamin E was used as a reference compound in the same concentration range as the test compounds. A control solution, without the tested compound, was prepared in the same manner as the assay mixture. All the analyses were done in triplicate. The degree of discolorisation indicates the free-radical scavenging efficiency of the substances.

The antioxidant activity of *R. alaternus* extracts was calculated as an inhibitory effect (*IE*%) of the DPPH radical formation as follows:

$IE\% = 100 \times (A_{517(\text{control})} - A_{517(\text{sample})}/A_{(517\text{control})}$, and expressed as IC_{50}. The IC_{50} value was defined as the concentration (in μg/ml) of the compound required to scavenge the DPPH radical by 50%.

2.7. XOD and superoxide-scavenging activity assay

Both the 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 absorbance at 290 nm as proposed by Cimanga et al. (1991), whilst the superoxide anion scavenging activity was detected spectrophotometrically by the nitrite method Russo et al. (2005). Briefly, the assay mixture consisted of 100 μl of the tested compound solution, 200 μl xanthine (X) (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 (5.5 mU/ml) 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 0.5 M HCl. The absorbance was read spectrophotometrically against a blank solution, prepared as described above but replacing XO 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 latter was calculated from the differential absorbance.

To detect the superoxide scavenging activity, 2 ml of the colouring reagent, consisting of sulphanilic acid solution (final concentration 300 μg/ml), N-(1-naphthyl) ethylenediamine dihydrochloride (final concentration 5 μg/ml) 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 Spectronic Helios Alpha, (Thermo-Fisher Scientific, Waltham, MA) spectrophotometer. For both inhibition of XO and superoxide anion scavenging activity, allopurinol was used as a positive control.

The dose-effect curve for each test compound was linearised by regression analysis and used to derive the IC_{50} values.

2.8. Cell culture

K562 cell line, obtained from the American Type Culture Collection (Rockville, MD) is a highly undifferentiated lineage (ATCCCCL-243) isolated from a Caucasian human with chronic myelogenous leukaemia (Kunzelmann, Toti, Freyssinet, & Meyer, 2002). Cells were cultivated in RPMI-1640 medium supplemented with 10% v/v foetal calf serum, 1% gentamycin and 2 mM L-glutamine as a complete growth medium. Cells were maintained in 25 cm^3 flasks with 10 ml of medium and were incubated at 37 °C in an incubator with 5% CO_2 in a humidified atmosphere. Every two days the cells were subcultured by splitting the culture with fresh medium.

2.9. Lipid peroxidation inhibitory activity

Lipid peroxidation was assayed by the measurement of malondialdehyde (MDA) according to the method of Ohkawa, Ohishi, and Yagi (1979). The cells (3×10^7 cells/ml) were exposed to various concentrations of each extract (100, 400 and 800 μg/ml) in the incubation medium for 2 h, followed by 70 μM H_2O_2-treatment for 2 h. The cells were washed with PBS, pelleted and homogenised in 1.15% KCl. Samples were combined with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% thiobarbituric acid. The mixture was brought to a final volume of 4.0 ml with distilled water and heated to 95 °C for 120 min. After cooling for 10 min on ice, 5.0 ml of a mixture of *n*-butanol and pyridine (15:1 v/v) were added to each sample, and the mixture was shaken vigorously. After centrifugation at 650 *g* for 10 min, the supernatant fraction was isolated and the absorbance was measured at 532 nm on a Spectronic Genesys 10-S, (Thermo Electron Corp., Madison, WI). Inhibitory activity towards lipid peroxidation was expressed as percentage inhibition and IC_{50} values.

2.10. Statistical analysis

Data were collected and expressed as the mean ± standard deviation of three independent experiments and analysed for statistical significance from control, using the Dunnett test (SPSS 11.5 Statistics Software; SPSS, Chicago, IL). The criterion for significance was set at $p < 0.05$. IC_{50} values, from the *in vitro* data, were calculated by regression analysis.

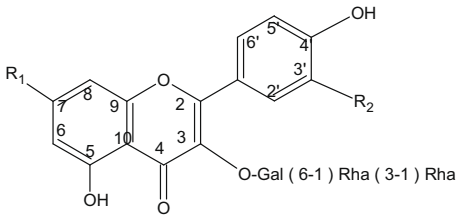
3. Results and discussion

3.1. Elucidation of the purified compounds

The protons and carbons were assigned from the combination of ^1H–^1H correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple band correlation (HMBC) data.

The compounds **1**, **2** and **3** were identified as kaempferol 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside, rhamnocitrin 3-*O*-[α-L-rhamnopyranosyl (1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside and rhamnetin3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside, respectively (Fig. 1).

The compounds isolated here were previously isolated from other plant species, particularly from the genus *Rhamnus*. The proposed structures were confirmed by comparison with those reported in the literature (Lin, Chung, Can, & Lu, 1991; Lin & Wei, 1994; Lu, Sun, Foo, McNabb, & Molan, 2000; Marzouk et al., 1999; Satake et al., 1993; Özipek, Calis, Ertant, & Rüedi, 1994).



	R_1	R_2
1:	OH	H
2:	OCH_3	H
3:	OCH_3	OH

Fig. 1. Chemical structures of compounds (**1**–**3**).

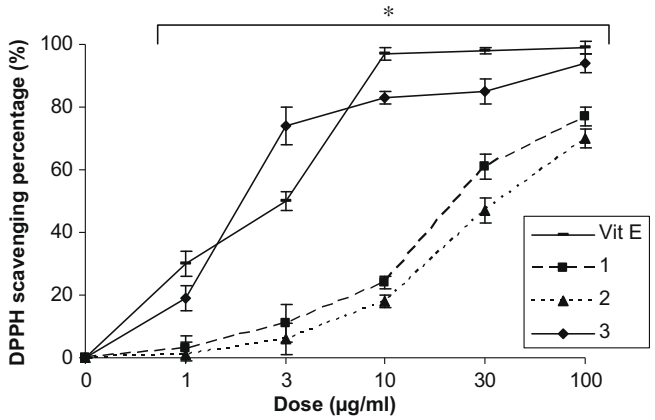


Fig. 2. DPPH radical scavenging activity of compounds **1**, **2** and **3**, expressed as percentages of inhibition (%), versus the positive control (Vitamin E). Symbols represent statistical significance from control (*$p < 0.05$).

3.2. DPPH radical-scavenging activity

The DPPH· test is largely used in plant or food biochemistry to evaluate the free radical-scavenging effect of specific compounds or extracts. This stable free radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule. In its radical form, DPPH· has a broad absorption band with a maximum at 517 nm, whilst if it is protonated by an antiradical compound, it loses this property (Lo Scalzo, 2008). In this assay, all test compounds effectively reduced the stable radical DPPH· to the yellow-coloured diphenylpicrylhydrazine and their scavenging effect was dose-dependent (Fig. 2). It was also found that compound **3** possesses the most potent DPPH radical-scavenging activity, with an IC_{50} value of 1.5 µg/ml, which was about three-fold more potent than Vitamin E (3 µg/ml) used as positive control. Based on the IC_{50} values, the potency of DPPH free radical-scavenging activity of the tested flavonoids was in the order of **3** (1.5 µg/ml) > **1** (23 µg/ml) > **2** (38 µg/ml).

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 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 both 3-, 5- and 7-hydroxyl groups. Results revealed that the radical scavenging activities of the tested flavonoids were correlated with the number and position of phenolic hydroxyl groups in the molecules. At various levels of hydroxyl groups and similar glycosylation, the radical-scavenging activity of the tested flavonoids tended to follow the above order. Indeed, compound **3**, the most potent DPPH· scavenger in this study, not only possesses the 2,3-double bond in conjugation with 4-oxo function in the C-ring, but also possesses 3′,4′-dihydroxy groups in the B-ring and another free 5-OH in the A-ring, which are amongst the essential structural elements for potent radical-scavenging activities of the flavonoids.

In the DPPH· test, the antioxidants reduce the DPPH radical to a yellow-coloured compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen-donating ability of the antioxidants (Bondent, Brand-Williams, & Bereset, 1997). The mechanism of reaction between antioxidant and DPPH· depends on the structural conformation of the antioxidant. Some compounds react very quickly with DPPH·, reducing a number of DPPH· molecules equal to the number of the hydroxyl groups (Bondent et al., 1997). Our results on the efficiency of flavonoids in inhibiting DPPH free radical are generally consistent with these criteria. Hence, compound **2**, showing the lowest potency, only satisfies the requirement of 5-hydroxyl substitution and the 2,3-double bond in conjugation with a 4-oxo function (flavone structure). The other more potent inhibitors (**3** and **1**) satisfy at least three of these requirements.

Finally, although a large number of antioxidant assays are available, the DPPH free radical is very stable and thus allows for easy handling and manipulation. Furthermore, its stability implies that a potential antioxidant will react with other well-known free radical entities, which are more unstable and therefore more reactive (Frum, Viljoen, & Van Heerden, 2007). Thus, an antioxidant candidate which proves promising in the DPPH antioxidant assay would provide an optimistic scaffold for prospective *in vivo* studies.

3.3. Evaluation of XOD activity and superoxide-scavenging effect

XOD is an enzyme with the capacity of catalysing the changing of hypoxanthine to xanthine. Afterwards, the xanthine is transformed into urate. During the reoxidation of XOD, molecular oxygen acts as an electron acceptor, producing superoxide radical and hydrogen peroxide. Consequently, XOD is considered to be amongst the important biological sources of superoxide radicals (Montoro, Braca, Pizza, & De Tommasi, 2005). Thus, in this study, we evaluate the XOD inhibitory effects of the studied flavonoids, as well as their superoxide-scavenging activities. Inhibition of XOD results in a decreased production of uric acid and a decreased production of superoxide anions.

From our results, compounds **1**, **2** and **3** showed high inhibition of XOD, with respective IC_{50} values of 18, 81 and 40 µg/ml, and their inhibitory effects were dose-dependent (Fig. 3). Compounds **2** and **3**, characterised by the absence of the 7-OH group revealed less action than that with the hydroxyl function at C-7 (compound **1**) (see Table 1). Thus, in the presence of a hydroxyl group in the C-7 position on the A-ring (**1**), flavonoids inhibit effectively the X/XO system. Our results showed also that the order of the $O_2^{\cdot-}$ production inhibition activity of the tested flavonoids was **2** (79 µg/ml) < **1** (42 µg/ml) < **3** (35 µg/ml) (Fig. 4). This suggested that the number of hydroxyl groups as well as their disposition increased the superoxide radical-scavenging activity of the tested flavonoids, furthermore the substitution of the hydroxyl groups by methoxyl groups reduced their activity. It was also observed that compound **3** still showed higher activity because it contains the ortho-(3′,4′) dihydroxyl groups. The inhibition of $O_2^{\cdot-}$ generation could be either due to scavenging activity or to inhibition of XOD (Cotelle et al., 1996).

Comparing the results obtained from the inhibition of XOD and the scavenging of the superoxide anions, it is clear that the three tested flavonoids showed activity in both tests, so that the inhibi-

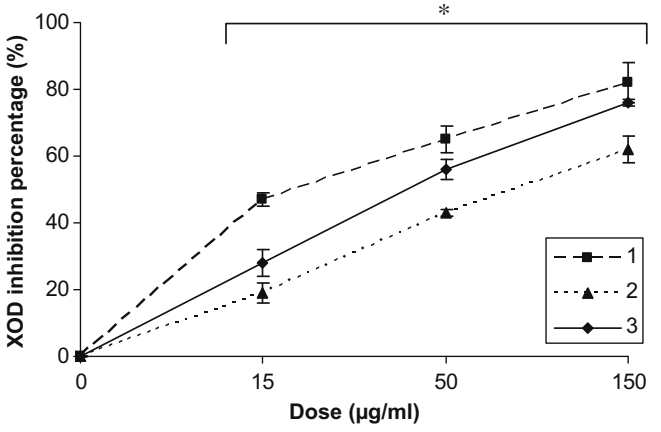


Fig. 3. Inhibition percentage of XOD activity in the presence of different concentrations of compounds **1**, **2** and **3**. Symbols represent statistical significance from control (*$p < 0.05$).

Table 1
Effect of the compounds **1**, **2** and **3** on lipid peroxidation inhibition, DPPH· and superoxide anion scavenging, and XOD activity. Comparative study of IC_{50}[a,b] (μg/ml) values.

Compound	Scavenging of:	Inhibition of:		
	DPPH·	Superoxide anions	XO activity	Lipid peroxidation
1	23 ± 3	42 ± 2	18 ± 2	180 ± 19
2	38 ± 2	79 ± 5	81 ± 7	320 ± 14
3	1.5 ± 0.1	35 ± 3	40 ± 4	106 ± 6
Vitamin E	3 ± 0.2	–	–	–
Allopurinol	–	37 ± 4	6 ± 0.7	–
Vitamin C	–	–	–	15 ± 2

(–): not done.
[a] Means of three experiments.
[b] Values obtained from regression lines. IC_{50} is defined as the concentration sufficient to obtain 50% of maximum inhibition or radical scavenging.

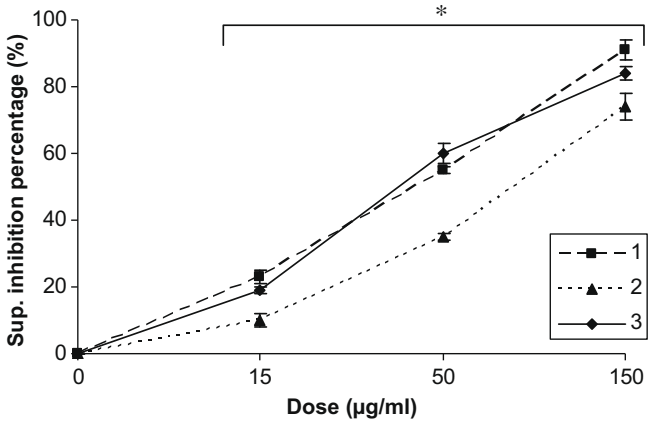


Fig. 4. Superoxide anion inhibition by compounds **1**, **2** and **3** isolated from *R. alaternus*, expressed as percentages of inhibition (%). Symbols represent statistical significance from control (*$p < 0.05$).

tion of the XOD system is strengthened by the simultaneous action on the superoxide anions. 7-Hydroxyflavonoids have been proposed to be potent inhibitors of XOD, which is implicated in the generation of reactive oxygen species (Cotelle et al., 1996). 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. Structure–activity relationships of flavonoids in the inhibition of XOD and in the scavenging of superoxide anion are not similar. The unsaturation in ring-C and the free hydroxyl group at C-7 enhanced the activity (compound **1**). It is proposed that the C-7 OH of flavonoids may take the place of the C-2 or C-6 OH of xanthine in the active site of the enzyme. A C-4′ OH or C-4′ OMe substitution on the 7-hydroxyflavones is not favourable to a fit in the active site (Cotelle et al., 1996). In the case of both inhibition of XOD activity and superoxide-scavenging activity, the superoxide concentration reduction is lower, so that the corresponding IC_{50} values of the flavonoid for superoxide are higher than those of uric acid.

Allopurinol, the positive control used in this study, is a powerful inhibitor of the XO enzyme and it is used as a medication in cases where it is necessary to inhibit XO enzyme action. In the literature, several flavonoids have been described as inhibitors of XO enzyme in a similar way to that presented by allopurinol (Da Silva et al., 2004). Several studies demonstrated the capacity of some flavonoid compounds to interact with XOD, diminishing its activity level in a competitive inhibitory action. In fact, many authors have demonstrated that flavonoids have a high capacity to inhibit XOD and they have verified the biological power of these compounds by structure–activity relationships studies (Lin, Chen, Chen, Liang, & Lin, 2002; Ponce, Blanco, Molina, Garcia-Domenech, & Galvez, 2000).

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 flavonoids exhibit interesting antioxidant properties expressed either by the capacity to scavenge free radicals (for compound **3**) or to inhibit XOD activity (for compound **1**). These findings were noteworthy because such compounds may be useful in the treatment of many kinds of diseases related to free radical oxidations. Notably, such compounds would be well-adapted to the pathogenesis of ischaemic injury, which is characterised 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 & Lucchesi, 1990), which produces $O_2^{\cdot-}$ 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 ischaemic tissues.

3.4. Anti-lipid peroxidation effect

Oxidative stress can damage many biological molecules: proteins and DNA are significant targets of cellular injuries. Another target of free radical attack in biological systems is the cell membrane lipids. Lipid peroxidation, an oxidative alteration of polyunsaturated fatty acids in the cell membranes, generates numerous degradation products. MDA, one of these products, has been studied widely as an index of lipid peroxidation and as a marker of oxidative stress (Janero, 1990). The measure of thiobarbituric acid reactive substances (TBARS) has been widely used in studies of anti-lipid peroxidation activity of natural phytochemicals in cultured cells (Wu & Ng, 2007).

Our results showed that inhibition of malondialdehyde (MDA) formation increases with increasing concentrations of test compounds. Indeed, the addition of H_2O_2 (70 μM) to the K562 cultured cells for 2 h significantly increased the extent of TBARS formation, compared to the control sample. However, as shown in Fig. 5, adding 100–800 μg/ml tested compounds to the cells significantly reduced TBARS formation, indicating significant anti-lipid peroxidation activities.

It can also be noticed that the three tested compounds showed protection against lipid peroxidation at all of the doses. The effect is more significant at higher doses with **3**, **1** and **2**, which show maximum inhibition effects of 89%, 85% and 72% at a concentration of 800 μg/ml, respectively. Based on the IC_{50} values, the potency of anti-lipid peroxidation activity was in the order of **3** (106 μg/ml) > **1** (180 μg/ml) > **2** (320 μg/ml).

Compound **3**, which displayed the best inhibitory effect against lipid peroxidation was capable of inhibiting TBARS formation by 49%, 68% and 89%, at concentrations of 100, 400 and 800 μg/ml,

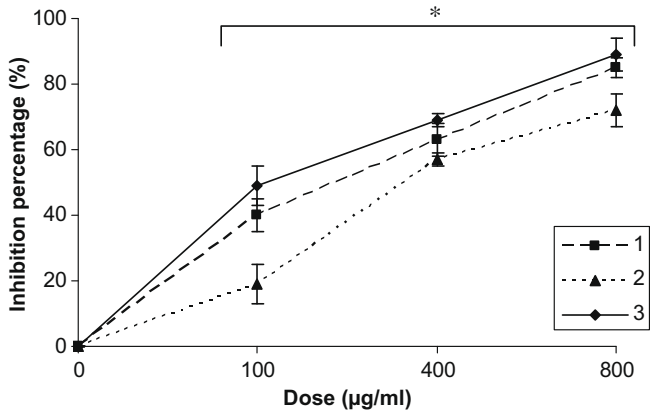


Fig. 5. Lipid peroxidation inhibitory activity in K562 cells treated with compounds **1**, **2** and **3**, isolated from *R. alaternus* and H_2O_2 (70 µM). Vitamin C was used as positive control for protection against H_2O_2 induced peroxidation. Cells treated by H_2O_2 (70 µM) alone were used as a control for lipid peroxidation induction. Symbols represent statistical significance from control (*$p < 0.05$).

respectively. Incubation of cells with vitamin C at 17 µg/ml resulted in 55% inhibition of lipid peroxidation.

Usually free radical scavengers inhibit lipid peroxidation (Lee, Shin, Hwang, & Kim, 2003). The potent anti-lipid peroxidation activity of tested compounds could be related to their structure properties. Indeed, 3′,4′,5′-hydroxy-substitution on the B-ring was excellent for protection against lipid peroxidation (Cotelle et al., 1996), as compound **3** (IC_{50} = 106 µg/ml) effectively prevented K562 cells from MDA formation, as compared to compounds **1** and **2**. Our results on the efficiency of flavonoids in inhibiting lipid peroxidation are partially consistent with the criteria previously shown by Bors et al. (2001). Hence, the less potent lipid peroxidation inhibitor **2** only satisfies the requirement of 5-hydroxyl substitution. The other more potent inhibitors satisfy at least two of these requirements.

The 7-hydroxyl group in combination with a 2,3-double bond, present in compound **3**, is known to improve antioxidant efficiency, and this may be the reason why this flavonoid was the most potent inhibitor of lipid peroxidation. The significant effects observed in this test substantiate the radical-scavenging activity of the tested compounds, and are in good agreement with the previous assays (DPPH· and superoxide anion-scavenging activities).

Lipid peroxides are potentially toxic and possess the capacity to damage mast cells. In fact, accumulation of lipid peroxides has been reported in atherosclerotic plaques, in brain tissues damaged by trauma or oxygen deprivation and in tissues poisoned by toxins (Middleton, Kandaswami, & Theoharides, 2000). In aerobic organisms, one of the major targets of ROS is the cellular biomembranes, where they induce lipid peroxidation. Under this process, not only the membrane structure and its function are affected, but also some oxidation reaction products. For example, malondialdehyde (MDA) can react with biomolecules and exert cytotoxic and genotoxic effects. In addition, high levels of lipid peroxides have been found in the serum of patients suffering from liver disease, diabetes, vascular disorders and tumours (Pezzuto & Park, 2002).

Substances termed antioxidants can influence the oxidation process through simple or complex mechanisms, including prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging (Ames, Shigrenaga, & Hagen, 1993). Mechanism of antioxidant action can generally include suppressing reactive oxygen species formation, either by inhibition of enzymes or by chelating trace elements involved in free-radical production, scavenging reactive species, and upregulating or protecting antioxidant defences.

In vivo, some of these ROS play a positive role such as energy production, phagocytosis, regulation of cell growth and intracellular signalling. ROS are not only strongly associated with lipid peroxidation resulting in deterioration of food materials, but also are involved in development of a variety of diseases, including ageing, carcinogenesis, coronary heart disease, diabetes and neurodegeneration (Moskovitz, Yim, & Choke, 2002). Cells have several antioxidant defence mechanisms that help to prevent the destructive effects of ROS. These defence mechanisms include antioxidative enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase and of small molecules such as glutathione and vitamins C and E. The efficiency of the antioxidant defence system is altered under pathological conditions.

The structure–antioxidant activity relationships of flavonoids in the aqueous or lipophilic system have been extensively reported (Burda & Oleszek, 2001; Natella, Nardini, Di Felice, & Saccini, 1999; Nenandis, Wang, Tsimidou, & Zhang, 2005). Generally, antioxidant activity depends on the number and positions of hydroxyl groups, other substituents and glycosylation of flavonoid molecules. The presence of certain hydroxyl groups on the flavonoid nucleus enhances antioxidant activity. Substitution patterns in the B-ring and A-ring, as well as the 2,3-double bond (unsaturation) and the 4-oxo group in the C-ring also affect antioxidant activity of flavonoids. Glycosylation of flavonoids diminishes their activity when compared to the corresponding aglycones.

The different classes of flavonoids present distinct pharmacological properties, which can be associated to cardiotonic, antiulcer, hepatoprotective, antioxidant, antiphlogistic, antineoplasic and antimicrobial activities (Susanti et al., 2007). In all of these processes, the flavonoids can act with specific enzymes and hormones. In addition, antioxidants also play an important role in the food industry, because excessive formation of free radicals can accelerate oxidation of lipids in foods and thereby decrease food quality.

In previous studies, we have evaluated the antioxidant and the free radical-scavenging activities of the two crude extracts used here to obtain the three flavonoids (**1**–**3**). The TOF and the methanolic extracts showed an important free radical-scavenging activity towards the DPPH radical, with respective IC_{50} values of 1 and 29 µg/ml (Ben Ammar et al., 2005). Furthermore, fifty percent inhibition of uric acid production was obtained at IC_{50} values of 173 and 200 µg/ml with, respectively, TOF and methanolic extracts. Likewise it appears from the IC_{50} values of superoxide anions measured in the presence of TOF and methanolic extracts (respectively 138 and 183 µg/ml) that TOF extract is the most potent superoxide scavenger (Ben Ammar et al., 2007a), as it was enriched in polyphenolic compounds compared with the methanolic extract (Ben Ammar et al., 2007b). The antioxidant and free radical-scavenging potential of the flavonoids (**1**–**3**) tested in this study could justify and explain the same activities obtained with the original extracts, as these flavonoids are the major components of the TOF and the methanolic extracts.

4. Conclusion

In this study, the antioxidant potential of flavonoids isolated from *R. alaternus* leaves was evaluated using *in vitro* DPPH·, superoxide anions, XOD and inhibition of H_2O_2-induced lipid peroxidation assays. The findings presented here showed that some 7-hydroxyflavonoids were potent inhibitors of XOD. It can be suggested that the 7-OH of flavonoids takes the place of the 2 or 6-OH of xanthine in the active site. It has also been confirmed that OH-substitution on the B-ring plays a crucial role in radical-scavenging activity in the DPPH· assay and on the inhibitory effect on peroxidation of cell lipids in the MDA test. Our study provides evidence that the tested flavonoids exhibit interesting antioxidant

properties, expressed either by their capacity to scavenge free radicals or to inhibit XOD. The investigation of such structure–activity interactions could yield important information, with regards to developing superior protocols for antioxidant therapy. Finally, the information presented here could be used as preliminary data and biologically more relevant experiments that will examine the therapeutic potential of the *R. alaternus* isolated compounds will be designed.

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