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Walnut (*Juglans regia*) leaf extracts are strong scavengers of pro-oxidant reactive species

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Abstract

The growing interest in the substitution of synthetic food antioxidants by natural ones has fostered research on vegetable sources and on the screening of raw materials, for identifying new antioxidants. In the present study, an ethanol:water (4:6) extract from *Juglans regia* leaves was evaluated for its putative *in vitro* scavenging effects on reactive oxygen species (ROS) [hydroxyl radical ($HO^{\bullet}$), superoxide radical ($O_2^{\bullet-}$), peroxyl radical ($ROO^{\bullet}$) and hydrogen peroxide (H_2O_2)] and reactive nitrogen species (RNS) [nitric oxide ($^{\bullet}NO$) and peroxynitrite anion ($ONOO^-$)]. The extract presented a potent scavenging activity against all the reactive species tested, all the IC_{50}s being found at the μg/mL level. IC_{50}s (mean ± SE) for the ROS $O_2^{\bullet-}$ and H_2O_2 were 47.6 ± 4.6, 383 ± 17 μg/mL, respectively. The oxygen radical absorbance capacity (ORAC) value obtained for $ROO^{\bullet}$ was 2.17 ± 0.22 μmol Trolox equivalents/mg extract. The IC_{50}s for $^{\bullet}NO$ and $ONOO^-$ were 1.95 ± 0.29 and 1.66 ± 0.10 μg/mL, respectively. The content of total phenolics was 270 ± 3 mg of gallic acid equivalents (GAE)/g of lyophilised extract.

These results showed that *J. regia* leaf extracts can be used as an easily accessible source of natural antioxidants.

Keywords: *Juglans regia*; Reactive oxygen species; Reactive nitrogen species; Antioxidant activity

1. Introduction

The prevention of the oxidative reactions in foods, pharmaceuticals and cosmetics and the management of oxidative stress-related diseases are some of the potential applications of antioxidants. The most widely used antioxidants, to prevent the oxidation of lipids in foods, are butylated hydroxyanisole (BHA), propyl gallate and 2-*tert*-butylhydroquinone (TBHQ) (Moure et al., 2001). However, there has been growing concern over the safety of some of the commercial antioxidants because several studies documented the mutagenesis and carcinogenesis potential associated with some synthetic antioxidants (Ito et al., 1986; Witschi, 1986), although other authors reported anti-carcinogenic effects (Williams, Iatropoulos, & Whysner, 1999). Therefore, the extraction and characterisation of natural antioxidants are of considerable interest. Natural antioxidants, such as tocopherols, ascorbic acid and plant polyphenols have gained increasing importance.

Walnut (*Juglans regia* L.) is a deciduous tree native in southeastern Europe of the Juglandaceae family. The walnut fruits are a highly nutritious food, which are rich in oil composed of unsaturated fatty acids. Antioxidant effects of isolated polyphenols obtained from walnuts have been previously reported (Fukuda, Ito, & Yoshida, 2003). Scavenging of hydroxyl radicals and superoxide radicals is documented for water and methanol extracts of the kernel of *J. regia* (Ohsugi et al., 1999). Walnut liqueur, obtained with green walnuts, also presents antioxidant activity which was correlated with its polyphenolic composition (Alamprese, Pompei, & Scaramuzzi, 2005). Walnut leaf has been widely used in folk medicine for the treatment of skin inflammations, hyperhidrosis and ulcers and for its antidiarriec,

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anti-helmintic, antiseptic and astringent properties (Bruneton, 1999; Proenca da Cunha, Silva, & Roque, 2003).

No studies concerning the scavenging effect of reactive oxygen species (ROS) or reactive nitrogen species (RNS) have been reported for *J. regia* leaves.

The objective of the present study is thus to assess the *in vitro* scavenging activity of pro-oxidant reactive species exerted by the *J. regia* leaf ethanol:water (4:6) extract, namely on the ROS [hydroxyl radical ($HO^{\bullet}$), superoxide radical ($O_2^{\bullet-}$), peroxyl radical ($ROO^{\bullet}$) and hydrogen peroxide (H_2O_2)] and the RNS [nitric oxide ($^{\bullet}NO$) and peroxynitrite anion ($ONOO^-$)].

2. Materials and methods

2.1. Plant material

J. regia leaves were collected during Summer, July 2003, in Mirandela, Northern Portugal, in a highland with high thermal amplitude. The leaves were dried at room temperature. Voucher specimens were preserved in our laboratory for further reference.

2.2. Chemicals

Dihydrorhodamine 123 (DHR), 4,5-diaminofluorescein (DAF-2), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), nitroblue tetrazolium chloride (NBT), lucigenin, diethylenetriaminepentaacetic acid (DTPA), β-nicotinamide adenine nucleotide (NADH), phenazine methosulfate (PMS), ferric chloride anhydrous ($FeCl_3$), thiobarbituric acid (TBA), mannitol, manganese dioxide, deoxyribose (DR), Folin reagent, gallic acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH) and rutin were purchased from Sigma (St. Louis, MO, USA). Azodiisobutyramidine dihydrochloride (AAPH), histidine and trolox were obtained from Fluka Chemie GmbH (Steinheim, Germany). Fluorescein sodium salt and dimethylformamide were obtained from Aldrich (St. Louis, MO, USA). Trichloroacetic acid was obtained from Panreac (Barcelona, Spain). Ethanol (96%) was purchased from Aga (Portugal). Dimethylsulfoxide (DMSO) was obtained from Ridel-de Haën (Germany). Hydrogen peroxide (30%), sodium phosphate, potassium phosphate, sodium bicarbonate, sodium nitrite, sodium hydroxide, sodium chloride, potassium chloride, ethylenediaminetetraacetic acid (EDTA) disodium salt, hydrochloric acid, sodium carbonate and ascorbic acid were obtained from Merck (Darmstadt, Germany).

2.3. Preparation of ethanol:water extracts

The dried leaves (2 g) were grounded (500 μm) and extracted five times (10 min, 500 rpm, 40 °C) with ethanol:water (4:6) solution (5 × 50 mL) and filtered with a glass filter funnel (G4) (Our preliminary studies indicated that the walnut extract obtained with this solvent mixture presented a higher free radical scavenging activity than the extracts obtained with other solvents). The extracts were gathered and the ethanol was evaporated under reduced pressure at 40 °C in a rotavapor (Büchi, RE 111), followed by lyophilisation (Labconco, FreeZone 4.5, USA).

2.4. Determination of total phenolics

The amount of total phenolics in the extract was determined using the Folin Ciocalteu colorimetric method, according to a described procedure (Wang, Lee, & Peng, 1997). The contents are expressed as milligrams of gallic acid equivalents (GAE) per gram of lyophilised extract. The measurements were performed in triplicate.

2.5. $O_2^{\bullet-}$ scavenging assay

$O_2^{\bullet-}$ was generated by the NADH/PMS system and the $O_2^{\bullet-}$ scavenging activity was determined spectrophotometrically in a microplate reader (Synergy HT, BIO-TEK), by monitoring the effect of the tested extract on the $O_2^{\bullet-}$ induced reduction of NBT (Fernandes, Toste, Lima, & Reis, 2003). The effects are expressed as the percentage inhibition of the $O_2^{\bullet-}$-induced NBT reduction to formazan. Trolox and ascorbic acid were used as positive controls. Each study corresponded to four experiments, which were performed in triplicate.

2.6. $HO^{\bullet}$ scavenging assay

$HO^{\bullet}$ was generated by a Fenton system (ascorbic acid/$FeCl_3$–EDTA/H_2O_2). Deoxyribose (DR) is degraded to malonaldehyde when exposed to $HO^{\bullet}$, which generates a pink compound with thiobarbituric acid, at low pH under heating (Fernandes et al., 2003; Halliwell, Guteridge, & Aruoma, 1987). The effects are expressed as the percentage inhibition of the DR degradation to malonaldehyde. Mannitol was used as the positive control. Each study corresponded to four experiments which were performed in duplicate.

2.7. $ROO^{\bullet}$ scavenging assay

$ROO^{\bullet}$ was generated by thermodecomposition of AAPH in a microplate reader (Synergy HT, BIO-TEK) at 37 °C. The $ROO^{\bullet}$scavenging activity was measured by monitoring the decay in fluorescence, due to the oxidation of fluorescein, according to a described procedure, termed the oxygen radical absorbance capacity (ORAC) (Huang, Ou, Hampsh-Woodill, Flanagan, & Prior, 2002), with modifications (Fernandes, Costa, Toste, Lima, & Reis, 2004). The effects are expressed as the relative trolox equivalent ORAC value, which is calculated by the following equation, where AUC represents the area under the curve:

$$\text{Relative ORAC value} = [(AUC_{sample} - AUC_{blank})/(AUC_{trolox} - AUC_{blank})] \times \text{Trolox molarity/extract concentration (μg/mL)}.$$

Ascorbic acid was used as the positive control. Each study corresponded to four experiments which were performed in triplicate.

2.8. H_2O_2 scavenging assay

The H_2O_2 scavenging activity was measured using a chemiluminescence methodology, by monitoring the H_2O_2-induced oxidation of lucigenin, in a microplate reader (Synergy HT, BIO-TEK), according to a previously described procedure (Costa, Gomes, Reis, Lima, & Fernandes, 2005). The effects are expressed as the percentage inhibition of the H_2O_2-induced lucigenin oxidation. Ascorbic acid was used as the positive control. Each study corresponded to four experiments which were performed in triplicate.

2.9. $^{\cdot}NO$ scavenging assay

$^{\cdot}NO$ was generated by decomposition of NOC-5. The $^{\cdot}NO$ scavenging activity was measured by monitoring the oxidation of the non-fluorescent 4,5-diaminofluorescein (DAF-2) to the fluorescent triazolofluorescein by $^{\cdot}NO$, in a microplate reader (Synergy HT, BIO-TEK), using the method of Nagata, Momose, and Ishida (1999), with modifications (Gomes, Costa, Lima, & Fernandes, 2006). The effects are expressed as the percentage inhibition of the $^{\cdot}NO$–induced DAF-2 oxidation. Rutin was used as the positive control. Each study corresponded to four experiments which were performed in triplicate.

2.10. $ONOO^-$ scavenging assay

The synthesis of $ONOO^-$ was carried out according to a described procedure (Beckman, Chen, Ishiripoulos, & Crow, 1994). The $ONOO^-$scavenging activity was measured by monitoring the oxidation of the non-fluorescent dihydrorhodamine 123 (DHR) to the fluorescent rhodamine 123 by $ONOO^-$ using the method of Kooy, Royall, Ischiropoulos, and Beckman (1994), with modifications (Gomes et al., 2006). The effects are expressed as the percentage inhibition of the $ONOO^-$ induced DHR oxidation. Ascorbic acid and cysteine were used as positive controls. In another set of experiments, the assays were performed in the presence of 25 mM $NaHCO_3$, in order to simulate the physiological conditions with high CO_2 concentrations *in vivo* (Whiteman, Ketsawatsakul, & Halliwell, 2002). Each study corresponded to four experiments which were performed in triplicate.

3. Results and discussion

3.1. Total phenols

The total phenolic content of the *J. regia* ethanol:water (4:6) extract was 270 ± 3 mg GAE per g of lyophilised extract (mean ± SE). The extract yield was 0.804 g, which corresponds to a recovery of 20.1%. Several polyphenols have been identified on *J. regia* leaves, namely cinnamic acids (3-caffeoylquinic acid, 3-*p*-coumaroylquinic and 4-*p*-coumaroilquinic acids) and flavonoids (quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside and a kaempferol derivative). The main constituent was found to be quercetin 3-galactoside (Amaral et al., 2004).

3.2. Scavenging of ROS

The occurrence of ROS in foods is inevitable due to their biological nature. ROS are mainly responsible for the initiation of oxidation reaction in foods. ROS react with lipids, proteins, sugars and vitamins, producing undesirable volatile compounds, destroying essential fatty acids, amino acids and vitamins and producing carcinogens. These reactive species change the functionalities of proteins, lipids and carbohydrates by forming oxidised dimers and trimers. ROS make food products less acceptable or unacceptable to consumers and lower the overall nutritional, chemical and physical qualities of food during storage and marketing (Choe & Min, 2006). In biological systems, oxidative stress, resulting from an imbalance between the generation of ROS and the antioxidant defense capacity of the cell affects major cellular components, including lipids, proteins and DNA. Continuous overproduction of ROS and/or the decrease in antioxidant defences may contribute to the development of several diseases, such as cancer, rheumatoid arthritis, diabetes and neurological disorders as reviewed by Valko et al. (2007).

The walnut leaf extract presented a remarkable capacity to scavenge all the tested ROS, all the IC_{50}s being found at the μg/mL level with the exception of $HO^{\cdot}$ (Table 1). The IC_{50} (mean ± SE) for $O_2^{\cdot-}$ was 47.6 ± 4.6 μg/mL, while ascorbic acid and Trolox presented IC_{50}s of 0.028 ± 0.005 μg/mL and 452 ± 9 μg/mL, respectively. The *J. regia* (ethanol:water) extract showed a concentration dependent inhibition of the $O_2^{\cdot-}$-induced reduction of NBT (Fig. 1). The scavenging activity of $O_2^{\cdot-}$ has been reported for quercetin 3-galactoside (Zou, Lu, & Wei, 2004), 3-caffeoylquinic acid (Nakatani et al., 2000), quercetin 3-rhamnoside (Moharram, Marzouk, Ibrahim, & Mabry, 2006) and quercetin (Marzouk, Moharram, Haggag, Ibrahim, & Badary, 2006). Although $O_2^{\cdot-}$ is not a potent pro-oxidant *per se*, it appears to be a key starting point of oxidative stress. $O_2^{\cdot-}$ can be formed by gamma irradiation, pulsed electric field, microwave and ohmic processing of foods (Choe & Min,

Table 1
$O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$ scavenging activities of the *Juglans regia* leaf extract (IC_{50}, mean ± SE)

Extract/compound	$O_2^{\cdot-}$ (μg/mL)	H_2O_2 (μg/mL)	$HO^{\cdot}$ (μg/mL)
Juglans regia	47.6 ± 4.6	383 ± 17	–
Trolox	452 ± 9	–	–
Ascorbic acid	0.028 ± 0.005	200 ± 22	–
Mannitol	–	–	913 ± 48

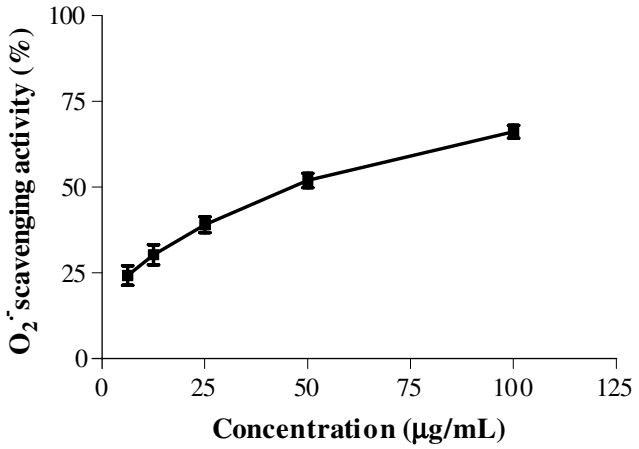

Fig. 1. $O_2^{\cdot-}$ scavenging activity of the *Juglans regia* leaf extract. Each point represents the values obtained from four experiments, which were performed in triplicate (Mean ± SE).

2006). $O_2^{\cdot-}$ is produced readily by multiple processes *in vivo* and leads to the generation of many other oxidants. It is catalytically reduced by superoxide dismutase to H_2O_2. H_2O_2 can be also produced by radiolysis of water. *Per se*, H_2O_2 is less reactive oxidant than $O_2^{\cdot-}$. The *J. regia* (ethanol:water) extract inhibited the H_2O_2-induced oxidation of lucigenin in a concentration dependent manner (Fig. 2). For this reactive species, the extract exhibited an IC_{50} of 383 ± 17 μg/mL. Ascorbic acid presented an IC_{50} of 200 ± 22 μg/mL in this assay. Quercetin, which is present in this plant, was previously found to be an effective scavenger of H_2O_2 (Farombi & Fakoya, 2005).

In the presence of iron and other metal ions, $O_2^{\cdot-}$ and H_2O_2 can react to form the more potent and toxic HO·, through the Fenton reaction (Halliwell & Guteridge, 1990). HO· is also formed by UV-induced homolytic fission of the oxygen-oxygen bond of H_2O_2 and after high energy radiation of gamma rays on water (Choe & Min, 2006).

HO· is the strongest oxidant and is mainly responsible for the initiation of lipid oxidation. Lipid peroxidation is generally thought to be a major mechanism of cell injury in aerobic organisms subjected to oxidative stress as reviewed by Catalá (2006). Mannitol was the positive control for the HO· scavenging assay and exhibited an IC_{50} of 913± 48 μg/mL. In this assay, although the *J. regia* (ethanol:water) extract inhibited the hydroxyl-induced degradation of deoxyribose (Fig. 3) in a concentration dependent manner, the IC_{50} was not calculated. The maximum observed inhibitory effect was around 50% for 1000 μg/mL and no other concentrations were tested due to low solubility in the reaction media. The plant polyphenols quercetin 3-galactoside, quercetin 3-rhamnoside and quercetin have previously shown protective effect against HO· (Chen, Zhu, Hu, & Zhu, 2002; Cos et al., 2002; Moharram et al., 2006).

A decrease of malonaldehyde formation was observed in the absence of EDTA, which is an indicator of iron-chelating activity. Indeed, the capacity to chelate transition metal ions gives rise to stable complexes that, by entrapping metals, prevent these from participating in free radical generation. The metal chelating ability of flavonoids can be formed between 5-OH and 4-oxo groups or between 3′-OH and 4′-OH groups (Wang et al., 2006). Therefore, the components of the extract can exert their effect involving these mechanisms.

The ORAC value obtained represents the ROO· absorbing capacity and was 2.17 ± 0.22 μmol Trolox equivalents/mg extract, which represents around 55% of the trolox value (4). Ascorbic acid presented an ORAC value of 1.24 ± 0.42 (Table 2). ROO· is formed by a direct reaction of triplet oxygen with alkyl radicals in fatty acid oxidation. This radical produces hydroperoxide (ROOH) by abstracting oxygen from other molecules. Most hydroperoxides are stable at room temperature. However heat, UV light or transition metals accelerate homolysis of hydroperoxydes and produce ROO· (Choe & Min, 2006). The antioxidant

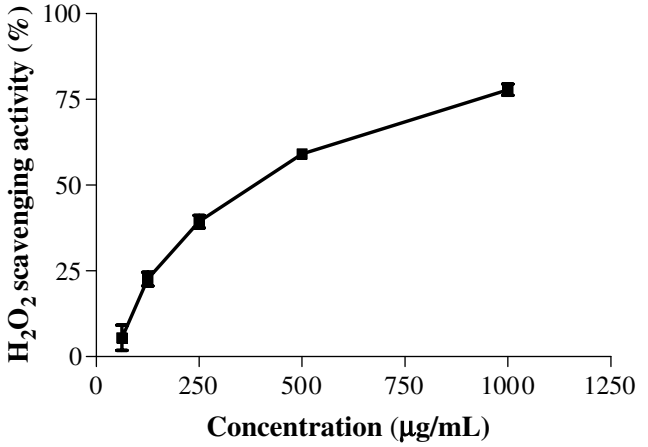

Fig. 2. H_2O_2 scavenging activity of the *Juglans regia* leaf extract. Each point represents the values obtained from four experiments, which were performed in triplicate (Mean ± SE).

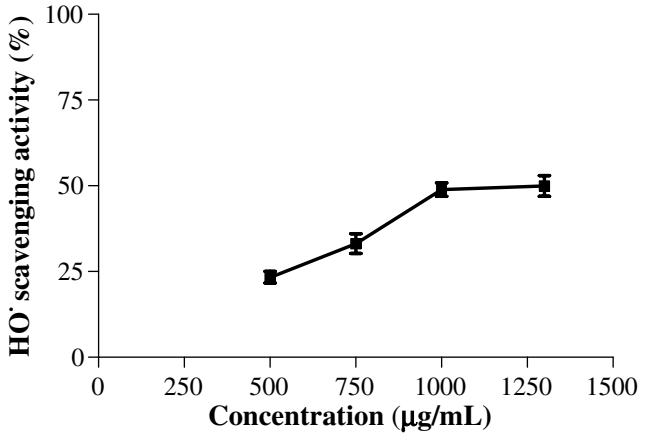

Fig. 3. HO· scavenging activity of the *Juglans regia* leaf extract. Each point represents the values obtained from four experiments, which were performed in triplicate (Mean ± SE).

Table 2
ROO· scavenging activity of the *Juglans regia* leaf extract (mean ± SE) evaluated by the ORAC assay

Extract/compound	ORAC (μmoles Trolox/extract concentration μg/mL)
Juglans regia	2.17 ± 0.22
Trolox	4
Ascorbic acid	1.24 ± 0.42

activity for *p*-coumaric acid, quercetin, quercetin 3-galactoside and quercetin 3-rhamnoside was previously confirmed by the ORAC assay (Aaby, Hvattum, & Skrede, 2004; Lau et al., 2007).

3.3. Scavenging of RNS

The major pathway for ·NO production in uncured meats is via nitric oxide synthase. ·NO may then react with food born $O_2^{\cdot-}$ to form $ONOO^-$, a strong oxidising and nitrating species (Wink & Mitchell, 1998). Once formed, $ONOO^-$ could lead to the onset of rancidity, loss of colour and alterations in protein functional properties (Brannan, Connolly, & Decker, 2001).

·NO has been found to play a critical role in numerous physiological processes, as well as in the pathophysiology of many human diseases. Its toxicity is not only related to the levels of ·NO generation but is also highly dependent upon the levels and types of other species which react with ·NO, converting it into toxic oxidants and nitrating agents. The reactivity of RNS may have profound effects on the biological activity of numerous molecules (Elserich, Patel, & O'Donnell, 1998).

The walnut leaf extract also exerted a potent scavenging activity against RNS. IC_{50}s for ·NO and $ONOO^-$ were 1.95 ± 0.29 and 1.66 ± 0.10 μg/mL, respectively. The positive controls presented IC_{50}s of 0.86 ± 0.06 (rutin) for ·NO and IC_{50}s of 0.46 ± 0.03, (cysteine) and 0.21 ± 0.01 μg/mL (ascorbic acid) for $ONOO^-$ (Table 3). For the latter reactive species, a slight reduction of the scavenging activity of the extract was observed in the presence of 25 mM $NaHCO_3$ (IC_{50} = 2.01 ± 0.18 μg/mL). No modification was found for cysteine (IC_{50} = 0.42 ± 0.06 μg/mL) while ascorbic acid showed a decrease in activity (IC_{50} = 0.34 ± 0.04 μg/mL). It has been reported that physiological concentrations of CO_2 can modulate $ONOO^-$ reactivity due to the fast reaction between these two compounds, yielding $\cdot NO_2$ and $CO_3^{\cdot-}$, which are the main responsible radicals for the nitration and oxidation reactions that are usually observed *in vivo* (Squadrito & Pryor, 1998). Following the results of the present study it may be expected that the *J. regia* leaf extract is also an effective scavenger for $\cdot NO_2$ and $CO_3^{\cdot-}$ since it maintained its scavenging effectiveness in the presence and absence of HCO_3^-.

Table 3
·NO and $ONOO^-$ scavenging activities of the *Juglans regia* leaves extract (IC_{50}, mean ± SE)

Extract/compound	·NO (μg/mL)	$ONOO^-$ Absence of $NaHCO_3$ (μg/mL)	$ONOO^-$ Presence of 25 mM $NaHCO_3$ (μg/mL)
Juglans regia	1.95 ± 0.29	1.66 ± 0.10	2.01 ± 0.18
Rutin	0.86 ± 0.06	–	–
Ascorbic acid	–	0.21 ± 0.01	0.34 ± 0.04
Cysteine	–	0.46 ± 0.03	0.42 ± 0.06

The extract inhibited the ·NO induced oxidation of DAF-2 to triazolofluorescein (Fig. 4) and the $ONOO^-$ induced oxidation of DHR (Fig. 5) in a concentration dependent manner. *J. regia* leaf (ethanol:water) extract was more active in scavenging RNS, comparatively, to the other studied reactive species. Noteworthy, Haenen, Paquay, Korthouwer, and Bast, 1997 have reported that

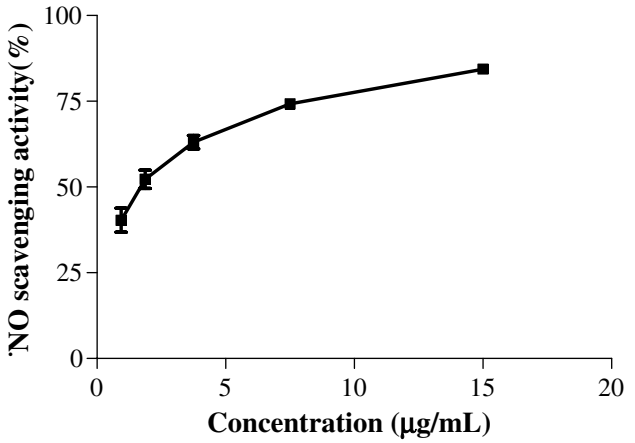

Fig. 4. ·NO scavenging activity of the *Juglans regia* leaf extract. Each point represents the values obtained from four experiments, which were performed in triplicate (Mean ± SE).

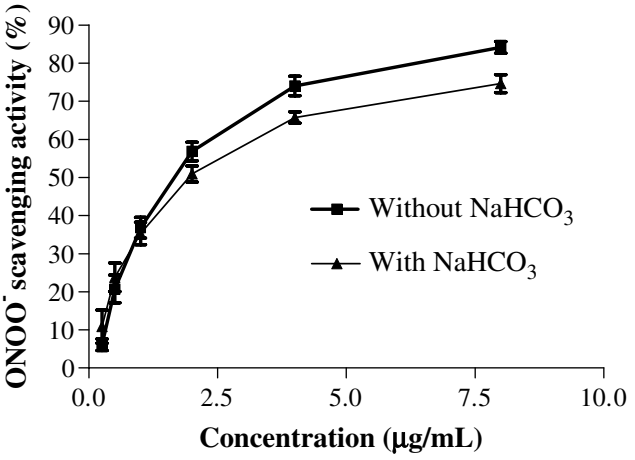

Fig. 5. $ONOO^-$ scavenging activity of the *Juglans regia* leaf extract in the presence and in the absence of $NaHCO_3$. Each point represents the values obtained from four experiments, which were performed in triplicate (Mean ± SE).

quercetin was the most potent flavonoid in scavenging $ONOO^-$ and the catechol group and the hydroxyl group at position 3 gave the highest contribution to this effect.

4. Conclusions

The studied *J. regia* (ethanol:water) extract was shown to be very effective against the evaluated pro-oxidant species. The scavenging effects of the studied reactive species have been described for some of the polyphenols that have been identified in *J. regia* leaves. These data imply that at least part of the observed antioxidant activity may be a result of the extract's phenolic compounds.

The results of the present study showed that the *J. regia* leaf can be used as an easily accessible source of natural antioxidants. The studied extract might be helpful in the prevention of lipid peroxidation and the protection of food, excipient bases and medicines from oxidative damage. Furthermore, the observed antioxidant activity can at least partially justify the therapeutic use of *J. regia* leaves in inflammatory diseases. Nevertheless, its potential toxicity should be addressed before any possible application on a practical scale.

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