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Antihypoxic and antioxidant activity of *Hibiscus esculentus* seeds

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RESUMEN

Actividad antihipóxica y actividad antioxidante de semilas de *Hibiscus esculentus*

La actividad antihipóxica y antioxidante de semillas de *Hibiscus esculentus* fue investigada empleando ocho ensayos in vitro. La actividad antihipóxica fue investigada en dos modelos, uno de caracter hemínico y otro circulatorio. Los efectos fueron pronunciados en ambos modelos de hipoxia. Los efectos antihipóxicos fueron dependientes de la dosis. Los resultados indican que los extractos tienen un efecto protector contra la letabilidad inducida por hipoxia en ratones. Los extractos mostraron actividad antioxidante en algunos modelos. El IC_{50} para la actividad captadora de radicales fue 234 $\pm$ 8.9 $\mu g\ ml^{-1}$. Los extractos muestran una débil actividad captadora de óxido nítrico comprendida entre 0.1 y 1.6 mg ml^{-1}. Los extractos muestran una débil capacidad quelatante de Fe^{2+}. El IC_{50} fue de 150 $\pm$ 13 $\mu g\ ml^{-1}$. Los extractos también muestran una baja actividad antioxidante en modelos con ácido linoleico aunque fueron capaces de eliminar peróxido de hidrógeno en una manera dependiente de la concentración. La concentración de compuestos fenólicos totales en cada extracto fue determinado como equivalents de ácido gálico y el contenido total de flavonoides fue calculado como equivalentes de quercitina para la curva de calibración. Los efectos farmacológicos pueden ser atribuidos, al menos en parte, a la presencia de fenoles y flavonoides en el extracto.

PALABRAS CLAVE: Actividad antihipóxica – Actividad antioxidante – DPPH – Flavonoides – Fenoles – Hibiscus esculentus.

SUMMARY

Antihypoxic and antioxidant activity of *Hibiscus esculentus* seeds

The antihypoxic and antioxidant activities of *Hibiscus esculentus* seeds were investigated employing eight in vitro assay systems. Antihypoxic activity was investigated in two models, haemic and circulatory. The effects were pronounced in both models of hypoxia. The antihypoxic effects were dose-dependent. The results indicated that the extracts have a protective effect against hypoxia induced lethality in mice. The extracts showed antioxidant activity in some models. IC_{50} for DPPH radical-scavenging activity was 234 $\pm$ 8.9 $\mu g\ ml^{-1}$. The extracts showed weak nitric oxide-scavenging activity between 0.1 and 1.6 mg ml^{-1}. The extracts showed weak Fe^{2+} chelating ability. IC_{50} were 150 $\pm$ 13 $\mu g\ ml^{-1}$. The extracts also exhibited low antioxidant activity in the linoleic acid model but were capable of scavenging hydrogen peroxide in a concentration dependent manner. The total amount of phenolic compounds in each extract was determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve. Pharmacological effects may be attributed, at least in part, to the presence of phenols and flavonoids in the extracts.

KEY-WORDS: Antihypoxic activity – Antioxidant activity – DPPH – Flavonoids – Hibiscus esculentus – *Phenols.*

1. INTRODUCTION

Free radicals cause the oxidation of biomolecules (e.g., protein, amino acids, lipid and DNA) which leads to cell injury and death (McCord 2000). Moreover, the oxidative stress caused from an imbalance between the generation and the neutralization of free radicals by antioxidant mechanisms is responsible for many human diseases including aging, cancer and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's diseases (Ebrahimzadeh *et al*. 2008a). Their deteriorative effects can be diminished by natural antioxidants available in foods. Also, oxidative reactions limit the shelf life of fresh and processed food stuffs and are a serious concern in the food industry (Sokmen *et al*. 2004). Synthetic antioxidants such as butylhydroxyanisole (BHA) or butylhydroxytoluene (BHT) are used to decelerate these processes. However, due to their unstable and highly volatile nature, they have frequently brought about some questions regarding their safety and efficiency ever since their first introduction into the food industry (Nabavi *et al*. 2008a). Consequently, the need to identify alternative natural and safe sources of food antioxidants arose and the search for natural antioxidants, especially of plant origin, has notably increased in recent years (Goli *et al*. 2005; López *et al*. 2007). *Hibiscus esculentus L*. (Okra), a tropical to subtropical plant that is widely distributed from Africa to Asia, Southern European and America (Oyelade *et al*. 2003) belongs to the family of Malvaceae. Its Persian name is known as "bamye". It is a common vegetable in most regions

of Greece, Turkey and Iran, especially in the northern region. It is available all year-round, with a peak season during the summer months (Purewal and Rhandhawa 1947). Okra is primarily a southern vegetable garden plant, grown for its immature pods, which are consumed when cooked either alone or in combination with other foods. The seeds of the mature okra are roasted and ground and used as a coffee substitute in Turkey (Calısır *et al*. 2004). The quality of the seed protein is high. The seeds of mature pods are sometimes used for chicken feed and have been used on a small scale for the production of oil (Oyelade *et al*. 2003). Studies have confirmed okra seeds as a good source of oil and protein (Karakoltsidis and Constantinides 1975). Okra seed oil is rich in unsaturated fatty acids such as linoleic acid (Savello *et al*. 1980) and its fruit is a rich source of α-Tocopherol (Ching and Mohamed 2001) which are essential for human nutrition. In addition, the Okra seed has high contents of minerals including Ca, Cu, Fe, Mg, Zn, P (Akpanabiatu *et al*. 1998). There are no reports on the antihypoxic activity of this plant. The aim of this study was to determine the antihypoxic and antioxidant activities in *H.esculentus* seed extracts in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

2. MATERIALS AND METHODS

2.1. Plant materials

The seeds of *H. esculentus* were collected from the dashte-naz area (Panbe-Choole village) north of Sari, Iran, in summer, 2007. After identification of the plant by Dr. Bahman Eslami, a voucher (No. 358) has been deposited in the Faculty of Pharmacy herbarium.

2.2. Freeze-dried extract

Seeds were dried at room temperature and coarsely ground before extraction. A known amount of seeds was extracted at room temperature using the percolation method with methanol. The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained, which was then freeze-dried for complete solvent removal.

2.3. Animals

Animals used in the experiments were male mice, mass 22–25 g, kept under standard conditions in an animal house (water and food *ad libitum*, 12 h dark and light cycle). The following groups of animals were used in the study: control groups and 3 experimental groups for each model, haemic and circulatory hypoxia. Each group comprised 7 animals. Controls were treated with a vehicle (5% DMSO in water) in the same volume as the treated animals (0.1 ml per 10 g). No effects of the vehicle were observed. All the experimental procedures were conducted in accordance with the NIH guidelines for the Care and Use of Laboratory animals.

2.4. Haemic hypoxia

Thirty minutes after i.p. administration of the extract, 250, 500 and 1000 mg kg^{-1}, $NaNO_2$ (360 mg kg^{-1}) was applied *i.p.* to each mouse and antihypoxic activity was estimated as the latent time of evidence of hypoxia in minutes according to the method of Roshtina and Ostrovskaya (Roshtina and Ostrovskaya 1981). Antihypoxic activity was expressed relative to the control.

2.5. Circulatory hypoxia

Thirty minutes after i.p. administration of the methanol extract, 250, 500 and 1000 mg kg^{-1}, NaF (150 mg kg^{-1}) were applied *i.p.* to each mouse and the antihypoxic activity was estimated in minutes as the latent time of evidence of hypoxia (Krasteva *et al*. 2004). Antihypoxic activity was expressed relative to the control.

2.6. Maximum non-fatal dose

Different doses of extract were injected into separate groups of seven. After 48 h, the highest dose that did not induce mortality was considered the maximum non-fatal dose (Hosseinzadeh and Sadati 2003).

2.7. Determination of total phenolic compounds and flavonoid content

Total phenolic compound contents were determined by the Folin-Ciocalteau method (Ebrahimzadeh et al. 2008a,b). The extract sample (0.5 ml) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 ml of 75 g l^{-1} sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoids were estimated using the method of Ordonez et al. (Ebrahimzadeh et al. 2008a,b). Briefly, a 0.5 mL solution of plant extract in methanol were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer). Total flavonoid contents were calculated as quercetin from a calibration curve.

2.8. DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for the determination of the free radical-scavenging activity of the extracts (Ebrahimzadeh and Bahramian 2009a; Nabavi

et al. 2008a). Different concentrations of extract were added, at an equal volume, to a methanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated three times. Vitamine C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of the sample, which is required to scavenge 50% of DPPH free radicals.

2.9. Reducing power determination

The reducing power of *H. esculentus* was determined according to the method of Yen and Chen (Ebrahimzadeh *et al*. 2008a,b; Nabavi *et al*. 2009a). 2.5 ml of extract (25-800 $\mu g ml^{-1}$) in water were mixed with a phosphate buffer (2.5 ml, 0.2M, pH6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as a positive control.

2.10. Assay of nitric oxide-scavenging activity

For the experiment, sodium nitroprusside (10 mM) in phosphate-buffered saline was mixed with different concentrations of each extract dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without extract but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as a positive control (Ebrahimzadeh *et al*. 2008a,b; Nabavi *et al*. 2008a).

2.11. Metal chelating activity

The chelating of ferrous ions by *H. esculentus* was estimated by the method of Dinis et al., (Ebrahimzadeh *et al*. 2008c). Briefly, the extract (0.2-3.2 mg/ml) was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine– Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of the extract/ standard. Na_2EDTA was used as a positive control.

2.12. Determination of Antioxidant Activity by the FTC Method

This method was adopted from Osawa and Namiki (Dehpour *et al.* 2009). Twenty mg/mL of samples dissolved in 4 mL of 95% (w/v) ethanol were mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL) and distilled water (3.9 mL) and kept in screwcap containers at 40°C in the dark. 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate were then added to 0.1 mL of this solution. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured and it was measured again every 24 h until the day when the absorbance of the control reached its maximum value. The percent inhibition of linoleic acid peroxidation was calculated as: (%) inhibition = 100 − [(absorbance increase of the sample/absorbance increase of the control) × 100]. All tests were run in duplicate and analyses of all samples were run in triplicate and averaged. Vit C and BHA were used as positive controls.

2.13. Scavenging of hydrogen peroxide

The ability of the extracts to scavenge hydrogen peroxide was determined according to the our recently Publisher paper (Ebrahimzadeh et al. 2009b). A solution of hydrogen peroxide (40 mM) was prepared in a phosphate buffer (pH 7.4). (0.1 − 1 mg ml^{-1}) extract in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows: % Scavenged $[H_2O_2] = [(A_o - A_1)/A_o] \times 100$ where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and standard (Ebrahimzadeh *et al*. 2009b).

2.14. Statistical analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p < 0.05$) and the means separated by Duncan's multiple range test. The EC_{50} values were calculated from linear regression analysis.

3. RESULTS AND DISCUSSION

There are literature data affirming that the administration of sodium fluoride (substance that induces circulatory hypoxia) increases the blood histamine content and decreases the oxygen carrying capacity (Sumina *et al*. 1978). A significant protective effect on other forms of hypoxia such as hypobaric hypoxia has been reported by *Ginkgo biloba* regarding an extract that contains flavonoids (Karcher *et al*. 1984). Our results may

be supported by other literature data which show that flavonoids increase cerebral blood flow and possess antihypoxic activity. The mechanism of this protective action may be due in part to the antioxidant activity of quercetin (Beck *et al*. 1986; Meli *et al*. 1990; Mora *et al*. 1990). The maximum non-fatal doses of methanolic extract of *H. esculentus* seeds was 2.5 g kg^{-1}. The methanol extract showed a very good protective effect against hypoxia. It produced significant and dose dependent effects on both Haemic and Circulatory hypoxia (Table 1).

The total phenolic content was 58.4 ± 2.6 mg gallic acid equivalent/g of extract powder by reference to the standard curve (y = 0.0054x + 0.0628, r^2 = 0.987). The total flavonoid content was 23.9 ± 1.2 mg quercetin equivalent/g of extract powder by reference to the standard curve (y = 0.0063x, r^2 = 0.999). This plant showed high total phenol and flavonoid contents. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources and they have been shown to possess significant antioxidant activities (van Acker *et al*. 1996). Studies have shown that increasing levels of flavonoids in the diet could decrease the occurence of certain human diseases (Hertog *et al*. 1993).

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (Lee *et al*. 2003). DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen –or electron– donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Dehpour *et al*. 2009). It was found that the radical- scavenging activity of extract increased with increasing concentration. IC_{50} for DPPH radical-scavenging activity was 234 ± 8.9 μg ml^{-1}. The IC_{50} values for Ascorbic acid, quercetin and BHA were 1.26 ± 0.11, 1.32 ± 0.07 and 13.49 ± 1.04 μg ml^{-1}, respectively. The phenol and flavonoid contents of this plant may lead to its good DPPH-scavenging activity.

Fe (III) reduction is often used as an indicator of electron- donating activity, which is an important mechanism in phenolic antioxidant action (Nabavi *et al*. 2009b). In this assay, the presence of reductants (antioxidants) in the samples would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability.

Fig. 1 shows the dose– response curves for the reducing powers of the *H. esculentus* seed extract. It was found that the reducing powers of extracts also increased with an increase in their concentrations. The extract exhibited a fairy good reducing power at 25 and 800 μg ml^{-1}. In lower doses, 25 and 50 μg ml^{-1}, the extract showed better activity than vitamin C, but in higher doses, it was not comparable with Vit C ($p < 0.05$). (e.g. absorbance was 1.32 ± 0.06 vs. 1.94 ± 0.1 in 800 μg ml^{-1}, for extract and control, respectively). It was evident that *H. esculentus* did not show reductive potential and could not serve as electron donors for terminating the radical chain reaction.

The extracts also showed weak nitric oxide-scavenging activity between 0.1 and 1.6 mg ml^{-1}. The procedure is based on the principle that, sodium nitroprusside in an aqueous solution at physiological pH spontaneously generates nitric oxide (NO) which interacts with oxygen to produce nitrite ions that can be estimated using the Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to a reduced production of nitrite ions. The % inhibition increased with increasing concentrations of the extract. IC_{50} was 970 ± 48 for *H. esculentus* vs. 17.01 ± 0.03 μg ml^{-1} for quercetin. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada *et al*. 1991). The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health.

Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in humans and thereby improves quality of life and overall survival of some diseases such as Thalassemia major (Hebbel *et al*. 1990). In addition, brain iron dysregulation and its association with amyloid precursor protein plaque formation are implicated in Alzheimer's disease (AD) pathology and so iron chelation could be considered

Table I
Antihypoxic activity of methanol extract of Hibiscus esculentus seeds on two models of brain hypoxia

Haemic hypoxia		Circulatory hypoxia	
Dose (mg kg^{-1})	**Activity (%)[a]**	**Dose (mg kg^{-1})**	**Activity (%)[a]**
Control	10.55 + 0.33	Control	9.38 + 0.03
250	12.5 + 0.41[ns]	250	11.63 + 0.43 ***
500	15.67 + 0.44***	500	15.43 + 0.44***
1000	22.18 + 0.33***	1000	18.89 + 0.31***

[a] Data are expressed as mean ± *SD* (n = 7). *** $p < 0.001$ compared to control. [ns] $p > 0.05$.

a rational therapeutic strategy for AD (Reznichenko *et al*. 2006). Foods are often contaminated with transition metal ions which may be introduced by processing methods. Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry (Halliwell 1997). These processes can be delayed by iron chelation and deactivation. The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (Ebrahimzadeh *et al*. 2008c; Nabavi *et al*. 2008b). Because Fe^{2+} also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe^{2+} concentration in Fenton reactions affords protection against oxidative damage. The chelating of ferrous ions by the extract was estimated by the method of Dinis *et al*., (Ebrahimzadeh *et al*. 2008 a,b,c; Nabavi *et al*. 2008a). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complex decreases. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The absorbance of Fe^{2+} -ferrozine complex decreased dose-dependently, i.e. the activity increased upon increasing concentrations from 0.2 to 3.2 mg ml^{-1}. *H. esculentus* seed extract showed good Fe^{2+} chelating ability. IC_{50} was 150 ± 13 μg ml^{-1}. EDTA showed very strong activity (IC_{50} = 18 μg ml^{-1}). Metal chelating capacity was significant since the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation (Duh *et al*. 1999). It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Gordon 1990). *H. esculentus* extract showed good Fe^{2+} chelating ability. IC_{50} was 150 ± 13 μg ml^{-1}.

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Especially, linoleic acid and arachidonic acid are targets of lipid peroxidation (Yu 2001). The

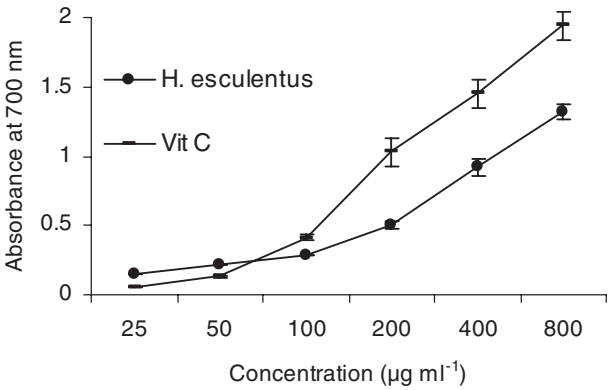

Figure 1
Reducing power of *H. esculentus* seeds methanol extract. Vitamin C was used as positive control.

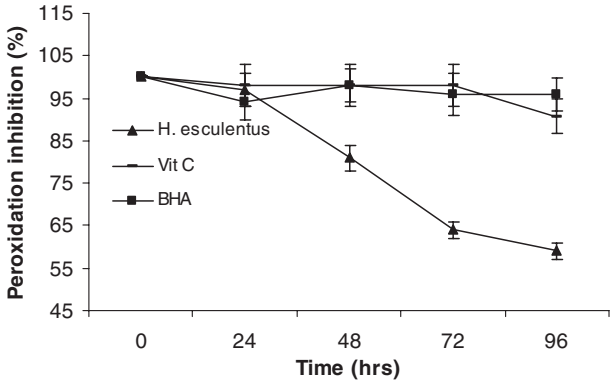

Figure 2
Antioxidant activity of *H. esculentus* in FTC method at different incubation times. Plant methanolic extract (0.4 mg/ml), Vit C and BHA (0.1 mg/ml).

inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (Nabavi *et al*. 2008a). Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. The tested extract did not exhibit any antioxidant activity (peroxidation inhibition). There were significant differences between the extract and controls ($p < 0.001$) (Figure 2).

Scavenging of H_2O_2 by *H. esculentus* extracts may be attributed to their phenolics, and other active components which can donate electrons to H_2O_2, thus neutralizing it to water (Halliwell and Gutteridge 1990). The *H. esculentus* extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. IC_{50} for H_2O_2 scavenging activity was 180.9 ± 11.3 μg ml^{-}. The IC_{50} values for Ascorbic acid and BHA were 21.4 and 52.0 μg ml^{-1}, respectively. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems.

4. CONCLUSIONS

Our studies indicate that the methanol extract of *H. esculentus* seeds has a low acute i.p. toxicity and a remarkable antihypoxic effect in both models of circulatory and haemic hypoxia. It is therefore very promissing for further pharmacological and biochemical experiments, which will be focused on evaluating the mechanism of antihypoxic activity. It also exhibited good but different levels of antioxidant activity in some of models studied. The extracts had good reducing power and nitric oxide scavenging activity. Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed. **The** antihypoxic and antioxidant activity of *H. esculentus* make it a suitable candidate for the prevention and/or treatment of strokes.

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