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ORIGINAL ARTICLE

Antihypoxic, nephroprotective and antioxidant properties of hydro-alcoholic extract of loquat flowers

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Summary. *Objectives:* Loquat is well known Chinese medicinal herb with numerous traditional usages. The fact of the promising effect of loquat on different diseases is approved by a plethora of recent studies. *Methods:* In the present study antihypoxic, antioxidant and nephroprotective activity of hydro-alcoholic extract of *Eriobotrya* japonica flower was an investigated employing different in vivo and in vitro assay system. The chemical analysis of *Eriobotrya* japonica flowers, using atomic absorption spectroscopy, highlighted the presence of eight elements. Gallic acid, rutin and quercetin were determined by HPLC/DAD. *Results:* Results indicated that the hydro-alcoholic extract has a protective effect against hypoxia induced lethality in mice. Extract at doses of 200 and 400 mg/kg/day for 10 consecutive days, i.p. gave nephroprotection by changing blood urea nitrogen (BUN), serum urea and creatinine levels. Extract showed good antioxidant and antihemolytic activity in all studied models. Present study demonstrates hydro-alcoholic extract of *Eriobotrya japonica* as a plant with high biological properties. *Conclusion:* In conclusion, loquat flowers are potential sources of antioxidant, anti-hypoxic and nephroprotective agents.

Key words: Free radical, *Eriobotrya japonica,* gentamicin, rutin

Introduction

Loquat, *Eriobotrya japonica* (Lindley), is a member of the Rosaceae family and its edible fruit is used. From ancient time, Japanese farmers cultivate loquat tree and so it has been become an important edible fruit and has considerable economic importance at a regional level in Iran. Its fruit is called "biwa", "Japanese medlar" or "Japanese plum". They also exist in northern India, the Mediterranean region, England, Madagascar and North, Central and South America (1). Loquat is often used in herbal remedies and traditional medicine for treatment of cough and asthma (1). In Iran it is called "azgil-e-zhaponi" and used as edible fruit in jellies and jams and has some uses in Iranian traditional medicine. Different parts of loquat tree have been used for their anti-inflammatory effect and in treatment of cough, tumors, liver problems, chronic bronchitis (1, 2), nephropathy, diabetes, as a tissue factor inhibitor and inhibitor of Nuclear Factor kappa B, p38 mitogen-activated protein kinase, and ERK (2). Oleanolic acid, ursolic acid and amygdalin has been reported from the flower of *Eriobotrya japonica* Lindl (3). Within this study, antioxidant, antihemolytic, antihypoxic and nephroprotective effects of *E. japonica* flowers hydro-alcoholic extract were assessed.

Methods

Chemicals

Ferrozine, Linoleic acid, Trichloroacetic acid, 1, 1-Diphenyl-2-picryl hydrazyl, Potassium ferricyanide, Sodium nitrite, Sodium fluoride, and Hydrogen perox-

ide were purchased from Sigma Chemicals Co. (USA). Gallic acid, Quercetin, Butylated hydroxyanisole, Vitamin C, Sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, EDTA and Ferric chloride were purchased from Merck (Germany) and Gentamicin was purchased from Daru-pakhsh Co (Iran). All other chemicals were of analytical grade or purer.

Sample preparation

Loquat flowers were collected from Panbeh Chuleh village at December 2009, near the Caspian Sea, in Mazandaran, Iran. Loquat flowers were transported to the laboratory and kept at < 4°C within 24 h prior to sample preparation.

Determination of metal content

Instrumentation and analytical procedures

Dried and ground samples were ash-dried overnight at 400-420°C in a Vitreosil crucible. Two grams of ash were dissolved in a 1:3 mixture of hydrochloric and nitric acids diluted to 50 mL with distilled water and used for analysis by means of an atomic absorption spectrometer Perkin Elmer AAS 100 (Wellesley, MA).

Preparation of extract

Plant powder was extracted by percolation method using ethanol/distilled water (70/30) for 24 h at room temperature (4). Extract was filtered and concentrated under reduced pressure at 40°C using a rotary evaporator.

Determination of total phenolic compounds and flavonoid content

Total phenolic compound contents were determined by the Folin-Ciocalteau method (5). 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 sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Result was expressed as gallic acid equivalents. Total flavonoids content was estimated as previously described (6). Briefly, 0.5 mL solution of extract in methanol was 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. Total flavonoid content was calculated as quercetin from a calibration curve.

Assay of putative biological active components

Gallic acid

A Knauer series liquid chromatography system comprising degasser, pump, auto- sampler, thermostatted column compartment, and diode array detector was used. The column used was a C_{18} reversed phase Kingsorb 5 mm (250×4.6 mm). Mobile phase eventually adopted for this study was methanol/ water/ orthophosphoric acid (20/ 79.9/ 0.1) and the flow rate was 1.0 mL/min. Absorption wavelength was selected at 210 nm. The column was operated at 30°C. The sample injection volume was 20 μL (7).

Quercetin and Rutin

Chromatographic analysis was carried out by the column used was a C_{18} reversed phase Kingsorb (250 × 4.6 mm) packed with 5μm diameter particles. The mobile phase was methanol/ acetonitrile/ water (40/15/ 45) containing 1% acetic acid. This mobile phase was filtered through a 0.45 μm membrane filter (Millipore), then deaerated ultrasonically prior to use. Quercetin was quantified by DAD following HPLC separation 368 nm for quercetin and 257 for rutin, respectively. Flow rate and injection volume were 1.0 mL/min and l0 μL, respectively (8).

Free radical scavenging activity

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the sample (9). Different concentrations of sample were added, at an equal volume, to ethanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. Vitamin C, BHA and quercetin were used as standard controls.

Metal chelating activity

Briefly, the sample (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 (10).

Assay of nitric oxide-scavenging activity

For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of sample 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 positive control (11, 12).

Scavenging of hydrogen peroxide

Briefly, a solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Sample (0.1-1 mg/ mL) in distilled water (1.4 mL) was added to a hydrogen peroxide solution (0.6 mL, 40 mM). The absorbance of sample at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide (13).

Antihemolytic activity of extract

Antihemolytic activity of extract against H_2O_2 induced hemolysis

Briefly, Erythrocytes from male rat blood were separated by centrifugation and washed with phosphate buffer (pH 7.4). Erythrocytes were then diluted with phosphate buffered saline to give 4% suspension. 1g of samples/mL of saline buffer was added to 2 mL of erythrocyte suspension and the volume was made up to 5 mL with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 mL of H_2O_2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H_2O_2 in the reaction mixture was adjusted to bring about 90% hemolysis of blood cells after 240 min. After incubation the reaction mixture was centrifuged at 1500 rpm for 10 min and the extent of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation (14).

Animal

The study was performed on male NMRI mice of approximately the same age-group and body weight (2-3 weeks; 20-25 g), housed in ventilated animal rooms at a temperature of 24 ± 2 ˚C with a 12 h light/dark cycle and 60 ± 5 % humidity. All experiments were performed according to the norms of the ethical committee (DM42/2004-A).

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 (15).

Anti hypoxic activity

Haemic hypoxia

Twenty four mice were divided into four groups each containing six mice. Control group was treated with 0.9% (w/v) saline solution. Thirty minutes after i.p. administration of extract 125, 250 and 500 mg/kg, $NaNO_2$ (360 mg/kg) was applied i.p. to each mouse and antihypoxic activity was estimated as the latent time of evidence of hypoxia in minutes (15).

Circulatory hypoxia

Twenty four mice were divided into four groups each containing six mice. Groups were treated with 0.9% (w/v) saline solution. Thirty minutes after i.p. administration of extract 125, 250 and 500 mg/kg, NaF (150 mg/kg) was applied i.p. to each mouse and the antihypoxic activity was estimated in minutes as the latent time of evidence of hypoxia (15).

Nephroprotective assay

Experimental protocol

Animals were randomly divided into four groups of 10 animals each. Group I was kept as normal control receiving isotonic saline (0.5 mL, i.p.) for 8 consecu-

Table 1. Amount of trace elements in the plants by AAS Analysis (μg/g)

Sample	Yield %	Cr	Fe	Cu	Zn	Ni	Mn	Pb	Cd
E. japonica	35	ND	2.02	2.24	13.4	4	5.18	ND	ND

Note: Values are averages of three independent measurements having a precision of ± 1%.

Table 2. Amount of phytochemicals the plants by HPLC-DAD (mg/g of extract)

Sample	Rutin	Quercetin	Gallic acid
E. japonica	*56.0 ± 2.18*	*1.40 ± 0.06*	*2.41 ± 0.10*

tive days, and animals of groups II, III and IV were administered gentamicin, manufactured by Daru-Pakhsh Co., Iran (100 mg/kg/day, i.p.) for 8 consecutive days, which is well known to produce significant nephrotoxicity in mice. Injections of gentamicin were made daily at 08:00 hours to minimize the circadian variation in nephrotoxicity (16). Animal of Group II and III received extract (200 and 400 mg/kg/day, i.p.) and group IV received isotonic saline (0.5 mL, i.p.) for 10 consecutive days. After the last application, animal were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg) given intraperitoneally. Blood samples were collected via retro-orbital puncture in plain plastic tubes, left to stand at 48 °C for 1 hour, and centrifuged (900 g for 15 min at 5°C) to separate serum.

Biochemical analysis

Blood urea nitrogen (BUN), Ceratinine (Cr) and serum urea concentration was assessed as markers of nephrotoxicity. BUN, Cr and serum urea were determined spectrophotometrically from serum samples using commercially available kits (Sigma).

Statistical analysis

The values are presented as Mean ± SD. Differences between group means were estimated using a one-way ANOVA followed by Duncan's multiple range test. Results were considered statistically significant when $p<0.05$.

Results and Discussion

Table 1 presents the elemental analysis in ash of *E. japonica* flower by AAS technique. The concentration of various elements analyzed in the present work decreases in the order: Zn>Mn> Ni> Cu> Fe. Cells need to trace elements and their deficiencies may cause different diseases (17). Tuzcu et al. (18) report nephroprotective activity of zinc picolinate on cisplatin-induced renal injury. Therapeutic effect of copper in anemia, kinky hair syndrome and anti-inflammatory activity has been proved (17). It is well known that manganese compounds have antioxidant role. The daily requirement for an adult man is 10-15, 12-15 and 2-3 mg/d for Fe, Zn and Cu, respectively (17).

The total phenolic content of *E. japonica* was 115.23 ± 5.41 mg gallic acid equivalent/g of extract powder. Also, total flavonoid content of *E. japonica* was 30.77 ± 1.44 mg quercetin equivalent/g of extract powder. The amount of rutin, quercetin and gallic acid determined by HPLC/DAD were showed in table 2. IC_{50} for DPPH radical-scavenging activity was 145.7± 6.11. The IC_{50} values for vitamin C, quercetin and BHA were 5.05 ± 0.1, 5.28 ± 0.2 and 53.96 ± 3.1 μg/mL, respectively. DPPH is well known stable nitrogen-centered free radical. Any substances with hydrogen or electron donating activity can change it color can be considered as radical scavengers (19). Phenol and flavonoid contents of this plant, especially high rutin content, seem to have a crucial role in its good DPPH-scavenging activity.

In metal chelating model, extract showed weak activity with IC_{50} = 822.8 ± 32 μg/mL. EDTA showed better activity (IC_{50} = 18 μg/mL). Chelation therapy reduces iron-related complications in man and can improve life quality and overall survival in some diseases such as thalassemia, cancer, HIV or Wilson's disease (20). Also iron chelators showed that have antimalarial activity through the mechanism of preventing iron from essential metabolic pathways of the intra-erythrocytic plasmodium (21). In other hand many studies have demonstrated that iron chelators show anticancer activity (22). A growing body of evidences show potent transition metals chelating ability of phytochemicals with scarce side effect (23-25). Ferrozine can quantitatively

form complexes with Fe^{2+} monitored by forming red color. Chelator agent prevent from ferrozine-iron complex formation. 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.

Excessive NO production has direct in numerous disease states including renal failure, burn, cancer and neurodegenerative disease and so, elimination of the excess NO could have beneficial effects (26). The *E. japonica* flower extract showed potent nitric oxide-scavenging activity (IC_{50} =76.5 ± 2.9 μg/mL vs. quercetin with IC_{50} = 20 ± 0.01 μg/mL). It is may correlate with high flavonoid compound especially rutin and quercetin. Nitric oxide scavenger compounds challenge with oxygen, and reduce nitrite ions formation and also can restrict nitric oxide mediated damages (26).

In another models, *E. japonica* extract showed potent H_2O_2 scavenging activity (IC_{50} was 320.7 ± 16.03 μg/mL). The IC_{50} values for vitamin C and BHA were 21.4 ± 1.1 and 52 ± 2.6 μg/mL, respectively. Although hydrogen peroxide is not very toxic, But it can give rise to hydroxyl radicals formation in the cell and causes cytotoxicity. Thus, elimination of excessive H_2O_2 is important throughout cells. *E. japonica* activity is originated from presenting flavonoid compounds in the extract specially rutin.

Tested extract showed good activity in hemoglobin-induced linoleic acid system (figure1). Cell membrane is rich in unsaturated fatty acids that are main target of free radical attacks (6, 27-29). Erythrocytes

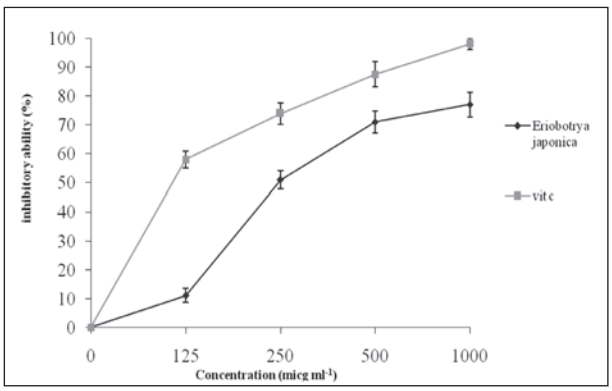

Figure 1. Antioxidant activities of *Eriobotrya japonica* against linoleic acid peroxidation induced by hemoglobin. Each value is expressed as mean 3 standard deviation positive control (Vitamin C).

membranes lipids have been known as prime targets lipid peroxidation (30, 31). Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (32, 33). Lipid peroxidation caused by hydroxyl radical (34). They act by hydrogen atoms robbing from the membrane lipids (35, 36). Potent lipid peroxidation inhibitory activity of extract correlates with high amount of rutin and quercetin.

E. japonica extract showed potent antihemolytic activity (IC_{50} was 258.2 ± versus. 235± 9.1 μg/mL for vitamin C). Good antihemolytic effect of hydro-alcoholic extract of loquat flowers maybe result of high rutin content.

The maximum non-fatal dose of extract was 4 g/kg. A statistically significant antihypoxic activity of the extract (doses 500 mg/kg) was established in the experimental model of haemic and circulatory hypoxia in mice ($P<0.001$ vs. control). The effect was found to be dose-dependent in a range of 125-500 mg/kg for haemic and circulatory hypoxia (Table 3). Previous studies demonstrated that intraperitoneal administration of sodium fluoride increases histamine in blood and decreases the oxygen carrying potential (15). Results of present study may be leaning on other literature data that phytochemicals such as flavonoids increase cerebral blood flow and show antihypoxic effect. The mechanism of antihypoxic effects can be due in part to the antioxidant effects of phytochemicals such as rutin (15).

The results show that administration of gentamicin at dose of 100 mg/kg/day for 8 consecutive days brought a significant increase in BUN, serum creatinine and urea and extract in 200 and 400 mg/kg/day has recovery effects (Table 4). Many studies show that hydroxyl radicals play an important role in production of gentamicin-induced nephrotoxicity. Fenton's reac-

Table 3. Anti-hypoxic activity of E. japonica in the different tests

Group	Doses (mg/kg)	sodium nitrite test (min)	sodium fluoride test (min)
Control		9.50 ± 0.30	9.36 ± 0.32
E. japonica	125	10.74 ± 0.604 *	10.64 ± 0.45**
E. japonica	250	13.00 ± 0.87 **	12.23 ± 0.16****
E. japonica	500	16.68± 1.50 ****	14.51 ± 0.56****

*Each group represents the Mean ± SD (n = 10). *P >0.05, ** P< 0.05, *** P< 0.01 and****P<0.001 vs. control*

Table 4. Effect of extract on serum creatinine, serum urea and blood urea nitrogen levels in gentamicin-induced renotoxic mice.

Groups	Serum creatinine μmol/l	Serum urea mg/dl	Blood urea nitrogen mg/dl
Gentamicin control (100 mg/kg, i.p.)	34.88 ± 4.27	119.56 ± 2.63	54.99 ± 1.22
Normal	24.05 ± 3.12	59.683 ± 2.30	27.45 ± 1.07
Extract-treated (200 mg/kg, i.p.)	35.45 ± 1.36 [a]	107.00 ± 4.25 [c]	50.00 ± 1.98 [c]
Extract-treated (400 mg/kg, i.p.)	28.94 ± 0.57 [b]	70.91 ± 3.48 [b]	33.13 ± 1.62 [b]

Values are Mean ± SD (n = 10). Data for normal animals are considered as base-line data; there was no significant base-line difference between the groups. [a] P<0.01 versus control group; [b] P<0.001 versus control group; [c] P>0.05 versus control group

tion induced oxidative injuries and aminoglycoside-iron complexes formation that have been suggested to be the major mechanisms in the progression of gentamicin-induced nephrotoxicity (16). Previously it has been reported that gentamicin in vitro model enhances the hydrogen peroxide production by renal cortical mitochondria and that iron ion chelators and scavengers of hydroxyl radicals ameliorate renal damage induced by gentamicin (16). Many studies show that oxidative and nitrosative stress play an important role in the ensuing renal injuries (37) especially in renal injuries induced by aminoglycoside antibiotics. On the other hand it has been reported that nitric oxide scavenger could be better than inducible nitric oxide synthase inhibitors as a curative intervention (37). In present study we show that the extract has good antioxidant activity especially nitric oxide scavenging activity. BUN and serum creatinine and urea levels were augmented indicating glomerular injury (38). However, the combined intraperitoneally administration of *E. japonica* with gentamicin to mice resulted in significant reduction in the elevated levels of BUN and serum creatinine and urea. These results could be in accord with several other researches, which reported that, polyphenolic compound (39), partially prevented the increase in BUN and serum creatinine and urea levels induced by gentamicin.

Conclusion

In present study, phytochemical constituents and pharmacological activities of flowers extract of loquat have been reported. Potent pharmacological activity of *Eriobotrya japonica may* correlate with high flavonoid content specially rutin in the extract. These results can be useful as a starting point of view for further applications of this plant or its constituents in pharmaceutical preparations after performing clinical researches.

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