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REZA SHARAFATI CHALESHTORI

FARHAD SHARAFATI CHALESHTORI

MAHMOUD RAFIEIAN

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## Biological characterization of Iranian walnut (*Juglans regia*) leaves

Reza SHARAFATI-CHALESHTORI<sup>1</sup>, Farhad SHARAFATI-CHALESHTORI<sup>2</sup>,

Mahmoud RAFIEIAN<sup>2</sup>

<sup>1</sup>Department of Food Hygiene, Research and Science Branch, Islamic Azad University, Tehran - IRAN

<sup>2</sup>Medical Plants Research Center, Shahrekord University of Medical Sciences, Shahrekord - IRAN

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**Abstract:** In recent years, emphasis has been placed on the use of natural materials in the control and treatment of various infections, as some chemically synthesized drugs have undesirable side effects. In this study, the ethanol extract of Iranian walnut leaves was examined for antibacterial activities on *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sanguinis*, and *Actinomyces viscosus* using the microdilution method. Total phenols, flavonoids, and flavonols were also determined colorimetrically. The minimum inhibitory concentrations (MIC) for ethanolic extract ranged between 15.6 and 187.5 mg/mL and minimum bactericidal concentrations (MBC) ranged between 31.25 and 250 mg/mL. Total phenols were  $410 \pm 14.43$  mg/g gallic acid equivalent, and total flavonols and flavonoids were  $270 \pm 22.33$  and  $330 \pm 12.21$  mg/g rutin equivalent, respectively. These findings show that Iranian walnut leaves have antibacterial effects on the 4 examined bacteria and may be a suitable alternative remedy for protection and treatment of dental plaque due to these microorganisms.

**Key words:** Antimicrobial effects, *Juglans regia*, dental plaque, phenols, flavonoids, flavonols

### Introduction

The genus *Juglans* (family Juglandaceae) comprises several species and is widely distributed throughout the world. Green walnuts, shells, kernels and seeds, bark, and leaves are used in the pharmaceutical and cosmetic industries (1,2). Leaves are easily available in abundant amounts. Walnut leaves are considered to be a source of healthcare compounds and have been intensively used in traditional medicine for the treatment of venous insufficiency, hemorrhoids, hypoglycemia, diarrhea, and fungal or microbial infections (3).

Natural antioxidants, such as phenolic compounds, are gaining importance due to their

benefits for improving human health, decreasing the risk of degenerative diseases by reduction of oxidative stress, and inhibiting macromolecular oxidation (4). Some studies have demonstrated the antimicrobial activities of walnut products; however, information about *Juglans* leaves is almost nonexistent (5). In addition to antioxidant activity, several studies have demonstrated the antimicrobial activity of phenols and/or phenolic extracts (6,7), making them a good alternative to antibiotics and chemical preservatives.

Dental caries is a public oral health problem and an infectious-contagious disease implying an imbalance of normal molecular interactions between the tooth surface/subsurface and the adjacent microbial biofilm (8). Dental caries still remains a dental health

problem. A prevalence of 48% to 95% was reported in children 8-13 years old while 53.4% was reported in a group of 15-year-old adolescents (9). The side effects of synthetic antimicrobial agents are high and the tolerance to these agents is increasing. Therefore, a search for alternative drugs is essential. In recent decades, an increasing tendency toward the use of natural substances instead of synthetic ones has been observed.

The aim of the present study was to determine the phenolic compounds and to evaluate the antimicrobial activities of Iranian walnut leaves against 4 dental plaque microorganisms.

## Materials and methods

### Plant material and extraction

Walnut leaves were collected in the spring of 2009 in Chaharmahal and Bakhtiari Province, Iran, from the branches exposed to sunlight. Voucher specimens of the collected leaves were confirmed and deposited at the herbarium of the Medical Plants Research Center of Shahrekord University of Medical Sciences, Iran (No. 203).

The walnut leaves were cut into small pieces and dried in the shade (22 °C) for 48-72 h. Dried leaves were ground into a fine powder using a homogenizer. A hydroalcoholic extract of the leaves was prepared by the maceration method, using a ratio of 1 g of walnut leaves to 5 mL of EtOH (80%) for 48 h. Flasks were shaken several times. The extract was filtered and the solvent was evaporated using a rotary evaporator. The extract was then dried at 40 °C and powdered. A stock concentration of 200 mg/mL of dry extract in DMSO was prepared, sterile-filtered (0.22 µm), and stored in the dark at 4 °C.

### Total phenol determination

The amount of total phenolic compounds in the walnut leaf extract was determined colorimetrically with Folin-Ciocalteu reagent using the method described by McDonald et al. (10) with some modifications. Mixed with the Folin-Ciocalteu reagent (1:10 dilution with distilled water) and aqueous Na<sub>2</sub>CO<sub>3</sub> (0.4 ml, 7.5%) was 0.5 mL of the extract or gallic acid (standard phenolic compound). The mixtures were allowed to stand for 30 min and the

total phenols were determined by spectrophotometer (Unico UV-2100, USA) at 765 nm. A standard curve was prepared using 12.5, 25, 50, 62.5, 100, and 125 mg/L solutions of gallic acid in methanol and water (60:40, v/v). Total phenol values were expressed in terms of gallic acid equivalent (mg/g), which is a common reference compound. The experiment was repeated 3 times.

### Total flavonoid and flavonol determination

The amount of total flavonoids in the walnut leaf extract was determined using the colorimetric method as described by Chang et al. (11) with some modifications. Mixed with 1.5 mL of methanol (60%), 1 mL of 2% aluminum chloride, and 6 mL of 5% potassium acetate was 1 mL of the extract or rutin (standard flavonoid compound), and the mixtures remained at room temperature for 40 min. The absorbance of the reaction mixture was then measured at 415 nm with a double beam spectrophotometer (Unico UV-2100, USA). The calibration curve was prepared using rutin solutions at concentrations of 25, 50, 100, 250, and 500 ppm in methanol.

Similarly, the aluminum chloride colorimetric method was employed for flavonol determination, but the incubation period was 150 min and the absorbance of the reaction mixture was determined at 440 nm (12). The experiments were repeated 3 times. Total flavonoids and flavonols were expressed in terms of rutin equivalent (mg/g), which is a common reference compound.

### Antimicrobial activity

Microorganisms of the Persian Type Culture Collection (PTCC) were obtained from the Iranian Research Organization for Science and Technology (IROST), Iran. *Streptococcus mutans* (PTCC: 1683), *Streptococcus salivarius* (PTCC: 1448), and *Streptococcus sanguinis* (PTCC: 1449) were cultured aerobically, and *Actinomyces viscosus* (PTCC: 1202) anaerobically, at 37 °C in blood agar. A 0.5 McFarland standard was used to create inoculum densities of  $1.5 \times 10^8$  cfu/mL in PBS, using the direct suspension method (13) for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Amikacin (30 µg, Merck, Darmstadt, Germany) was used as a positive antimicrobial reference.

The MIC was determined by the microtiter broth method (14) in sterile, flat-bottom 96-well polystyrene plates. Serial dilution techniques were used to determine the MIC of the extract at concentrations of 7.8 to 250 mg/mL after 18 h of incubation (5  $\mu$ L bacterial suspension + 95  $\mu$ L Mueller Hinton broth + 100  $\mu$ L serial 2-fold dilution extract). The negative control group was "Mueller Hinton broth + bacterial suspension" and the positive control group was "bacterial suspension + Mueller Hinton broth + antibiotic amikacin." Serial dilutions (1.25-2000  $\mu$ g/mL) of chlorhexidine mouth rinse (Iran Najo, Tehran, Iran) were also prepared as described above. All tests were performed in triplicate. Optical density readings were taken using an ELISA reader (State Fax 2100, Awareness Technology, USA) at 450 nm, 0 and 18 h after inoculation. Results were reported as MIC for growth at 18 h after inoculation. The lowest concentration that did not permit any visible growth when compared with the control was considered as the minimum inhibitory concentration. The contents of all of the tubes that showed no visible growth were cultured on Mueller Hinton agar and incubated at 37 °C for 24 h. The minimum bactericidal concentration was considered as the lowest concentration that could not produce a single bacterial colony. All of the tests were repeated 3 times.

## Results and discussion

### Total phenols, flavonoids, and flavonols

The total phenolic content of Iranian walnut (*Juglans regia*) leaf extract was  $410 \pm 14.43$  mg/g gallic acid equivalent. Total flavonoids and flavonols of the extract were  $330 \pm 12.21$  and  $270 \pm 22.33$  mg/g rutin equivalent, respectively.

Phenols, flavonoids, and flavonols are becoming the subject of medical research. They have been reported to possess many useful properties, including antiinflammatory activity, estrogenic activity, enzyme inhibition, antimicrobial activity, antiallergic activity, antioxidant activity, vascular activity, and cytotoxic antitumor activity (15).

The phenols and flavonoids of walnut leaves are likely responsible for the antibacterial activities of the extract derived from this plant (2). The use of walnut leaves for mild skin inflammation has

been approved by the German Commission E (14). Recently, Pereira et al. (1) reported the identification and quantification of 10 phenolic compounds in walnut leaves, namely, 3- and 5-caffeoylquinic acids, 3- and 4-*p*-coumaroylquinic acids, *p*-coumaric acid, quercetin 3-galactoside, quercetin 3-pentoside derivative, quercetin 3-arabinoside, quercetin 3-xyloside, and quercetin 3-rhamnoside.

Delineation of the possible mechanism of action of flavones and flavonoids is hampered by conflicting findings. Flavonoids lacking hydroxyl groups on their  $\beta$ -rings are more active against microorganisms than are those with the -OH groups (16). This finding supports the idea that their microbial target is the membrane. Lipophilic compounds would be more disruptive of this structure. However, several authors have also found the opposite effect: the more hydroxylation, the greater the antimicrobial activity (17). It has been recognized that phenolic compounds are a class of antioxidant agents, which act as free radical terminators. Free radicals are involved in many disorders, such as neurodegenerative diseases, cancer, and AIDS. Antioxidants, through their scavenging power, are useful for the management of those diseases. The mechanisms of action of flavonoids are through scavenging or chelating processes (18,19). The antioxidant capacity of walnut polyphenols has already been described. Anderson et al. (20) reported the in vitro inhibition of human plasma and low density lipoprotein (LDL) oxidation by a walnut extract containing ellagic acid, gallic acid and flavonoids. Flavonoids can also protect cells by acting as free radical scavengers, inhibiting DNA damage and mutagenicity (21).

### Antibacterial activity

The ethanolic extract of the walnut leaves was screened for its antimicrobial properties against dental plaque microorganisms. The MIC and MBC values for the tested bacteria (Table) were determined as an evaluation of the antimicrobial activity of the extract and the chlorhexidine mouth rinse (0.2%).

Recently, Darmani et al. (22) reported the growth inhibition of various cariogenic bacteria (*Streptococcus mutans*, *Streptococcus salivarius*, *Lactobacillus casei*, and *Actinomyces viscosus*) by walnut aqueous extracts. The most sensitive was *A. viscosus*, followed by *S. mutans* and *S. salivarius*, with

Table. Antimicrobial activity of Iranian walnut leaf extract and chlorhexidine mouth rinse on dental plaque microorganisms.

Bacteria	Leaf extract		Chlorhexidine mouth rinse	
	MIC (mg/mL)	MBC (mg/mL)	MIC ( $\mu\text{g}/\text{mL}$ )	MBC ( $\mu\text{g}/\text{mL}$ )
<i>Streptococcus mutans</i>	125	250	62.5	125
<i>Streptococcus salivarius</i>	15.6	31.25	62.5	125
<i>Streptococcus sanguinis</i>	15.6	31.25	15.6	31.25
<i>Actinomyces viscosus</i>	187.5	250	15.6	31.25

*L. casei* being the most resistant. In the present study, using ethanolic extract, the most sensitive organisms were *S. salivarius* and *S. sanguinis*. Furthermore, the MIC and MBC values of the walnut leaves were more than those of the chlorhexidine mouth rinse (0.2%). Natural products may be a rich source of anti-infective agents. For example, flavonoids show antimicrobial activity, and quercetin and other related compounds act essentially by enzyme inhibition of DNA gyrase (1). Some researchers have reported a synergy between naturally occurring flavonoids and other antibacterial agents against resistant strains of bacteria. Examples of these include epicatechin gallate and sophoraflavanone G. At least one group has demonstrated a synergy between flavonoids and antibacterial activity. Others have synthetically modified natural flavones and analyzed them for antibacterial activity. For example, Wang et al. complexed 5-hydroxy-7, 4-dimethoxyflavone with a number of transition metals and showed that this process could increase antibacterial activity (23).

The results obtained in this study show that walnut leaves could be used as an easily accessible source of natural bioactive compounds to inhibit the growth

of different gram-positive bacteria responsible for dental plaques and oral hygiene problems. Walnut leaves may also constitute a good source of healthy compounds, namely phenols, suggesting that they can be useful in the prevention of diseases in which free radicals are implicated. Further studies should be developed to identify the molecules responsible for this bioactivity.

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### Corresponding author:

Mahmoud RAFIEIAN

Medical Plants Research Center,

Shahrekord University of Medical Sciences,

Shahrekord - IRAN

E-mail: rafieian@yahoo.com

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