

Antimicrobial Activity of Walnut Leaf Extract against Gram-Negative Bacteria

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Abstract

Nowadays, hospital-acquired infections caused by multidrug resistant bacterial strains are of great concern. Thus, the development of alternative natural antimicrobial compounds is more necessary than ever. Walnut (*Juglans regia* L.) leaves are an easily available source of natural compounds with antioxidant and antimicrobial properties. Due to these particular features, we evaluated the antimicrobial effect of the walnut leaf methanolic extract at three different concentrations (30, 50 and 100 mg/L) against the two clinical strains *Pseudomonas aeruginosa* and *Escherichia coli* using the disc diffusion method. The results showed that both clinical strains were sensitive to all the extract concentrations tested. However, the diameter of the inhibition zone corresponding to 100 mg/L of extract concentration for *P. aeruginosa* was smaller than that for *E. coli*. These results demonstrate the potential of walnut leaves as an economical alternative source of antimicrobial agents.

1. Introduction

In the previous century, the discovery of antibiotics as miracle drugs against various microbial infections was very promising (Abdallah 1930; Ngwoke et al. 2011; Cheng et al. 2014; Roose-Amsaleg et al. 2016; Gupta et al. 2017; Ruiz et al. 2017). But unfortunately, their unreasonable and irrational use soon gave rise to the development of highly resistant clinical strains of *Staphylococcus aureus*, *Staphylococcus pyogenes*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Helicobacter pylori*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Tuberculosis*, *Escherichia coli*, *Klebsiella pneumoniae*, etc. to the most commercially known antibiotics (Abdallah 1930; Doern et al. 1986; Appelbaum 1992; Olayinka et al. 2004; Ormerod 2005; Manges et al. 2006; Ndip et al. 2008; Boyanova 2009; Ngwoke et al. 2011; Webster et al. 2011; Gavarić et al. 2015; Roose-Amsaleg et al. 2016; Gupta et al. 2017; Ruiz et al. 2017). In general, bacteria can genetically transmit and acquire resistance to antibiotics. This fact is very concerning because many patients in hospitals who have suppressed immunity encounter the new bacterial strains which are multidrug resistant. Consequently, new and high mortal infections can occur in hospitals (Nascimento et al. 2000). So, the development of new antimicrobial drugs is an urgent need. However, despite progress in producing new synthetic antibiotics from several decades back, no new classes of antibiotics were discovered and all commercially produced ones were modifications of existing molecules. Therefore, there is no obvious hope to obtain new antibiotic compounds in the near future (Abdallah 1930). Regarding these problems as well as side effects of chemical antibiotics on the human and environment and their high cost in developing countries, the use of alternative antibiotic compounds seems so necessary (Abdallah 1930; Zakavi et al. 2013; Roose-Amsaleg et al. 2016; Delaviz et al. 2017; Cheraghali et al. 2018).

In recent years, the interest in using natural antimicrobial compounds, especially extracted from plants, has considerably increased so as to produce more effective antibiotics with less side effects and reduced cost (Fernández-Agulló et al. 2013; Tawiah et al. 2016; Delaviz et al. 2017; Cheraghali et al. 2018). Several studies have shown that medicinal plants are rich in natural compounds such as alkaloids, glycosides, saponins, terpenoids (mono- and sesquiterpenes, steroids, etc.) and phenolics (tannins) with strong

antimicrobial activity (Abdallah 1930; Cheng et al. 2014; Devkota et al. 2015; Delaviz et al. 2017). These natural antimicrobial agents are secondary metabolites that are normally accumulated in the cells of the different parts of the plants (Abdallah 1930; Cheng et al. 2014; Tawiah et al. 2016). Leaves have been found to contain the highest antimicrobial activity and are generally preferred for therapeutic purposes (Tawiah et al. 2016).

Among medicinal plants, walnut (*Juglans regia* L.) is remarkable due to the healing properties of its different parts. The genus *Juglans* (family Juglandaceae) comprises several species and is widely distributed all over the world (Zakavi et al. 2013). The Persian or common walnut (*J. regia* L.) is the best-known member and, in addition to some provinces of Iran such as Fars, Hamedan, Kohgiluyeh, Boyer-Ahmad and Lorestan, it also grows in many areas of the world (Pereira et al. 2007). Several by-products derived from the walnut tree including green walnuts, shells, kernels, seeds, barks and leaves are used in the food, pharmaceutical and cosmetic industry (Pereira et al. 2007; Zakavi et al. 2013). Walnut leaves are widely used in traditional medicine for the treatment of venous insufficiency and hemorrhoids and for their antidiarrheic, anti-parasitic, depurative and astringent properties (Van Hellemont 1986; Bruneton 1993; Wichtl et al. 1999) as well as reducing blood glucose and improving diabetes (Mohammadi et al. 2011). Moreover, keratolytic, antifungal, hypoglycemic, hypotensive, anti-scorfulous and sedative activities have been reported (Valnet 1992; Girzu et al. 1998). Dry walnut leaves are also frequently used as infusions in some European countries, especially in rural areas (Fernández-Agulló et al. 2013; Delaviz et al. 2017). The antimicrobial activity of the walnut tree is related to its chemical composition (Zakavi et al. 2013). In walnut leaves, naphthoquinones and flavonoids are considered the major phenolic compounds. Juglone (5-hydroxy-1,4-naphthoquinone) is the characteristic compound of *Juglans* spp. (Pereira et al. 2007). This phenolic compound is present in all parts of the walnut including fresh and dry leaves (at vestigial amounts for dry leaves) and is known for its antimicrobial effect (Pereira et al. 2007; Fernández-Agulló et al. 2013). Other reports revealed that leaves from walnut contained monoterpenes and sesquiterpenes which are antibacterial secondary metabolites (Zakavi et al. 2013). Moreover, some research groups have reported the antimicrobial effect of walnut leaf methanolic extract against some gram-positive and negative bacteria and antifungal activity (Pereira et al. 2007; Delaviz et al. 2017).

Unfortunately, many clinical isolates of *P. aeruginosa* and *E. coli* are highly resistant to most chemical antibiotics and their outbreak in hospitals is very dangerous for hospitalized patients, especially for those who have suppressed immune system. For this reason, we decided to provide a new alternative organic compound against the nosocomial infections caused by them. Since walnut (*Juglans regia*) is native to Iran and is planted in most provinces of the country, its leaves are easily available in abundant amounts. Therefore, proving antibacterial activity for the leaves of Iranian walnut cultivars can suggest a new, economical and effective source of antibiotic compounds. In this study we evaluated the antimicrobial activities of the methanolic extracts of Persian walnut leaves (Tuyserkan cultivar) against the two clinical strains *P. aeruginosa* and *E. coli*.

2. Materials And Methods

2.1. Materials

Juglans regia L. leaves were obtained from Tuyserkan cultivar of walnut tree and collected in the summer of 2020 in central areas of Iran (western part of the central province). The two clinical strains of *Pseudomonas aeruginosa* and *Escherichia coli* were obtained from the Shohadaye Yaft Abad Hospital in Iran. These strains were isolated from burn injuries and urinary tract infections (UTI) of patients who had been hospitalized for more than 48 h. The bacterial stocks were maintained at 4°C on Luria Bertani (LB) agar (tryptone 1% (w/v), yeast extract 0.5% (w/v), NaCl 1% (w/v) and agar 2% (w/v)). All chemicals for the medium preparation, gentamicin, dimethyl sulfoxide (DMSO) and methanol were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of walnut leaf extract

The collected walnut leaves were washed, dried at 25° C (in dark room) and powdered. Then, 50 g of the prepared powder was added into 250 mL of 80% methanol and extracted under stirring at 200 rpm for 48 h at room temperature and filtered through Whatman paper no. 4. The obtained extract was freeze dried and stored in a refrigerator at 4°C. For the experiments, the freeze-dried extract was redissolved in DMSO to the concentrations of 30, 50 and 100 mg/L and sterilized by a 0.45-µm syringe filter.

2.3. Antimicrobial activity

The activity of the extracts was evaluated using the Kirby-Bauer method. For this, each bacterium was first sub-cultured in LB broth medium at 37°C for 18 h. From these cultures, bacterial suspensions containing 1.5×10^8 colony-forming units of bacteria were prepared based on a 0.5 McFarland standard. Then, a sterile cotton swab was dipped into each standardized bacterial suspension and streaked on the surface of solid LB medium in Petri plates to ensure an even distribution of the inoculum. The plates were allowed to dry for 3–5 min. Afterwards, sterile filter paper disks 5 mm in diameter were placed on the plate at regular distances and pressed firmly onto the agar with a sterile forceps to ensure a complete contact with it. A volume of 20 µL of the different concentrations of walnut leaf extract was added to the filter discs on the plates. Under the same conditions, two filter discs were impregnated with 20 µL of DMSO as a negative control and gentamicin (20 mg/mL) as a positive control. The plates were then incubated at 37°C for 18 h. The zone of inhibition corresponding to each sample was measured using a ruler, with an accuracy of ± 0.5 mm and recorded to the nearest size in mm. The results were expressed in terms of the diameter of the inhibition zone. The lowest concentration of walnut leaf extract showing a clear zone of inhibition was considered as the minimal inhibitory concentration (MIC). These experiments were done in five different plates for each strain of *P. aeruginosa* and *E. coli* to minimize the error.

2.4. Statistical Analysis

The one-way analysis of variance (ANOVA) was used to determine the significance ($p \leq 0.05$) of the data obtained in all tests.

3. Results

The methanolic extract of walnut leaves was screened for its antimicrobial properties against *P. aeruginosa* and *E. coli*. The diameters of the inhibition zones for different concentrations of the extracts (30, 50 and 100 mg/L), negative control (DMSO), and positive control (gentamicin 20 mg/mL) are presented in Table 1. According to the results, the different concentrations of the extracts had significant antimicrobial effects against both strains ($p < 0.0001$). However, the antimicrobial activities for the concentrations of 30 and 50 mg/L were very similar and for the highest concentration (100 mg/mL) it increased significantly against the two strains. Also, the highest inhibition zones were achieved against *E. coli* (Fig. 1, Table 1). The average zone of inhibition of the positive control (gentamicin) was much higher than that of the highest concentration (100 mg/mL) of the extract tested. This can be due to rawness of our methanolic extract of walnut leaves. The crude plant extracts may contain other constituents that do not possess antibacterial properties. Besides, the crude methanolic extract of walnut leaves was not able to diffuse through the agar as well as gentamicin because it contains larger molecules than gentamicin, a standard processed and purified antibiotic, and diffusion rate is inversely proportional to molecule size. All data were presented as the mean values of five measurements. In Fig. 2 the antimicrobial activity of the samples against the two clinical strains *P. aeruginosa* and *E. coli* by the disc diffusion method is illustrated.

Table 1
Average diameter of the inhibition zone of different concentrations of the methanolic extract of walnut leaves on the tested bacteria (mm).

Microorganism	Average diameter of the inhibition zone (mm)				
	[Extract] (mg/L)				
	30	50	100	Positive control	Negative control
<i>E. coli</i>	9.2 ± 0.9	9.8 ± 1.0	12.1 ± 2.1	0.0 ± 0.0	29.0 ± 1.0
<i>P. aeruginosa</i>	8.6 ± 1.1	8.8 ± 1.0	10.4 ± 0.7	0.0 ± 0.0	24.8 ± 0.9

4. Discussion

Hospital-acquired infections (HAI) are one of the serious problems of hospitals and medical centers and the most important causes of patients' dissatisfaction during the course of a treatment. HAI or nosocomial infections are defined as cases in which the patient had not been affected at the time of hospitalization or at its incubation period, and acquires it in hospital at least 48 h after admission (Rajakaruna et al. 2017; Marion-Sanchez et al. 2019). These infections, in addition to patients, affect all hospital staff and patients' companions and cause a longer duration of the patient hospitalization and the increase of diagnostic and therapeutic costs (Stone et al. 2005; Rajakaruna et al. 2017). The most common reported nosocomial infections are urinary and respiratory tract infections and cutaneous

infections, including surgical and burn wounds. Usually, the clinical strains *P. aeruginosa* and *E. coli* cause such infections (Bennett 1974; Mayon-White et al. 1988; Wurtz et al. 1995; Baviskar et al. 2019).

P. aeruginosa acts as an opportunistic pathogen in people with immunodeficiency and burn injuries and is resistant to many chemical antibiotics (Al-Wrafy et al. 2017; Azam and Khan 2019). Some strains of *E. coli* can also produce gastrointestinal, urinary tract infections and even cause meningitis. This bacterium is the most important cause of urinary tract infections, especially in women (Bélanger et al. 2011; Smith and Fratamico 2017; Mellata et al. 2018). Many strains of this bacterium are resistant to penicillin and cephalosporins by producing beta-lactamase enzymes (Nikolić et al. 2018). Nowadays, one of the most important medical approaches is reducing the duration of the treatment. Since the infectious diseases that are caused by highly drug resistant *P. aeruginosa* and *E. coli* strains usually increase the duration of the treatment; in this research, we tried to provide a new alternative compound, derived from a medicinal plant, against the nosocomial infections caused by them.

According to the results of many studies, walnut leaves due to their content in compounds such as juglone and phenolics can be used as an easily available source of natural compounds to inhibit the growth of some bacterial strains. Pereira et al. (2007) reported that walnut leaf aqueous extracts inhibited only the growth of gram-positive bacteria such as *Bacillus cereus*, *S. aureus* and *Bacillus subtilis* but gram-negative bacteria including *E. coli*, *P. aeruginosa* and *K. pneumoniae* were resistant to it (Pereira et al. 2007). Fadi Qa'dan et al. (2005) revealed the antimicrobial activity of walnut leaf extracts against *Propionibacterium acnes*, *S. aureus* and *Staphylococcus epidermidis* (Qa'dan et al. 2005). In 2010, Coban and Bivik studied the antimicrobial activity of ethanol extracts of walnut leaves against *E. coli*, *S. aureus*, *S. epidermidis*, *B. cereus*, *Micrococcus luteus*, *Salmonella typhimurium*, *S. pneumoniae*, *Enterococcus faecalis*, *Bacillus thuringiensis*, *Serratia marcescens*, *Pseudomonas extorquens*, *Proteus* sp., *Saccharomyces cerevisiae*, *Candida albicans*, *Candida glabrata*, *Candida utilis* and *Candida tropicalis*. Their results showed that the ethanol extracts of walnut leaves inhibited the growth of nine gram-positive bacteria and all the examined yeasts but did not show any antimicrobial effect against the three gram-negative bacteria tested (i.e., *E. coli*, *S. pneumoniae* and *S. marcescens*) (Biyik 2010). In addition, Sharafati et al. (2011) showed that walnut leaves could be used to inhibit the growth of different gram-positive bacteria responsible for dental plaques and oral hygiene problems (Zakavi et al. 2013).

Despite the aforementioned studies, in 2014, Enitan et al. reported the antibacterial activity of the methanolic leaf extract of *Plukenetia conophora* Mull. arg. (African walnut) against gram-negative *P. aeruginosa* and *E. coli* isolated from urinary tract infections. Using the well diffusion method with 100 µL of extract, they found the highest zone of inhibition for an extract concentration of 200 mg/mL (18 mm for *P. aeruginosa* and 12 mm for *E. coli*). For extract concentrations of 150, 100, 50 and 25 mg/mL *E. coli* did not show any zone of inhibition, while *P. aeruginosa* did (14, 12, 10 and 8 mm, respectively). The results obtained for *P. aeruginosa* were similar to that of the present study, while those of *E. coli* were worst, despite they used 100 µL and we only 20 µL (Table 2) (Enitan et al. 2014). Also, in 2018, Ioana Nicu et al. observed an inhibition zone of 11 mm against *P. aeruginosa* and of less than 8 mm against *E. coli* for 200 µL of concentrated extract of walnut leaves. Thus, despite using higher extract concentrations,

their results were worse than that of the present study (Table 2) (Nicu et al. 2018). Moreover, Mirza and et al (2019) showed that gram-negative bacteria were more resistant than the gram positive ones to zinc oxide nanoparticles derived from *Juglen regia* (walnut) leaves (Mirza et al. 2019).

Table 2
Comparison of agar diffusion test results on *P. aeruginosa* and *E. coli* in different studies.

[Extract] (mg/L)	Test sample (μ L)	Inhibition zone ($\emptyset$ mm)		Reference
		<i>P. aeruginosa</i>	<i>E. coli</i>	
5 total phenols (mg GAE/mL)	200	11	8	Ioana Nicu et al. 2018
25	100	8	0	Enitan and et al. 2014
30	20	8.6	9.2	Present study
50	100	10	0	Enitan et al. 2014
	20	8.8	9.8	Present study
100	100	12	0	Enitan and et al. 2014
	20	10.4	12.1	Present study
150	100	14	0	Enitan and et al. 2014
200	100	18	12	Enitan and et al. 2014

The antimicrobial activity obtained in present study for the walnut leave extract (Tuyserkan cultivar) suggests its use as an alternative source of treatment against microbial infections caused by *P. aeruginosa* and *E. coli*. In conclusion, it is very likely that the synergistic action of this extract and conventional antibiotics will be more effective against the multidrug resistant strains of *P. aeruginosa* and *E. coli*.

Statements & Declarations

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Competing Interests

“The authors have no relevant financial or non-financial interests to disclose.”

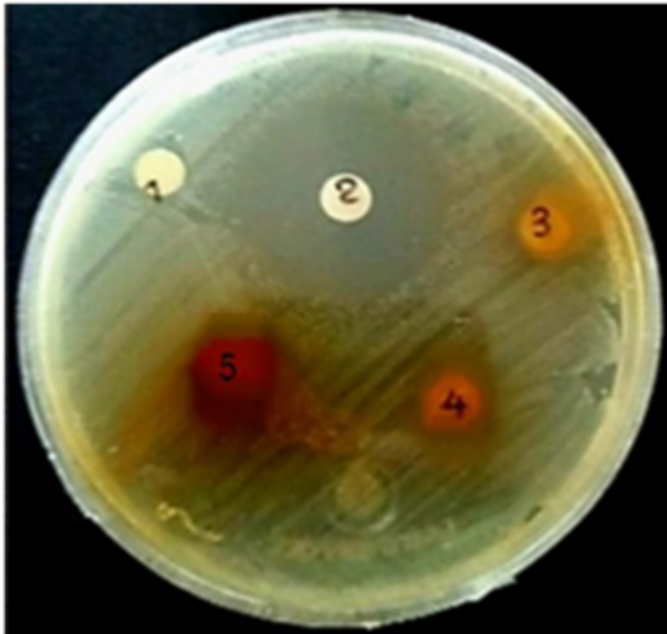
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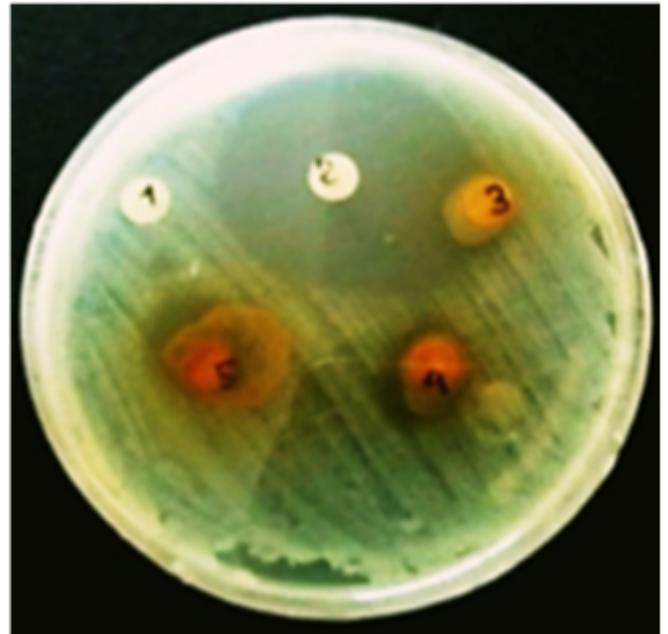
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Figures



P. aeruginosa



E. coli

Figure 1

The antibacterial effect of walnut leaf extract (discs 3 to 5 correspond to the concentrations of 30, 50 and 100 mg/L, respectively), DMSO (disc 1) and gentamicin (disc 2) against *P. aeruginosa* and *E. coli* after 18 h of incubation