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Antibacterial Activity of the Hydro-Alcoholic Extract of *Juglans regia* L. Stem Bark on Human Bacterial Infection

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ABSTRACT

Aims Bovine mastitis continues to be the most costly disease to the dairy farmers. It dominates in Iran as one of the most prevalent diseases in dairy cattle among the dairy farms. Mastitis treatment with antibiotics leads to the development of antibiotic resistant strains and consumer health problem.This study was performed for the first time to analyze *in vitro* effects of hydro-alcoholic extract of *Juglans regia* L. stem bark on 6 mastitis pathogens.

Materials & Methods the susceptibility of 6 strains of bacteria (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Streptococcus* spp., *Pasteurella multocida and Mannheimia haemolytica*) were analyzed against hydro-alcoholic extract of *Juglans regia* L. stem bark with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods.

Findings Hydro-alcoholic extract did not have antibacterial effects on *E. coli and K. pneumoniae.* Minimum inhibitory concentration for *S. aureus, P. multocida, M. haemolytica and Streptococcus* spp. was 62.5mg/ml of hydro-alcoholic extract. There was not any significant response with concentrations below 100mg/disc on *S. aureus, Streptococcus species, P. multocida and M. haemolytica.* Minimum bactericidal concentration of this extract was 100mg/ml in all isolates.

Conclusion *Juglans regia* L. have some antibacterial effects on *S. aureus, P. multocida, M. haemolytica* and Streptococcus species.

Keywords Mastitis, Bovine; Disk Diffusion Antimicrobial Tests; Juglans

CITATION LINKS

[1] Glossary of Indian medicinal plants (including the supplement) [2] Indigenous drugs of India [3] Potters new cyclopedia of botanical drugs and preparations [4] Handbook of energy crops [5] Fungistatic action of juglans [6] Antibacterial effect of juglansregia bark against oral pathologic bacteria [7] Medicinal Plants [8] Dorland's Illustrated Medical Dictionary [9] Sedative effect of walnut leaf extract and juglone, an isolated constituent [10] Quince (Cydoniaoblonga Miller) fruit (pulp, peel, and seed) and Jam: Antioxidant activity [11] Effect of plant flavonoids on immune and inflammatory cell function [12] Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs and goats [13] In vitro phytochemical screening and antibacterial activity of aqueous and methanolic leaf extracts of TridaxProcumbens against bovine mastitis isolated Staphylococcus aureus [14] Proteomic approach to understanding antibiotic action [15] The natural toxin juglone causes degradation of p53 and induces rapid H2AX phosphorylation and cell death in human fibroblasts [16] Total phenols, antioxidant potential and antimicrobial activity of walnut (Juglansregia L.) green husks [17] Walnut (Juglansregia L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivarsFood ChemToxicol [18] Performance standards for antimicrobial disk susceptibility tests: approved standard [19] Antibacterial activities of some plant extracts used in Indian traditional folk medicine [20] Antimicrobial resistance trends in medical centers using carbapenems: report of 1999 and 2000 results from the MYSTIC program (USA) [21] Antioxidant and antimicrobial attributes and phenolics of different solvent extracts from leaves, flowers and bark of Gold Mohar [Delonixregia (Bojer ex Hook.) Raf] [22] In-vitro antimicrobial effects of some selected plants against bovine mastitis pathogens [23] In-vitro antimicrobial activity screening of some ethnoveterinary medicinal plants traditionally used against mastitis, wound and gastrointestinal tract complication in Tigray Region, Ethiopia [24] In-vitro anthelmintic activity of stem bark of Juglansregia L. J Chem Pharm Res [25] GC-MS study of stem bark extract of Juglansregia L. Res J Pharm Biol Chem Scie

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Introduction

Juglandaceae family includes a valuable and medicinally useful species, *Juglans regia* Linn, commonly known as walnut, is a large and deciduous tree abundantly found in Baluchistan, South-East of Iran, the Caucasus, forests of Himalayas in India [1], Armenia, and other temperate regions [2]. The root, stem bark, leaves, seeds, cotyledons and seed oil are used to treat a variety of health complaints [3].

The stem bark is reported to be alterative, anthelmintic, astringent, bactericide, depurative, digestive, diuretic, laxative, detergent, stimulant, tonic and insecticidal [1] and walnut is used in cases of herpes, eczema, scrofula, and ulcers [3]. The dried bark of the tree is used as a tooth cleaner [4]. Bactericidal properties of the Juglans were reported by Ahmad *et al.* [5]. *Juglans regia* L. stem bark contains chemical constituents like β-sitosterol, ascorbic acid, juglone, folic acid, gallic acid, regiolone, and quercetin-3-α-L-arabinoside [6, 7] and is also known useful for the treatment of fungal infections, including ringworm infections of the skin [8].

Keratolytic, antifungal, hypoglycaemic, hypotensive, anti-scrofulous and sedative activities of *Juglans regia* L. have also been described [9]. Phytochemicals, such as phenolic compounds, are considered beneficial for human health, decreasing the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecular oxidation [10]. They have been shown to possess free radical-scavenging and metalchelating activity in addition to their reported anticarcinogenic properties [11].

Mastitis is an inflammation of mammary gland regardless of the cause, with economical and health consequences. Many infective agents have been implicated as causes of mastitis; among the major pathogens are *S. aureus*, *E. coli*, Streptococcus strains and *Enterobacter aerogens* [12].

Clinical and subclinical cases of mastitis are routinely treated with antimicrobials both intra-mammarily and parenterally. The use of antimicrobials over long periods has triggered the development of multidrug resistant strains, which has resulted in the use of increasing doses of antimicrobials, causing the danger of increasing amounts of drug residues in milk, a potential biohazard [13].

Increasing of antibiotic-resistant bacteria, side effects of chemical antibiotics, and high costs in developing countries cause the growing emphasis of plant studies on the field of microbiology. Antimicrobial activity of walnut tree comes from its chemical composition. *Juglans regia* L. bark have been analyzed chemically adequately in several studies, and in agreement among most of them, with respect to the essential oil composition, the major components are phenolic compounds, terpenoids, alkaloids, flavonoids, and steroids [14]. It is reported that leaves of *Juglans regia* L. contain monoterpenes and sesquiterpenes, and the bark contains ketones like juglone, regiolone, sterol, and flavonoid [15].

The antimicrobial capacity of different extracts of *Juglans regia* L. was screened against Gram positive and Gram negative bacteria, and fungi by Oliveria *et al*. All the extracts inhibited the growth of Gram positive bacteria were *Staphylococcus aureus* and the most susceptible one was MIC of 0.1mg/ml [16]. In another study, walnut leaves selectively inhibited the growth of Gram positive bacteria (*B. cereus*, *B. subtilis* and *Staphylococcus aureus*) and *B. cereus* was the most susceptible one (MIC 0.1mg/ml). Gram negative bacteria (*E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) and fungi (*Candida albicans* and *Cryptococcus neoformans*) were resistant to the extracts at 100mg/ml. Walnut leaves were also submitted to antibacterial assays using clinical isolates of Staphylococcus spp. [17].

According to an increasing demand for the development of new antimicrobial agents, especially in common veterinary and human infections and as there is no previous study on veterinary infections due to pathogenic microbes, this study was performed for the first time to analyze *in vitro* effects of hydro-alcoholic extract of *Juglans regia* L. stem bark on 6 mastitis pathogens.

Materials & Methods

Collection of *Juglans regia* L. and bacteria

The stem bark of *Juglans regia* L. was obtained from a local market and was confirmed by an expert colleague in School of Pharmacy.

The bacteria (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae*, *Streptococcus* spp., *Pasteurella multocida* and *Mannheimia haemolytica*) were isolated in

Microbiology Laboratory of Veterinary Medicine Faculty, Shahid Chamran University, Iran, from milk samples during several months.

Preparation of hydro-alcoholic extract of *Juglans regia* L.

Juglans regia L. stem bark was ground to powder (100g), solved in 500ml of ethanol 80% (400ml of ethanol 96% + 100ml of water) and was placed in room temperature for 48 hours. The solvent was filtered by Whatman filter paper and then concentrated in vacuum at 40°C by means of a rotary evaporator (Heidolph; Germany). The obtained residue was stored until future tests at 4°C.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The disc diffusion method (by the National Committee for Clinical Laboratory Standards [18] protocol) was used to evaluate the susceptibility of studied bacteria. For susceptibility testing, 5 serial dilutions of 1000, 500, 250, 125, and 62.5mg/ml of each bacteria were prepared in sterile distilled water. Sterile antibiotic assay discs (Padtan Teb Co.; Iran) were impregnated with 30µl of the reconstituted extract and were dried completely at 37°C. Each disc was gently pressed down to ensure complete contact with the agar inoculated with bacteria that was adjusted to the 0.5 McFarland standard equivalents to 10^7CFU/ml in Mueller Hinton Agar (Merck; Germany).

Sterile distilled water discs were used as negative control. Standard antibiotic discs were used as positive control (all were examined previously). The inoculated plates were incubated at 37°C for 18 hours. Antimicrobial pattern was evaluated by measuring the zone of inhibition (mm) against the test bacteria [19]. The assays were performed 3 times for each bacterium.

MIC was determined by broth dilution method. 1ml of 24h cultured of test organisms (10^7CFU/ml) adjusted to McFarland turbidity standard were incubated in serial dilution ranging from 1000 to 62.5mg/ml of plant extracts in distilled water at 37°C for 24h. The concentration of the lowest dilution with no detectable bacterial growth was considered as MIC. Absence of growth was confirmed by absence of turbidity and by inoculating into agar [20].

0.1ml of culture medium from each broth dilution assay plate showing no apparent growth was aspirated and sub-cultured in fresh Mueller Hinton Agar. After incubation at 37°C for 24h, the least concentration showing no visible growth on subculture was taken as the MBC.

Data analysis

Obtained data from each dilution of plant extract and MIC of various bacteria were entered into SPSS 16 statistical software. Mean and standard deviation were used to report data.

Findings

M. haemolytica and *Streptococcus sp*., were susceptible to Gentamycin (10µg per disc), *S. aureus,* was susceptible to Erythromycin (15µg per disc), *E. coli* was susceptible to Trimethoprim Sulfamethoxazol (25µg per disc) and *P. multocida* and *K. pneumoniae* were susceptible to Tetracycline (30µg per disc); So, these antibiotics were selected as positive control.

Hydro-alcoholic extract did not have antibacterial effects on *E. coli and K. pneumoniae*. Minimum inhibitory concentration for *S. aureus*, *P. multocida*, *M. haemolytica* and *Streptococcus* spp. was 62.5mg/ml of hydro-alcoholic extract (Figure 1).

There was not any significant response with concentrations below 100mg/disc on *S. aureus,* Streptococcus species*, P. multocida* and *M. haemolytica*. Minimum bactericidal concentration of this extract was 100mg/ml in all isolates (Figure 2).

Figure 1) Mean of inhibitory zone (millimeter) of different dilutions of *Juglans regia* L. stem bark hydro-alcoholic extracts against 6 mastitis bacterial isolates to measure minimum inhibitory concentration

Bacteria	Positive Control	62.5mg/ml	125mg/ml	250mg/ml	500mg/ml	1000mg/ml
S. aureus	Erythromycin	-	9.1±3.2	12.0±3.5	16.2±3.5	18.4±2.8
E. coli	SXT	-	-	-	-	-
K. pneumoniae	Tetracycline	-	-	-	-	-
P. multocida	Tetracycline	-	10.0±2.1	13.2±3.1	16.0±3.2	17.1±3.7
M. haemolytica	Gentamicine	-	12.2±4.3	14.1±3.5	16.1±3.3	16.6±4.3
***Streptococcus* spp.**	Gentamicine	-	11.4±3.2	13.0±2.6	10.2±3.1	12.0±2.1

Figure 2) Mean of inhibitory zone (millimeter) of different dilutions of *Juglans regia* L. stem bark hydro-alcoholic extracts against bacterial isolates to measure minimum bactericidal concentration

Bacteria	Positive Control	100mg/ml	125mg/ml	250mg/ml	500mg/ml	750mg/ml
S. aureus	Erythromycin	12.1±2.5	14.0±3.1	16.2±3.5	18.3±3.6	19.2±3.7
P. multocida	Tetracycline	8.6±2.2	10.1±3.9	14.2±2.7	17.0±3.2	17.2±3.8
M. haemolytica	Gentamicine	10.1±3.8	12.0±3.1	14.0±3.5	15.2±2.2	17.3±3.6
***Streptococcus* spp.**	Gentamicine	9.6±2.4	11.1±3.2	13.3±2.5	15.1±3.2	16.6±3.2

Discussion

The study was conducted with the objective of assessing traditionally used medicinal plants in livestock and evaluating the antibacterial activity of stem bark of *Juglans regia* L. on bacterial isolates from mastitis milk. The MIC values indicate that the *Juglans regia* L. extract is potent against some of bacteria. The lowest value observed was 100mg/ml on *S. aureus* while the highest value was 750mg/ml for all sample bacteria except *E. coli* and *K. pneumoniae*. The extract of *Juglans regia* L. stem bark exhibited the highest inhibition zone against the gram-positive bacteria, *S. aureus*, with MIC of 100mg/ml.

Juglans regia L. stem bark hydro-alcoholic extract was totally ineffective against *E. coli* and *K. pneumoniae*. The antibacterial activity of the plant was relatively higher in gram-positive than gram-negative bacteria. MIC of 100mg/ml and 125mg/ml were registered for gram-positive and gram-negative bacteria, respectively.

There was a dose dependent inhibition on the studied microorganisms suggesting the importance of in-depth study of this herbal medicinal plant and supports the knowledge of the herbalists [21]. Several studies have been carried out in order to use of medicinal plants in the treatment of microbial infections such as *in vitro* antimicrobial activity screening of some medicinal plants traditionally used against oral pathogenic bacteria [7], ethno-veterinary for bovine mastitis [22], wound and gastrointestinal tract complication [23], fungiatatic [5] and anthelmintic activity of Juglans [24], All of which have achieved positive results.

The antibacterial property of the plant material may be due to the presence of n-octedecane, n-hexadecanoic acid (palmitic acid), 9-E-Hexadecenoic acid, tetracotane, 4,8,12,16 tetramethylheptadecane-4-olide, n-heptadecanoic acid, 1-iodohexadecane, stearic acid, oleic acid, erucic acid and Di-n-octyl phthalate [25].

It is worth mentioned that extraction technique and the choice of solvent could affect the extraction of active principle and, hence, affect the results. Continuous and progressing researches need to be conducted to prove the safety, efficiency and to determine the types of compounds responsible for the antibacterial effects of *Juglans regia* L.

Conclusion

Juglans regia L. have some antibacterial effects on *S. aureus*, *P. multocida*, *M. haemolytica* and Streptococcus species.

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Ethical Permission: None declared by authors.

Conflicts of Interests: The authors declare that there are no conflicts of interests.

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References

1- Chopra RN, Nayar SL, Chopra RC, Asolkar LV, Kakkar KK, Chakre OJ, et al. Glossary of Indian medicinal plants (including the supplement). New Delhi: Council of Scientific & Industrial Research; 1956-92. p. 11.

2- Chopra RN. Indigenous drugs of India. Calcutta : Academic Publishers; 1933.

3- Wren RC. Potters new cyclopedia of botanical drugs and preparations. London: CW Daniel Company Ltd; 1988.

4- Duke JA. Handbook of energy crops. Lafayette: Purdue University, Center for New Crops & Plants Products; 1983. p. 269.

5- Ahmad S, Wahid MA, Bukhari AS. Fungistatic action of juglans. Antimicrob Agents Chemother. 1973;3(3):436-8.

6- Zakavi F, Golpasand Hagh L, Daraeighadikolaei A, Farajzadeh Sheikh A, Daraeighadikolaei A, Leilavi Shooshtari Z. Antibacterial effect of juglans regia bark against oral pathologic bacteria. Int J Dent. 2013;2013:1-5.

7- Joshi SG. Medicinal Plants. New Delhi: Oxford & IBH Publishing; 2007.

8- Dorland WAN. Dorland's Illustrated Medical Dictionary. 32nd edition. Philadelphia: Saunders and Co.; 2011.

9- Gırzu M, Carnat A, Privat AM, Fiaplip J, Carnat AP, Lamaison JL. Sedative effect of walnut leaf extract and juglone, an isolated constituent. Pharmaceut Biol. 1998;36(4):280-6.
10- Silva BM, Andrade PB, Valentão P, Ferreres F, Seabra RM, Ferreira MA. Quince (Cydonia oblonga Miller) fruit (pulp, peel, and seed) and Jam: Antioxidant activity. J Agric Food Chem. 2004;52(15):4705-12.
11- Middleton E. Effect of plant flavonoids on immune and inflammatory cell function. Adv Exp Med Biol. 1998;439:175-82.
12- Radostits OM, Gay CC, Hinchcliff KW, editors. Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs and goats. 10th edition. London: W.B. Saunders; 2007.
13- Dhanabalan R, Doss A, Jagadeeswari M, Balachandar S, Kezia E. In vitro phytochemical screening and antibacterial activity of aqueous and methanolic leaf extracts of Tridax Procumbens against bovine mastitis isolated Staphylococcus aureus. Ethnobot Leafl. 2008;12:1090-95.
14- Bandow JE, Brötz H, Leichert LI, Labischinski H, Hecker M. Proteomic approach to understanding antibiotic action. Antimicrob Agents Chemother. 2003;47(3):948-55.
15- Paulsena MT, Ljungman M. The natural toxin juglone causes degradation of p53 and induces rapid H2AX phosphorylation and cell death in human fibroblasts. Toxicol Appl Pharmacol. 2005;209(1):1-9.
16- Oliveira I, Sousa A, Ferreira IC, Bento A, Estevinho L, Pereira JA. Total phenols, antioxidant potential and antimicrobial activity of walnut (Juglans regia L.) green husks. Food Chem Toxicol. 2008;46(7):2326-31.
17- Pereira JA, Oliveira I, Sousa A, Valentão P, Andrade PB, Ferreira IC, et al. Walnut (Juglans regia L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivars Food Chem Toxicol. 2007;45(11):2287-95.
18- National Committee for Clinical Laboratory Standards (NCCLS). Performance standards for antimicrobial disk susceptibility tests: approved standard. 8th edition. Wayne, PA: National Committee for Clinical Laboratory Standards; 2003.
19- Bhattacharjee I, Chatterjee SK, Ghosh A, Chandra G. Antibacterial activities of some plant extracts used in Indian traditional folk medicine. Asian Pac J Trop Biomed. 2011;1(Suppl 2):S165-9.
20- Pfaller MA1, Jones RN, Biedenbach DJ, MYSTIC Program Study Group. Antimicrobial resistance trends in medical centers using carbapenems: report of 1999 and 2000 results from the MYSTIC program (USA). Diagn Microbiol Infect Dis. 2001;41(4):177-82.
21- Shabir G, Anwar F, Sultana B, Khalid ZM, Afzal M, Khan QM, et al. Antioxidant and antimicrobial attributes and phenolics of different solvent extracts from leaves, flowers and bark of Gold Mohar [Delonix regia (Bojer ex Hook.) Raf]. Molecules. 2011;16(9):7302-19.
22- Muhamed Mubarack H, Doss A, Dhanabalan R, Venkataswamy R. In-vitro antimicrobial effects of some selected plants against bovine mastitis pathogens. Hygeia J D Med. 2011;3(1):71-5.
23- Kalayou S, Haileselassie M, Gebre-egziabher G, Tiku'e T, Sahle S, Taddele H, et al. In-vitro antimicrobial activity screening of some ethnoveterinary medicinal plants traditionally used against mastitis, wound and gastrointestinal tract complication in Tigray Region, Ethiopia. Asian Pac J Trop Biomed. 2012;2(7):512-22.
24- Kale AA, Gaikwada SA, Kamblea GS, Deshpandea NR, Salvekara JP. In-vitro anthelmintic activity of stem bark of Juglans regia L. J Chem Pharm Res. 2011;3(2):298-302.
25- Kale AA, Gadkari TV, Devare SM, Deshpande NR, Salvekar JP. GC-MS study of stem bark extract of Juglans regia L. Res J Pharm Biol Chem Scie. 2012;3(1):740-3.