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Investigating the antimicrobial effects of *Withania somnifera* methanol extract prepared by ultrasonic waves on omit *Klebsiella pneumonia*

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ABSTRACT

Background and aims: Herbal medicines has been a major remedy in traditional medical systems from thousands of years and made a great contribution in maintaining human health and in preventing many infectious diseases**.** *K. pneumonia* is a bacterial negative intestinal bacillus and a member of *Enterobacteriaceae* family. This paper aims at investigating the antimicrobial effects of the methanol extract of wind cheese prepared by ultrasonic waves on the *K. Pneumonia***.**

Methods: All 12 isolates of K. pneumoniae isolated from urine culture of hospitalized patients (Amir al-Mu'minin Hospital, Zabol, southeastern of Iran) suffered from urinary tract infection during the years 2013 and 2014. In this study, the extract of W. somnifera obtained by ultrasonic waves. Then the minimum inhibitory concentrations and minimum bactericidal concentration were investigated to characterize the antibacterial activities of this extract.

Results: The result showing that plant extracts from *W. somnifera* had inhibitory effect against *K. pneumoniae*. The MIC values were also determined against all the tested bacteria. The highest MIC values of extract were found to be 5mg/ml against *K. pneumoniae* and four of MIC value for *K. pneumoniae* was 2.5 mg/ml. ($p<0.005$).

Conclusion: The obtained results showed that extraction by using ultrasonic method improved the efficiency and amount of the antimicrobial effects of the plant. So, ultrasonic waves can be used as a stimulus for increasing the antimicrobial effects of the plant.

Keywords: *Antimicrobial effects, Withania somnifera, Ultrasonic waves, Antimicrobial activity*

Original article

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INTRODUCTION

K. pneumonia is a bacterial negative intestinal bacillus and a member of Enterobacteriaceae family. It forms a part of human natural microflore (1). One third of sound people carry the intestinal form of the bacteria which is an opportunistic pathogen involved in hospital infections (2). It causes a high mortality rate by creating diseases such as urinal infections, pneumonia septic emboli, and abdominal infections in hospital patients. Such infections are rarely seen out of hospital (3).

The main point about this bacterium is its resistance to different antibiotics and its disseminations among infants with poisonous septic who suffer high mortality (4). Overconsumption of antibiotics in the last decades has caused such resistant bacteria to emerge (5). Extraction is the main step to purifying the active components of vegetable members. The common extract methods such as Soksile method are too time-consuming. Further, they need high amounts of different solvents (6). So, there is a great need for extract methods that are more economical, less time-consuming and more environments friendly (7). Ultrasonic method is a new extraction method in which waves higher than 20 kHz penetrate into the material and cause subsequent expansions and condensations that create pores in the plant. They can also destruct bio cells and facilitate materials exit (8).This is a very cheap, effective, and fast technique used in room temperature. In this method, temperature-sensitive compounds are less damaged. Its drawback is the need for using compulsory filtration (9).

The aim of this study was to investigating the antimicrobial effects of *Withania somnifera* methanol extract prepared by ultrasonic waves on omit *K. pneumonia* isolated from the UTI patients by microtiterplate method.

METHODS

Bacteria Separation

All 12 strains of *K. pneumoniae*, isolated from urine culture of hospitalized patients (Amir al-Mu'minin, Hospital, Zabol, southeastern Iran) suffered from urinary tract infection during years 2013 and2014 were evaluated. Isolated bacteria were identified by Gram's stain and standard biochemical tests

Preparation of Half-McFarland suspension

For preparation of microbial suspension, bacteria were removed from reserve environment to the planting environment (German Merc). Upon growth of bacteria colonies, the panting environment was rinsed with normal saline solution and thick microbial suspension was prepared. Then, some of the suspension was poured into sterile pipe containing normal saline and its dark ness was measured with spectrophotometer at wavelength of

Table 1. The MIC and MBC of the ultrasonic extract of wind *W.sominefera* against *K. pneumonia (mg/ml)* ($p<0.005$)

Bacterial code	MIC/MBC mg/ml	Bacterial code	MIC/MBC mg/ml
1	2.5 / 5	7	No growth
2	No growth	8	5 / 10
3	No growth	9	2.5 / 5
4	2.5/ 5	10	2.5 / 5
5	No growth	11	No growth
6	5/10	12	No growth

630nm. The suspension was diluted with normal saline solution until its darkness was equal to Half-McFarland solution. As a result, bacteria suspension with concentration of 10^8 cfu/ml was prepared.

Extract preparation and investigation of the antimicrobial effects of the plant extract

The wind cheese used in this study was gathered from Sistan and Baluchistan province. It was detected to be wind cheese by a researcher from the University of Zabol. The samples were cut and 10g of the dry plant powder was put into half-liter flasks containing 100ml of methanol. The contents of flasks were shaken 24 hours at room temperature bshaker device with speed of 130 rpm and were kept in ultrasonic carrier for 10 minutes and were filtered with Wattman paper. Separation of solution from extract was done by rotary device and vacuum pump. The obtained extract was weighed. The samples were used in fridge at 4 degrees of temperature to be used in antimicrobial tests.

The antimicrobial effect of the extract on *K. pneumonia*

Sensitivity of the bacteria samples with multiple resistances to the wind *W.sominefera* extract against *K. pneumonia* was analyzed by dilution method in broths. To this, seven broths of microtitre plates were injected to 100 ml of MHB. The first broth was added to 100ml of the diluted extract. Then, 100ml of the first broth was transferred to the second one and the same was done to the last broth. 100ml of the last broth was removed and 100ml of the microbial suspension with 107 units per ml was added to all broths. The mixture was kept 24 hours at temperature of 37 degrees. The first broth inhibiting the growth of bacteria after being positioned in the incubator was considered as MIC and for more precision, 10 ml of the light broths was transferred to Moller environment. After 24 hours, the first concentration removing 99.9% of the bacteria was regarded as MBC.

RESULTS

Inhibitory effects of plant extract from *W. somnifera*, against *K. pneumoniae* were demonstrated in Table 1 showing that plant extracts from *W. somnifera* had inhibitory effect against *K. pneumoniae*. The MIC values were also determined against all the tested bacteria. The highest MIC values of extract were found to be 5mg/ml against *K. pneumoniae* and four of MIC value for *K. pneumoniae* was 2.5 mg/ml. ($p<0.005$). ($P= 0.043$) in 48hr(table 1).

DISCUSSION

The results of this study showed the high antimicrobial effects of the ultrasonic extract so that 6 samples of bacteria were inhibited even in low concentrations of extract and the highest inhibitory concentration was 5 mg/ml.

Bokaeian, omit was to evaluated the effect of *W. somnifera* extracts on drug resistant *E. coli* strains isolated from clinical samples. The results showed that the isolated *E. coli* strains were sensitive to these antibiotics: erythromycin (52.94%), tetracycline (76.47%), ceftazidime (41.17%), cefixime (35.29%), penicillin (76.47%), ampicillin (58.82%) and nalidixic acid (41.17%). Examination of the herbal extracts showed that the highest maximum inhibitory concentration (MIC) against drug resistant *E. coli* was 200 ppm. The lowest MIC was 50 ppm, where three strains of *E. coli* were inhibited at this concentration (10).

In another study Bokaeian evaluated the antibacterial activity of leaf extracts of Winter Cheery (*W. somnifera*) against antibiotic-resistant isolates of *K. pneumoniae* isolated from the urinary tract infection patients. The isolates of *K. pneumoniae* were resistance to four of the agents including ceftazidime (60% of isolates) cefixime (60% of isolates), erythromycin (66.6% of isolates). The highest MIC values of extract were found to be 250 ppm against *K. pneumoniae* and MIC values for *K. pneumoniae* were 63 ppm (11).

Singariya et al (2012) showed that the aqueous extract of wind cheese forms inhibitory clouds with diametres of 11.17 and 7.33 against *Proteus merabilis* and *Agerobacterium tumefaciens*, respectively(12).

Javadian et al (2015) explored the antimicrobial effects of the wind cheese

essence on *P. aeruginosa* and *Acintobacter*. Results showed that the highest MIC was for *Acintobacter* with concentration of 100 ppm in which 4 samples were inhibited and the least amount was for *Acintobacter* with concentration of 50 ppm in which only 5 bacteria were inhibited. Lack of growth was seen for 3 *Acintobacter*samples. The results for *P. aeruginosa*showed that the MIC was 100 ppm for which 1 sample was inhibited while the least concentration was 12.5 ppm (13).

The results obtained by Bansod&Rei(2008) showed that the wind cheese extract with concentration of 100 micro gram per disk, an inhibitory cloud of 15mm was created against *Aspergillus fumigates*(14).

Rawat&Bisht explored the entimicrobial effects of wind cheese against gram bacteria and showed that the methanol extract of wind cheese could inhibit the growth of *Staphylococcus* aureus- resistant to antibiotics and *Entreococus*- with average inhibitory cloud diameter of 20.6 mm and 19.4 mm at concentration of 2mg/ml(15).

Mohkamiand Bidarnamani showed that the MIC of the wind cheese' aqueous extract against *S.aureus, S. Pyogenes, S. pneumoniae, H. alvei, S. saprophyticus, A. baumanii, E. faecalis, P.mirabilis,* and *S. marcescens* are 125, 250, 62.5, 250, 250, 250, 250, 125, and 125 ppm; respectively(16).

G.Muotar et al. (2013) showed the diameter of the inhibitory cloud of the Ethyl acetate extract against *S.aureus, MRSA, Microsporumgypseum, Candida albicans, Cryptococcus neoformans,* and *Trichophytonmentagrophytes* to be 13, 13, 13, 13, 22, and 0 mm respectively. The same amounts for methanol extract were 15, 14, 0, 0, 0, and 0 mm; respectively (17).

Mehrotra et al (2011) explored the antimicrobial effects of the aqueous extract of wind cheese against *S.aureus* and found the MIC to be 2.3 mg/ml and 5.2 microgram/ml (18).

The study of de Lima Silva , to define whether novel extraction methods such as microwave and ultrasound could obtain the most effective ethanolic extracts of *Ocimum gratissimum* as antibacterial agents. Maceration gave the extract with the broadest spectrum

of activity. Ultrasound methods yielded an efficient extract for use as a topical antiseptic (minimum inhibitory concentration (MIC) = 0.66 to 1.32 mg/ml for *Staphylococcus aureus* and Methicillin-resistant *S.aureus*(MRSA)). The most active extracts to treat vancomycin-resistant enterococci (VRE) infections were obtained by Soxhlet and microwave (MIC = 5.28 mg/ml) (19).

The study of Liu , ultrasonic power could improve TPC and antioxidant activity, but long time of extraction lowered antioxidant activity. The TPC increased from 22.34 to 27.87 mg GAE (gallic acid equivalents)/100 g (dry extracts) with increasing solvent polarity. The half inhibition concentration (IC(50,) μg/mL) of the radical scavenging activity of the chicory extracts ranged from 281.00 to 983.33 μg/mL. The content of caffeoylquinic acids of root extract, which was extracted by the optimal combination was 0.104%. Several extracts displayed antibacterial activities against *E. coli, S. aureus, B. thuringiensis, B. subtilis*, and *S.typhi*, while *Penicillium*sp. and *Aspergillus* sp. resisted against all the extracts. Combination of 70% ethanol v/v, 24-h impregnation time, 3 sonication rounds, and 300-W ultrasonic input power was found to be the optimal combination for the chicory extract yield, TPC, antioxidant activity, and antibacterial activity (20)

The study of Thongson research was to determine the antimicrobial activity of conventional and high-intensity ultrasound-assisted (HI-US) solvent-extracted Thai spices, including ginger (Zingiber officinale Rose), finger root (Bosenbergia pandurate Holtt) and turmeric (Curouma longa Linn). The result show that application of HI-US reduced time of extraction to 5 min, compared with the 24 h required for conventional extraction and maintained antimicrobial activity against *Salmonella* but slightly reduced activity against *Listeria* (21)

The study of Kavitha, use of Ultrasonic assisted method to enhance the yield of bioactive components in which protein which is our area of concentration is included. The concentration of protein present were estimated and was found to be 142 μg/ml , 228μg/ml,360μg/ml,356μg/ml for

conventional,10mins,20mins,and 30mins respectively. Protein was further purified by Biogel P30 column chromatography and characterized by SDS-PAGE. In this the mushroom subjected to 20mins Ultra sonication was found to have higher efficiency. The mixture obtained was used for anti-microbial study which was found effective against gram positive and gram negative bacteria like *S. aureus*-5021, *B. substillis*-2717,*P. aeruginosa*-2492, *K. pneumonia*-2957,*E. coli*-2810(22).The study of Moawad, was to determine the antimicrobial activity of conventional and high-intensity ultrasound-assisted (HI-US) solvent-extracted Thai spices, including ginger (*Zingiber officinale* Rose), finger root (*Bosenbergia pandurate* Holtt) and turmeric (*Curouma longa* Linn).Extracts were obtained using hexane, isopropanol and a 7 : 3 isopropanol : hexane mixture as solvents with and without HI-US. The antimicrobial activity of the extracts was assayed against four strains each of *L. monocytogenes*and *S. Typhimurium* DT 104 using an agar dilution assay. Application of HI-US did not alter antibacterial activity against *S.Typhimurium*, but anti listerial activity of some HI-US spice extracts decreased. Solvent type affected antimicrobial efficacy of extracts with hexane producing the least antimicrobial activity. Finger root extracted with isopropanol–hexane and without HI-US had the best antilisterial effect whileHI-US–isopropanol finger root extract had the greatest antimicrobial efficacy against *S. Typhimurium* (23). The study of Abshora, evaluate the influence of extraction method and solvent type on extractable polyphenols and flavonoids in *Doum Hyphaene Thebaica* L. Mart. (Arecaceae) fruit, as well as to examine the antioxidant and antibacterial activities of the fruit extracts. His extraction procedures were performed separately in an ultrasonic bath or shaking water bath for 30 min (70 °C for ethanol and 60 °C for methanol) at agitation speed of 50 Hz and 70 rpm, respectively. The antioxidant potential of the extracts was investigated using β-carotene bleaching, 2, 2 - diphenylpicrylhydrazyl (DPPH) and reducing power abilityassays. In vitro antibacterial activity of the extracts against *S.aureus, L.monocytogenes, E. coli*, and *S. typhi* was assessed using

agar disc diffusion assay. Total polyphenol content (TPC) and total flavonoid content (TFC), as well as antioxidant capacity were maximized using methanol as the extraction solvent, particularly with the ultrasonic method. The half maximal inhibitory concentration (IC 50) values of the methanol/ultrasonic (MU), methanol/water bath (MW), ethanol/ultrasonic (EU), and ethanol/water bath (EW) extracts in the DPPH assay were 107.6 126.7, 172.7, and 196.3 µg/mL, respectively. The extracts showed strong antibacterial activity against *S.aureus* and *S. typhi*, while MU extract inhibited the growth of all pathogenic bacteria used in this study (24).

CONCLUSIONS

The obtained results showed that extraction by using ultrasonic method improved the efficiency and amount of the antimicrobial effects of the plant. So, ultrasonic waves can be used as a stimulus for increasing the antimicrobial effects of the plant. Yet, more precise results require the analysis of effective materials by HPLC method so that they can be used in clinical industries.

CONFLICT OF INTEREST

All authors disclose any financial and personal relationships with other people or organizations and the authors declare that there are not any potential conflicts of interest. I indicate here that any color photo in print is required.

Authors' Contributions

Study concept and design and Critical revision of the manuscript: Mohaddeseh Abouhosseini Tabari. Analysis and interpretation of data: Bibi Razieh Hosseini Farash. Drafting of the manuscript: Elham Moghaddas, Mohammad Amin Ebrahimi, Nilofar Nabavi Mousavi. Statistical analysis: Mohammad Reza Youssefi.

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REFERENCES

1. Varaldo PE, Biavasco F, Mannelli S, Pompei R, Proietti A. Distribution and antibiotic susceptibility of extraintestinal clinical isolates of *Klebsiella*, *Enterobacter* and *Serratia* species. Eur J Clin Microbiol Infect Dis. 1988; 4: 495–500

2. Bagley ST. Habitat association of *Klebsiella* species. Infect Control .1985; 6: 52–58.

3. Podschun R , Ullmann U. *Klebsiella* spp. as nosocomial pathogens: Epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev. 1998; 11: 589–603.

4.Chen LF, Chopra T , Kaye KS. Pathogens resistant to antibacterial agenst . Infect Dis Clin North Am. 2009; 23(4): 817-845.

5. Gootz TD. The global problem of antibiotic resistance. Crit Rev Immunol. 2010; 30(1): 79-93.

6. Luquede Castro MD , Garcia- Ayuso LE. Soxhlet extraction of solid materials An outdated technique with a promising innovative future. AnalyticaChimicaActa. 1988; 369: 1-10.

7. Zolfaghari B , Yekdane A. Recent developments in extraction methods of plant materials. Herbal Plan Quarter. 2010; 1, 51-55.

8. Luque-Garcia JL , Luque de Caster MD.Ultrasound : A Powerful tool for leaching. Tre in Analyt chem. 2003; 22: 41-47.

9.Vinatoru M. An overview of the ultrasonically assisted extraction of bioactive princilpes from herbs. Ultra chem. 2001; 8(3): 303-313.

10. Bokaeian M, Fakheri BA, Mahdi Nejad N , Zarei H, Saeidi S ,Mohasseli T. The Effect of *Withaniasomnifera* Extract on Drug Resistant Strains of *Escherichia coli*. Int J Infect. 2015 ; 2(2): e23036

11.Bokaeian M, Saeidi S, Shahi Z, Sahraei S, Zarei H , SohilBaigi G. Evaluation of Antibacterial Activity of *Withaniasomnifera* Leaf Extracts against Antibiotic-Resistant Isolates of *Klebsiella pneumoniae*. Int J Infect. 2014 ; 1(2): e21085

12. Singariya P, Kuma P , Mourya KK. Evolution of Antimicrobial Activity of Leaf extracts of Winter Cheery(*Withaniasomnifera*). Inter J of PharmTech Res.2012; 4(3): 1247-1253.

13. Javadian F, Nasiri AA, Arefnejad M, Kiani Z, Sargazi A, Sepehri Z , Arefi M. Investigating the antimicrobial effects of the wind cheese leaf essence on samples of *Pseudomonas aeruginosa* and *AcintobacterBumani*resistant to

different antibiotics. Zabol Med Sci J. 2015; 7(4).

14.Bansod S , Rai M. Antifungal Activity of Essential Oils from Indian Medicinal Plants Against Human Pathogenic *Aspergillusfumigatus* and *A. niger* .World J of Med Sci. 2008; 3 (2): 81-88.

15.Bisht P ,Rawat V.Antibacterial activity of *Withaniasomnifera* against Gram-positive isolates from pus samples.Ayu. 2014;35(3):330-2.

16.Zaynab M , Fatemeh B. Evolution of Antimicrobial activity of *Witjaniasomnifera* aqueous extract against human pathogens**.** Indian J of Fund and Appli Life Sci. 2014 ; 4(4): 2969-2973

17. G. Mwitari P, A. Ayeka P, Ondicho J, N. Matu E , C. Bii C. Antimicrobial Activity and Probable Mechanisms of Action of Medicinal Plants of Kenya: *Withaniasomnifera, Warbugiaugandensis, Prunusafricana* and *Plectrunthusbarbatus*. Published. 2013; 13.

18. MehrotraV ,Mehrotra S , Kirar V , Shyam R , Misra K , Kumar Srivastava A , Paul Nandi S. Antioxidant and antimicrobial activities of aqueous extract of *Withaniasomnifera* against methicillin-resistant *Staphylococcus aureus*. J Micro Biot Res. 2011, 1 (1): 40-45

19. de Lima Silva L, GieselHeldwein C, Brenner Reetz LG,Hörner R, de MoraesFábio DP, Duarte A, de Moraes Flores EM, Zanella R, Soares Pereira AM , Heinzmann BM. Influence of extraction method on antibacterial activity ofethanolic extracts of *Ocimumgratissimum*L. J of Med plan res. 2015; 9(7): 199-206.

20. Liu H, Wang Q, Liu Y, Chen G , Cui J. Antimicrobial and antioxidant activities of *Cichoriumintybus* root extract using orthogonal matrix design**.**J Food Sci.2013 ;78(2):M258-63.

.

21. Thongson C, Davidson PM, Mahakarnchanakul W, Weiss J. Antimicrobial activity of ultrasound-assisted solvent-extracted spices. Lett Appl Micro. 2004;39(5):401-6.

22. KavithaR, Damodharan N, Deborah U ,Blessing Bukola O. Ultrasonic-

Assisted Extraction and Antibacterial Activites ofProtein Recovred from White Button Mushroom (*Agaricusbisporus*) . Inter J of PharmTech Res. 2015; 8(5): 991-995.

23. Thongson C, Davidson PM, Mahakarnchanakul W, Weiss J. Antimicrobial activity of ultrasound-assisted solvent-extracted Spices. Lett in Appli Micro.2004; 39: 401–406

24. Aboshora W, Lianfu Z, Dahir M, Qingran M, Qingrui S, Jing L, Qaid M Al N , Ammar A. Effect of Extraction Method and Solvent Power on Polyphenol and Flavonoid Levels in *HyphaeneThebaica*LMart (Arecaceae) (Doum) Fruit, and its Antioxidant and Antibacterial Activities. Tro J of Pharmal Res. 2014; 13(12): 2057-2063