Antimicrobial activity of methanol and aqueous extracts of some medicinal plants against *Klebsiella pneumonia*

A. Gh. M. Al-Agha[1], N. J. M. Al-Khafaji and A. Kh. S. Al-Azawi
College of Veterinary Medicine/ University of Diyala

Abstract

The study was conducted to investigate the antimicrobial activity of methanol and aqueous extracts of *Melia azedarach* L. fruit; M. stem, *Lantana camara* flower, L. camara fruit, *Cordia dichotoma* fruit, Ricinus seeds, Pomegranate peel, Senna leaves, using well, and discs agar diffusion methods, in addition to find Minimum Inhibitory Concentration, and Minimum Bactericidal Concentration against *Klebsiella pneumonia* which was isolated from (Operation rooms in hospitals, Wounds, Cow's milk, Vegetable(Salts). Out of the two crude extracts, the methanol extract showed the highest activity. The well diffusion method was better than disc diffusion method as way to evaluate the sensitivity of microorganisms. Pomegranate peel extracts of both methanol and aqueous extract showed the highest anti- Klebsiella activity. The results justify their use in traditional herbal medicine for the management of bacterial based diseases.

Key words: sensitivity, antimicrobial; plant extract, Klebsiella.
e-mail: Al-khafaji@vetmed.uodiyala.edu.iq

الفعالية المضادة للجراثيم لمستخلصات الميثانول والماء لبعض النباتات الطبية ضد الكلبسيلة الرئوية

عبير غسان منذر الأغا، نزار جبار مصلح الخفاجي وعامر خزعل صالح العزاوي
كلية الطب البيطري/ جامعة ديالى

الخلاصة

هدفت الدراسة بحث الفعالية المضادة للكلبسيلة لمستخلصي الميثانول والماء لثمار السبحبح، سيقان السبحبح، أزهار النتانا، ثمار النتانا، ثمار البمبر، بذور الخروع، قشرة لب الرمان، أوراق السنا باعتماد طريقتي الحفر والأقراص، فضلا عن إيجاد التركيز الأدنى المثبط، والتركيز الأدنى القاتل للكلبسيلة المعزولة من (المستشفيات، الجروح، الحليب، والخضراوات). أظهرت النتائج ان مستخلص الميثانول هو الأفضل، وان طريقة الحفر كانت هي الأفضل كطريقة لتقيم الحساسية، فضلا عن ان مستخلصات لب الرمان كانت الأفضل كمضاد للكلبسيلة. وهذا يشير إلى إمكانية اعتمادها كأساس لعلاج الأمراض ذات الأساس الجرثومي.

الكلمات المفتاحية: حساسية، مضاد الجراثيم، مستخلصات النباتات، الكلبسيلة.

Introduction

The use of traditional medicinal plants for primary health care has steadily increased worldwide in recent years. Scientists are in search of new phytochemicals that could be developed as useful antimicrobials for treatment of infectious diseases. Many plants species reported to have pharmacological properties as they are known to possess various secondary metabolites like glycosides, saponins, flavonoids, steroids, tannins, alkaloids and terpenoids, which have been found in Vitro have antimicrobial properties (1, 2, 3). The ethanol extracts of 285 plant materials were screened for biological activities and revealed effective anti- bacterial, and a wide range of pharmacological activities (4). The aims of this study were to explore the antibacterial effects against *Klebsiella pneumoonia* of aqueous and methanol extracts of some famous and important medicinal plants, in addition to compare disc and well diffusion methods.

[1]The paper is part of MSc thesis of first author.

Materials and Methods

- **Test medicinal plants**

- **Isolation and identification of used *Klebsiella spp*.:** Urine samples from patients suffering or not from urinary tract infections were depended to isolate the bacteria by cultivation on MacConkey agar. The isolated bacteria were examined according to its morphological appearance and string test was noticed (Mucoid viscosity), then by biochemical test (IMVIC), followed by Api- 20E test. In addition to confirmation by PCR examination. All lead to obtain clear, pure *Klebsiella pneumonia* isolates which were depended in our studies.
- **Crude plant extracts preparation:** The fruits, leaves, flowers of plants were either purchased from local markets (Senna leaves) or collected from farms (Pomegranate fruit, Cordia fruit, Lantana flowers, Lantana fruits, Ricinus seeds, Melia fruits) during December 2015- may 2016, Baquba city, Diyala province, Iraq. They were air- dried under the shade at room temperature, ground into powder and extracted using Methanol, and water according to standard extraction methods (5).

- **Preparation of extracts**

- **Alcoholic extract:** Thirty-Forty grams of shade dried, powder of plant materials (Cordia fruit; Melia stem; Melia Fruit; Lantana flower; Lantana fruit; Senna leaves and pomegranate peel) were filled separately in the thimble and extracted successively with 250- 300 ml methanol (100%) using a Soxhlet extractor for 48 hours. Extracts then filtered using whatman No.1 filter paper (5). While Ricinus seeds extract prepared according to (6), in which the husks of the seeds were manually removed, the wet flesh then pressed by compressor to remove the oil present. The powder left, either macerated in distilled water or methanol to prepare the aqueous or methanol extract. All above extracts were concentrated using rotary evaporator. After complete solvent evaporation. Each of these solvent extracts was weighed and preserved at 4^{o}C in airtight bottles until further use (5).
- **Aqueous extract:** Thirty- Forty grams of shade dried, powder of above plants were put separately in 300 ml distilled water on magnetic stirrer and left for 24 hours, then filtered through double layer gauze, then through filter paper. The extracts were concentrated using rotary evaporator. After complete solvent evaporation. Each of these solvent extracts was weighed and preserved at 4^{o}C in airtight bottles until further use (5).
- **Sample reconstitution and preparation for use:** Standard concentrations were prepared by dissolving 0.1, 0.2, 0.3 g of dried extract each in 1 ml of methanol or water to obtain 100, 200, 300 mg/ml, and used as the test extracts for antimicrobial activity assay (7, 8).
- **Antimicrobial activity:** Antimicrobial activity was tested using a disc diffusion assay method described by (9, 10). Plant extracts were dissolved in saline. A loop of culture from stock agar was cultured on Muller Hinton agar overnight, and bacterial suspension in normal saline (10^5 CFU/ml) was prepared, and spread with a sterile swab into Petri- plates. Sterile discs (5mm) impregnated with plant extracts (100,200, 300 mg /ml) were places on the cultured plates and incubated for 24 hours at 37 ^{o}C. The solvent loaded disc without extracts in it served as control in the study. In addition, (Amoxiclav, AMC, Doxicyclin, DO, Cefexime, CFM) were used as standard antibiotic discs. The results were recorded by measuring the zones of growth inhibition. Clear inhibition zones around discs indicated the presence of antimicrobial activity. All data on antimicrobial activity were average of triplicate.
- **Agar Well diffusion method:** The modified agar Well diffusion method (11) was conducted to evaluate the antibacterial activities of the extracts. A freshly Colony

Forming Units (10^5 CFU/ml) was aseptically spread onto the surface of Muller Hinton agar and then left to dry for 30 min. Wells (6 mm in diameter) were made in media using a sterilized stainless steel borer. Each well was filled with 100 µl of the crude extract. The plates were left at room temperature for 30 min to allow diffusion of material in media. The controls were prepared using the same solvent. Plates were incubated at 37 ^{o}C for 18-24 hours. Inhibition zones in mm (including well diameter) around wells were measured. The antibacterial activity was expressed as the diameter of inhibition zones produced by the extracts against tested bacteria. All tests were performed in triplicate.

- **Determination of minimum inhibitory concentrations:** The minimum inhibitory concentration (MIC) of the extract was determined. A 16- hour culture was diluted with sterile physiological saline solution (0.9% w/v) sodium chloride) with reference to the 0.5 McFarland turbidometry to achievement the inoculum approximately equal to 10^5 CFU/ml (12). In the tube dilution assay, standard bacterial suspension (0.1ml of 10^5 CFU/ml) and 0.5 ml of different concentration of extracts (100, 200, 400, 800 and 1600mg/ml) were added to tubes containing 2 ml Nutrient broth. These tubes were incubated at 37 ^{o}C for 24 hours. The first tube of the series with no sign of visible growth was considered as the MIC. This process has been done three times (13).
- **Determination of Minimum Bactericidal Concentrations:** To determine the MBC, for each set of test tubes in the MIC assay, a loop full of broth was collected from the tubes without and visible growth and cultured at 37 ^{o}C for 18-24 hours. The highest dilution that yields no colony formation on solids medium was considered as MBC (14).
- **Data analysis:** In order to analyze data, multiple ways ANOVA was used to determine significant factors in production of inhibition zones (15).

Results

The results revealed that the inhibitory zone of methanol extract; Disc diffusion method against *Klebsiella pneumonia* was: the highest inhibitory zone obtained in Pomegranate peel extract. All extract showed inhibitory zones more than that of standard antibiotic disc used in experiment (Amoxiclav AMC; Doxicyclin DO; Cefexime CFM). The inhibition was concentration dependence. The narrowest zone was in 100 mg/ml of Ricinus seed extract and the widest zone was in 300 mg/ml of pomegranate peel extract (Table1).

Table (1) The diameter of inhibitory zone of methanol extract: Disc diffusion method

Extract	Concentration of extract: mg/ ml		
	100	200	300
Cordia Fruit	8.13 ± 1.53	8.38 ± 2.11	10.25± 2.01
Pomegranate peel	10.5 ± 2.07	17.25 ± 2.05	18.50 ± 0.82
Ricinus seed	7.00 ± 1.08	9.63 ± 1.48	9.88 ± 1.41
Lantana fruit	8.25± 1.38	9.25±1.98	11.00± 1.54
Senna	9.25 ± 0.75	9.88± 0.72	11.25 ± 2.70
Lantana Flower	9.00± 0.55	9.25 ± 0.71	10.00 ± 0.50
Control: AMC	6.75 ± 1.18		
: Do	5.88 ± 0.88		
: CFM	6 .00 ± 0.00		

Values are Mean ± S.E., the level of significant at P< 0.05.

The results of the study revealed that in aqueous extract, disc diffusion method, all extracts were exhibited wide zone against *Klebsiella pneumonia* in comparison with control discs. The inhibition was a concentration dependence. The widest zone 15± 3.25 in Pomegranate peel extract. The narrowest zone 6.25± 1.25 was in Lantana flower extract (Table 2).

Table (2) The diameter of inhibitory zone of Aqueous extract: Disc diffusion method.

Extract	Concentration of extract mg/ ml		
	100	200	300
Melia Stem	6.88 ± 1.56	7.25 ± 1.25	10.88 ± 2.41
Pomegranate peel	8.13 ± 1.33	13.75 ± 2.85	15.00± 3.25
Senna leaves	7.00± 0.68	8.00± 1.41	10.25± 1.11
Cordia Fruit	7.00± 1.35	7.88 ± 1.05	8.25 ± 0.66
Lantana Flower	6.25 ± 1.25	7.00± 0.41	7.75± 0.60
Melia Fruit	7.50± 0.61	7.88 ± 0.72	8.75± 2.07
Control	5.75 ± 0.75	6.00± 0.41	7.50 ± 0.29

Values are Mean ± S.E., the level of significant at P<0.05.

The results of the study revealed that all methanol extracts in Well diffusion method exhibited zones of inhibition wider than that of control. In 100 and 200 mg/ml the highest zone of inhibition was exhibited by pomegranate peel extract. While in 300 mg/ ml the widest zone was exhibited by Cordia fruit. The lowest zone of inhibition (6.50±1.50 mm) in Cordia fruit extract at 100 mg/ ml while the widest zoon of inhibition (21.75± 4.25 mm) was in Cordia fruit at 300 mg/ ml (Table 3).

Table (3) The diameter of inhibitory zone of methanol extract: Well diffusion method

Extract	Concentration of extract mg /ml		
	100	200	300
Cordia Fruit	6.50 ± 1.50	15.0 ± 0.00	21.75 ± 4.25
Pomegranate peel	13.0 ± 0.50	15.25 ± 4.75	17.25 ± 1.25
Ricinus seed	9.25 ± 0.75	13.25 ± 1.75	14.25 ± 0.75
Lantana Fruit	10.5 ± 3.50	11.00 ± 2.50	12.50 ± 4.00
Senna leave	7.25± 0.25	7.75 ± 1.75	10.50 ± 4.50
Control	6.75 ± 0.25	7.00 ± 1.00	7.75 ± 2.46

Values are Mean ± S.E., the level of significant at P<0.05.

The results of the study revealed that all extracts exhibited wider zones of inhibition than that of control. In 100, 200 and 300 mg/ ml concentration of extracts the widest zone of inhibition was exhibited by pomegranate peel. The lowest zoon of inhibition (5.00 ± 0.00 mm) was exhibited by lantana flower at 100 mg /ml while widest zone (19.5±0.00 mm) was exhibited by pomegranate peel extract (Table 4).

Table (4) The diameter of inhibitory zone of Aqueous extract: well diffusion method

Extract	Concentration of extract: mg/ml		
	100	200	300
Melia Stem	6.00 ± 0.00	8.00 ± 3.00	9.50 ± 0.00
Pomegranate peel	13.00 ± 0.50	14.0 ± 0.00	19.50 ± 0.00
Senna leave	8.00 ± 2.00	10.75 ± 0.25	11.00± 3.50
Cordia Fruit	6.25± 0.25	6.00 ± 1.00	7.00 ± 0.50
Lantana Flower	5.00± 0.00	7.25 ± 0.75	10.0 ± 3.50
Melia Fruit	6.0 ± 0.00	6.0 ± 1.00	7.25 ± 1.25
Control	5.00 ± 0.00	5.50 ± 0.500	6.25± 0.25

Values are Mean ± S.E., the level of significant at P<0.05.

The results revealed that in general methanol extract in well and disc diffusion methods show wider inhibitory zones than aqueous extracts. While in aqueous extract disc diffusion showed wider inhibition than in well diffusion methods (Tables 1, 4).

In methanol and aqueous disc diffusion methods, aqueous well diffusion method pomegranate peel exhibited the widest inhibition zone while in methanol extract, Well diffusion method Cordia fruit extract exhibited widest zone of inhibition (Tables 1, 4).

- **Minimum inhibitory concentrations (MIC):** In aqueous extracts Melia stem, and Melia fruit were exhibit no growth at 1600 mg/ ml. pomegranate peel at 400, 800, and 1600 mg/ ml. (Table 5).

Table (5) The Minimum inhibitory concentration of extract against *Klebsiella pneumonia*: Aqueous extract

Extract	Concentration of extract mg /ml				
	100	200	400	800	1600
Melia Stem	+ve	+ve	+ve	+ve	-ve
Pomegranate peel	+ve	+ve	-ve	-ve	-ve
Senna leave	+ve	+ve	+ve	+ve	+ve
Cordia Fruit	+ve	+ve	+ve	+ve	+ve
Lantana Flower	+ve	+ve	+ve	+ve	+ve
Melia Fruit	+ve	+ve	+ve	+ve	-ve

In methanol extracts, Cordia fruit, Ricinus seed, Lantana fruit, exhibit no growth at 800 and 1600 mg/ml, while Senna leave and Lantana flower exhibit no growth at 200, 400, 800, 1600 mg/ ml, Pomegranate peel extract exhibit no growth at 100, 2-00, 400, 800, 1600 mg/ml (Table 6)

Table (6) The Minimum inhibitory concentration of extract against *Klebsiella pneumonia:* methanol extract

Extract	Concentration of extract mg /ml				
	100	200	400	800	1600
Cordia Fruit	+ve	+ve	+ve	-ve	-ve
Pomegranate peel	-ve	-ve	-ve	-ve	-ve
Ricinus seed	+ve	+ve	+ve	-ve	-ve
Lantana Fruit	+ve	+ve	+ve	-ve	-ve
Senna	+ve	-ve	-ve	-ve	-ve
Lantana Flower	+ve	+ve	-ve	-ve	-ve

Minimum bactericidal concentrations were exhibited by Melia stem, pomegranate peel, Lantana flower at 1600 mg /ml of aqueous extract. While in methanol extract, Pomegranate peel, Lantana fruit exhibit at 800, 1600 mg/ ml, and Lantana flower at 1600 mg /ml (Table 7).

Table (7) Minimum Bactericidal Concentration

Type of extract	Extract	Concentration giving +ve result
Aqueous	Melia stem	1600 mg/ml
	Pomegranate peel	1600 mg/ml
	Lantana flower	1600 mg /ml
Methanol	Pomegranate peel	800 mg /ml
	Lantana fruit	1600 mg /ml
	Lantana flower	1600 mg /ml

Discussion

Extract of *Punica granatum* has been reported to have various medicinal values, antibacterial (16, 17). Pomegranate fruit extracts constituents possesses immense biological activities such as antibacterial (18, 20). These therapeutically activities are related to the presence of diverse 'phenolic compounds', tannins and anthocyanin; Inhibitory effect of pomegranate is always attributed to the pomegranate antioxidant activity that depends mainly on the phenolic and anthocyanin content of the fruit (20, 32). (33) reported, the highest antibacterial activity was recorded against *Klebsiella pneumonia* and among fungi high activity against *Aspergillus parasiticus* was recorded. The peel extract was shown no activities against *Salmonella typhi, Bacillus cereus* and *Aspergillus flavus.* Majority of the *Lantana camara* activity is due to bioactive compounds viz, flavones, isoflavones, flavonoids, isocatechins, alkaloids, tannin, saponins and triterpenoids. (34). (35), have reported that the leaves extract of *Lantana camara* be active against various gram positive and gram negative bacteria. The

essential oil of *Lantana camara* exhibited prominent antibacterial activity against all the bacterial strains tested. Gram positive *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* were the most sensitive strains to *L. camara* essential oil. Nevertheless, Gram negative *Klebsiella pneumonia* and *Pseudomonas aeruginosa* were not susceptible to the essential oil at lower concentration. (36). There has not been elaborate published work on the antimicrobial activity of fermented *Ricinus communis* extracts involving microbes (37). *Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris,* and *Staphylococcus aureus* were highly susceptible to both the methanol and water extracts of the seed (38). The essential oil showed strong antimicrobial activity against all microorganisms tested with higher sensitivity for *Bacillus subtilis, Staphylococcus aureus* and *Enterobacter cloacae* (39). The antimicrobial activity of *R. communis* essential oil is strictly connected to their chemical composition (40). *Melia azedarach* L. is found to possess numerous medicinal properties, such Antibacterial (41). The methanol extract of Senna leaves crude was the most active of the two crude extracts tested against bacterial organisms (42). The patterns of antimicrobial activity varied with the plant extract and the solvent used for extraction. The organic crude extracts showed more inhibition than the aqueous extracts on the average for all plants extracts tested (43). It is possible that the aqueous crude extracts may contain antimicrobial constituents insufficient for efficacy and which may explain why large amounts of the decoctions must be drunk by the patients (43). This observation is supported by (44), who also found out that, aqueous extracts showed little or no antimicrobial activity in contrast to those made using organic solvents. The antimicrobial effects can be attributed to the phytochemicals of the plants used in our study: In phenols and polyphenols include phenolic toxicity to microorganisms which include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (45); Quinones, they provide a source of a stable free radicals and are also known to complex irreversibly with nucleophilic amino acids in protein (46), often leading to inactivation of the protein and loss of function, they may also render substrates unavailable to the microorganism; Flavoines, flavoinoids and flavonols, they are known to be synthesized by plants in response to microbial infection (47) and found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (48); Tannins one of their molecular actions is to complex with proteins through so- called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (45). Tannins possess inhibitory activities and reported to be toxic to filamentous fungi, yeast and bacteria (49). Terpenoids and essential oils, activities of terpenenes or terpenoids have been demonstrated against bacteria (50). Lectins and polypeptides, Peptides are inhibitory to microorganisms are often positively charged and contain disulfide bonds. The formation of ion channels in the microbial membrane (51) or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors (52) could be their mechanism, of action. From literature search, the methanol extracts of leaves, flowers, stem and root of *Cassia alata* had been shown to have a broad spectrum of antibacterial activity after fractionating with petroleum spirit, dichloromethane and ethyl acetate. The studies of (53 and 54), reported that bioactive extracts of medicinal plants at moderate concentrations are active against unsusceptible bacteria. (46) Reported that flavonoids and terpenoids were highly present in all extracts that were screened for *S. didymobotrya* stem and root. Flavonoids have been associated with inhibition of cytoplasmic membrane functions as well as

inhibition of DNA gyrase enzyme and carrier protein activities. The extent of the antimicrobial activity was related to the extract of the plant part used. (43). These results are significant in early stages of drug research as reported by (55) that intermediate susceptibility results indicate that the therapy with the drug can be successful only if it is administered in larger doses or when the drug is concentrated at certain site as urinary tract or dermal. The variation could have affected the chemical composition of the plants and thus its antimicrobial activity. Therefore, the antimicrobial agents were either present in very little quantities or totally absent. This argument is in line with that of (56) who argues that seasonal variations and part of plant species harvested at different stages of growth and development, can affect chemical composition of the plants, and thus its biological activity. In most cases, maximum accumulation of chemical constituents occur at the flowering season and declines at the beginning of the fruiting stage and this can lead to either presence of chemicals in high or very low.

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