The inhibitory effect of celery, peppermint extracts and their mixture in comparison with amikacin on uropathogenic isolates: an *in vitro* study.

Abstract:

Urinary tract infections (UTIs) are regarded as amongst the most common serious health problems that affect millions of people each year. The antibacterial activity of two different plant extracts & their mixture at two different concentration (100 & 200 mg/ml) were carried out against two uropathogens Known as mainly bacterium induced urinary tract infections in humans & animals.

The dried extracts of celery seeds and peppermint leaves and their mixture were tested *in vitro* against six isolates of *E.coli* & three isolates of *K.peumoniae* by agar well diffusion & micro-diluted method. Amikacin (10µg) was used as reference antimicrobial. The plants extracts and their mixture showed closed related, potent antibacterial activity and inhibition patterns varied mainly according to the concentration of extracts. Moreover, the *E.coli* & *K.peumoniae* showed nearly same sensitivity towards the studied plants in culture media and micro-diluted plate. The MIC was measured using standard two-fold microdilution broth methodology at concentrations ranging from ٨ µg/ml to ٤٠٩٦ µg/ml. The MIC values of the ethanolic extracts of celery, peppermint & their mixtures were in the range of ٢٥٦ to ٥١٢ µg/ml against *E.coli* and ١٢٨ to ٥١٢ µg/ml against *K.pneumoniae*.

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Key words : antibacterial activity , celery , peppermint , uropathogenic isolates

التأثير التثبيطي لمستخلصي الكرفس والنعناع ومزيجهما مقارنة بعقار الاميكاسين على عزلات من اصابات الجهاز البولي : دراسة في المختبر

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الخلاصة :

تعد اصابات الجهاز البولي احد المشاكل الصحية الخطرة والشائعة والتي تصيب ملايين البشر سنويا . تم دراسة الفعالية المضادة للجراثيم لاثنين من النباتات الطبية ومزيجهما وعند تركيزين مختلفين وهما (١٠٠ ، ٢٠٠ ملغرام/مل) انجزت ضد ممرضين من ممرضات الجهاز البولي معروفة كمسببات مهمة لاصابات الجهاز البولي في الانسان والحيوان.

فحصت مستخلصات بذور الكرفس واوراق النعناع ومزيجهما ضد نمو ٦ عزلات من جرثومة الاشريكيا القولونية و٣ عزلات من جرثومة الكلبسيلا الرئوية بطريقتي حفر الاكار والتخفيف بالانابيب الدقيقة . الاميكاسين (١٠ مايكروغرام) استخدم كمادة قياسية . المستخلصات النباتية ومزيجهما اظهرا نتائج متقاربة تتمثل بفعالية مضادة للجراثيم قوية وتباين نمط التثبيط الذي كان معتمدا بصورة رئيسية على تركيز المستخلصات المستخدم. مع هذا فان جرثومتي الاشريكيا القولونية والكلبسيلا الرئوية اظهرا حساسية متقاربة أتجاه المستخلصات النباتية قيد الدراسة في الاطباق الزرعية والانابيب المختبرية .

تم حساب قيمة التركيز المثبط الادنى باستخدام طريقة Two fold microdiluted method بتراكيز تراوحت بين ٨ مايكروغرام/مل الى ٤٠٩٦ مايكروغرام/مل واظهرت نتائج تقدير التركيز المثبط الادنى للمستخلص الايثانولي لنباتي الكرفس والنعناع ومزيجهما انها كانت تقع بين ٢٥٦ الى ٥١٢ مايكروغرام/مل ضد نمو الاشريكيا القولونية و ١٢٨ الى ٥٥٦ مايكروغرام/مل ضد نمو الكلبسيلا الرئوية .

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الكلمات المفتاحية : الفعالية المضادة للبكتريا ، الكرفس ، النعناع ، عزلات الجهاز البولي

Introduction:

Urinary tract infections (UTI) are a widespread problem and considered the second most common type of infection in the body. There are estimated ١٥٠ million urinary tract infections per year world wide.[١,٢] Urinary tract infection is defined as the proliferation of active microorganisms inside the urinary channel which are harmful to their environment [٣] Most of the infections are caused by

bacteria normally present on the skin or in the intestinal tract that invade the urinary tract. UTI'S are more common in persons aged ٢٠-٥٠ years. Approximately ٩٥٪ of infections occur when bacteria ascend through the urethra and the bladder [٤,٥] A urinary tract infection (UTI) is very common infection that occurs when bacteria enter and multiply anywhere along the normally sterile urinary tract[٦].

Antibiotics are the beneficial route to treat UTI s. However, due to the rapid development of bacterial antibiotic resistance and side effects, alternate solutions for infection prevention are of great interest [٤] Medicinal plants are extensively used for the management and treatment of various diseases. Plant drugs are considered nontoxic and devoid of side effects [٧,٨] Medicinal plants have been used for centuries as remedies for human & animals diseases because of their therapeutic values. Hence, the quest for plants with medicinal properties ranging from antibiotics to antitumor continues to receive attention[٩] Use of indigenous plants as remedy against various diseases is increasing because synthetic drugs possess many side effects[١٠] Attention has been drawn to the antibacterial activity of plants and their metabolites due to the challenge of growing incidences of drug-resistant pathogens [١١] Celery, *Apium graveolens L.* is a plant from the family of Apiaceae that has been used as food and as medicine. A review of the literature indicates that celery has been cultivated for the last 3.000 years [12]This plant is native to the Mediterranean region that is also cultivated in other parts of the world [١٣]Seeds of the celery plant have been used in ayurvedic medicine and have been found useful in herbal medicine for the treatment of urinary calculi, gut diseases, relief of flatulence, various painful states, reduction of visceral spasms, and stimulation of the smooth muscle of the womb [١٤,١٥] Celery seeds have wide range of pharmacological activity as anti-rheumatism, sedative, antiseptic urinary tract, increased excretion of uric acid, blood pressure lowering, to some extent

against fungal diseases, diuretic, analgesic, anti-inflammatory, detoxification, anti-spasmodic, anti-bacteria, binding rules, anti-contractions, seizures, stomach tonic and is carminative [١٦]. *Mentha piperita* is a herb, which is commonly, used in folk medicine, in most part of the world for flatulent colic, appetite, to relieve abdominal pain, fever, nausea and vomiting and digestion [١٧]. Herbalists consider peppermint an astringent, antiseptic, antipruritic, antispasmodic, antiemetic, carminative, diaphoretic, mild bitter, analgesic, anticatarrhal, antimicrobial, rubefacient, stimulant, and emmenagogue [١٨] The development of microbial resistance to the available antibiotics and the increasing acceptance of traditional medicine as an alternative form of health care have led researchers to investigate the antimicrobial activity of medicinal plants[١٩,٢٠] So the aim of this work was to evaluate the antibacterial ability of two different plant extract and their mixture on the uropathogenic isolated that have been isolated from urinary tract infections *in vitro.*

Materials & Methods:

- **Plants materials & preparation of extracts :**

The plants materials used in this study including the seeds of celery plant & leaves of peppermint plant were purchased from local market; the air-dried plant materials were ground into fine powder and extracted with hot ethanol (٩٩٪). After filtration of total extracts, the extracts were evaporated to dryness in rotary evaporator apparatus at a low temperature (٤٠-٦٠°C) until all the solvent had been removed to obtain an alcohol free extract sample then weighed the yeild. [٢١]

- **Uropathogens strains :**

Urinary tract pathogens including six *Escherichia coli* isolates & three *Klbsiella pneumoniae* isolates were isolated from the urine of patients diagnosed with urinary infections in hospital of children in Al-Diwanyia city. All isolates were Identified by colony morphology , gram staining and biochemical characterization[٢٢]

- **Antibacterial activity :**

The antibacterial activity of celery and peppermint ethanolic extracts were tested by agar well diffusion method according to the method of Ahmad and Beg as follows: Bacterial suspension (١٠٦ CFU/ml) of *E.coli* ATCC ٢٥٩٢٢ & each one alone were prepared in normal saline from an overnight culture, compared with ٠.٥ McFarland tubes and cultured onto Muller Hinton agar. Celery extract, peppermint extract and their mixture were prepared in different concentrations (١٠٠, ٢٠٠ mg/ml) & tested for their antibacterial activity in culture media. Diluted ethanol (٤٠٪) used as negative control whereas Amikacin (١٠µg) used as positive standard to determine the sensitivity of the tested microorganism. Each test was performed three times. The plates were then incubated at ٣٧°C for ١٨-٢٤ hours and the mean diameter of inhibition zone was calculated. [٢٣]

- **Determination of Minimum inhibitory concentration :**

The MIC (minimum inhibitory concentration) of of celery extract, peppermint extract and their mixture each alone were evaluated using standard two-fold microdilution broth methodology. A stock solution of each extracts was serially diluted in ٩٦-wells microtiter plate with Mueller Hinton broth to obtain a concentrations ranging from ٨ µg/ml to ٤٠٩٦ µg/ml. A standardized inoculum for each bacterial strain was prepared so as to give an inoculum size of approximately ٥ x ١٠^{٥} CFU/ml in each well. Microtiter plates were then kept at ٣٧°C for an overnight incubation. Following incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacterial strain using reflective viewer. [٢٤]

Statistical analysis:

One way analysis of variance (ANOVA) was used to compare the means of different extracts & concentrations & positive & negative

control while least significance difference (LSD) test helped to compare the group means at ٥٪ level of significance[٢٥]

Results & Discussion:

Emergence of resistance among pathogenic bacteria against available antibiotics is becoming a great challenge to the current world. Thus, there is a great need to discover novel antibiotics. So traditional plants have been proved to be novel source in the search of antimicrobial compounds. In this connection, the present study goal to investigate the activity of two different plants extracts (celery & peppermint) & their mixture on the two important uropathogens isolates recorded as causative agents in urinary tract infections.

The ethanolic extracts of celery seeds, peppermint leaves & their mixture exhibited potent antibacterial activity against six *E.coli* isolates & three *K. peumoniae* isolates compared with negative control (diluted ethanol ٤٠٪) (Table ١, figure ١,٢,٣) were expressed as mean± standard error & this depending largely upon the extract concentration (١٠٠ or ٢٠٠mg/ml).there are no significant differences among two plant extracts and their mixture in their effectiveness against tested bacteria ($p<٠.٠٥$) at same concentration. In the same time there is a significant differences ($p<٠.٠٥$) in the effectiveness of two concentration of extracts used in this study that recorded according to the zone of inhibition around the hales in culture media this attributed mainly to the increase concentration of extracts accompanied by increase the active compounds or ingredients present in the plant extracts. Number of researchers from different countries investigated antimicrobial effectiveness of peppermint extracts and its constituents and reported that greatest activity of this plant which caused by

compounds neomenthol & carvacol [٢٦] .Singh & his colleagues (١٩٩٨)[٢٧] revealed that the peppermint extract was more active against gram positive organism than gram negative. In general , essential oils that present in the peppermint play important role in their antibacterial activity by cause damage to biological membrane due to their lipophilic characteristics.[٢٨] Also Studies by other researchers have also confirmed our results in this aspect. A study carried out by Koohsari and his colleagues (٢٠٠٦) reported that essential oil of peppermint plant had a strong antibacterial effect on *Escherichia coli*. .[٢٩] Bupesh & his colleagues(٢٠٠٧) indicated that leaf peppermint extracts by using four different solvents were found to have antibacterial effects against five pathogenic bacteria isolated from different types of infections. [٣٠] The principal active constituents of *Mentha piperita* are the essential oils, which comprise about ١.٢-١.٥ % of the herb. The oils are dominated by monoterpenes, mainly menthol, menthone, and their derivatives as isomenthone, neomenthol, acetylmenthol, pulegone. These essential oils have ability to inhibit bacteria. Its oils especially menthol have a broad spectrum antibacterial activity against Gram +ve and Gram -ve bacteria which found very sensitive to the oils[٣١] Menthol is the active compound in the peppermint and responsible saliently for the antibacterial characteristics of plant in addition to the spasmolytic nature, stimulates bile flow, reduces the tone in the esophageal sphincter, and facilitates belching. [٣٢] Nowadays, menthol is added in commercial tooth pastes to offer protection against oral microbial infections. [٣٣] In the same time, number of researchers investigates the antimicrobial potency of celery extracts & its constituents against wide number of microorganism in culture

media , Sipailiene and his colleagues reported that dried roots and leaves of celery were extracted with liquid carbon dioxide in the pilot plant scale equipment .The antimicrobial effect was assessed using agar diffusion method. It was found that all the investigated leaf extracts were effective inhibitors of *H. alvei, S. aureus, E. coli, Bac. cereus, E. faecalis* and *E. aerogenes*, however the extracts isolated from the roots were less effective; all of them possessed high activity only against *B. cereus* and *E. faecalis. C. freundii* and *P. vulgaris* were resistant against celery extracts isolated both from roots and leaves. The main constituents in the oil of roots were limonene, carvone and ٣-n-butylphthalide. The essential oil of leaves contained higher amount of limonene comparing to the roots, and very small amount of carvone.[٣٤] In another study Alshwaikh & his colleagues showed that watery extract from the celery inhibited the growth of various species of gram positive and gram negative bacteria that isolated from the UIT infection in the children under fifteen years old. [٣٥] Some studies had shown that Celery Seed oils can help as an herbal remedy today, Celery Seed is most commonly used as a natural diuretic, as well as a treatment for Urinary Tract Infections due to it is anti-bacterial properties.[٣٦]

Active extracts obtained by agar well diffusion assay were further subjected to determine the MIC required for the bacteriostatic effects by standard two-fold broth microdilution methodology, table (٢) represent the MIC values of two plants & their mixture against susceptible bacteria. All tested extracts showed significant differences in MIC values depending on the tested bacteria & type of plant extract.

Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of micro-organism to an antimicrobial agent and it monitors the activity of new antimicrobial agents. .[٣٧] It is plausible to conclude that Peppermint leaves & Celery seed extracts represent novel class of antimicrobial agents which may be of major impact for the treatment of urinary tract infections caused by these two types of bacteria. Further detection regarding their structural characterization, molecular mechanism of action and its toxicity profile would be of great importance to cope with the diseases which urgently need novel antimicrobial agents without problem of bacterial resistance.

Table (١): the inhibitory effect of celery, peppermint & their mixture compared with amikacin against uropathogenic bacteria *growth* in culture dishes.

Type of extract	Concentration	Zone of inhibition against isolates(mm)	
		E.coli	*K.pneumoniae*
celery extract	١٠٠ mg/ml	١٧.٤٨±٠.٩١ A	٢٠.١±٠.٣٣ A
	٢٠٠ mg/ml	٢٦.٨٢±١.٠٣ B	٢٦.٩٨±٠.٩ B
Peppermint extract	١٠٠ mg/ml	١٨.١٩±٠.٣١ A	١٩.١١±١.٠٨ A
	٢٠٠ mg/ml	٢٧.٢٥±٠.٥٢ B	٢٩.١٢±٠.٤٩ C
Celery & peppermint mixture	١٠٠ mg/ml	١٩.٩٥±١.٤٨ A	٢١.٢١±١.٠٩ A
	٢٠٠ mg/ml	٢٩.٦٨±١.٢٤ B	٣٠.٧٥±٠.٥٦ C
amikacin	١٠ µg	٢٨.٨٤±٢.١٢ B	٢٦.١٢±١.٤٢ B
Negative control	---	٠±٠ C	٠±٠ D

- The result denote to means of ٦ isolates of *E.coli* and ٣ isolates of *K.pnemoniae* ± standard error for mean
- In each well , the sample volume was ١٠٠ µl
- Values of inhibition zone in mm diameter including the diameter of well (٦mm) after ٢٤ hours incubation period.
- The similar letters refers to statistical non-significant differences whereas the different letters refers to significant differences at (p<٠.٠٥)

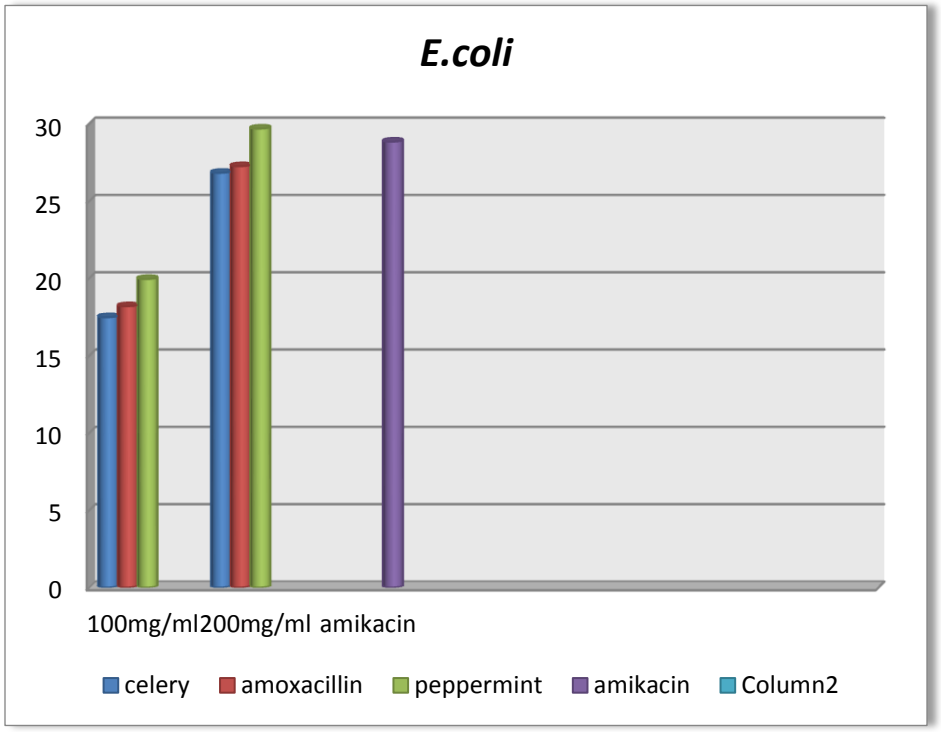

Figure (١): inhibitory effects of celery , peppermint and their mixture compared with amikacin against *E.coli* growth in culture dishes

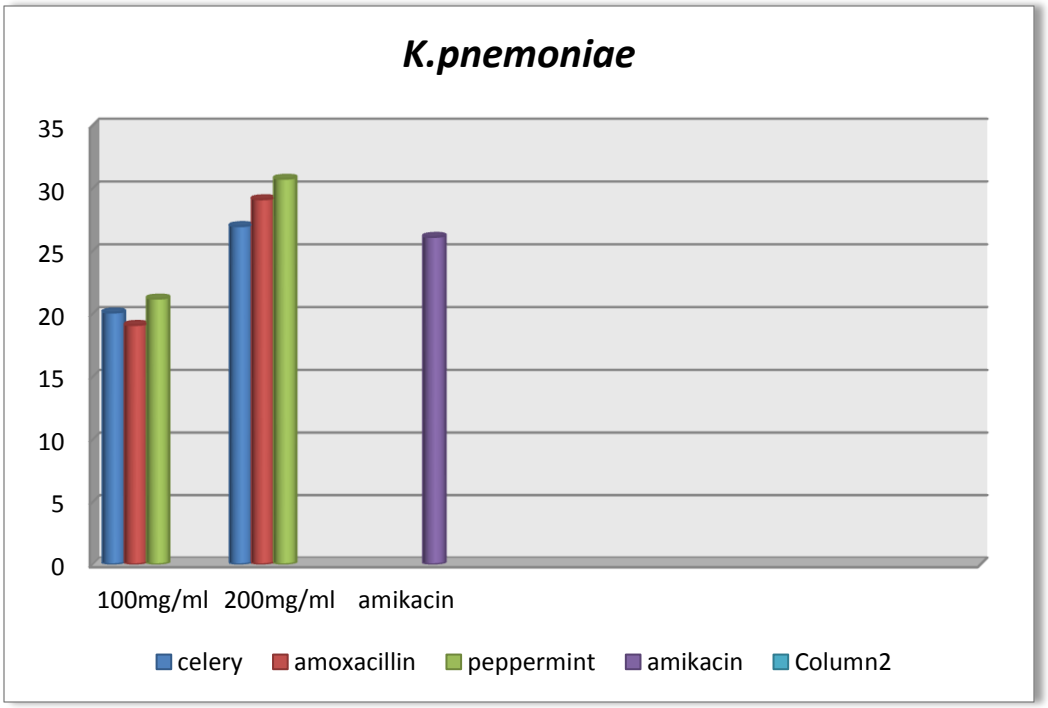

Figure (٢): inhibitory effects of celery , peppermint and their mixture compared with amikacin against *K.pneumoniae* growth in culture dishes

Table (٢) : The MIC values in (µg/ml) for celery , peppermint and their mixture against *some* uropathogens in microtubes.

Extract type	Type of bacteria	
	E.coli	***K.peumoniae***
Celery ethanolic extract	٥١٢	٢٥٦
Peppermint ethanolic extract	٢٥٦	٢٥٦
elery & peppermint mixture	٢٥٦	١٢٨

Values denote to the MIC value.

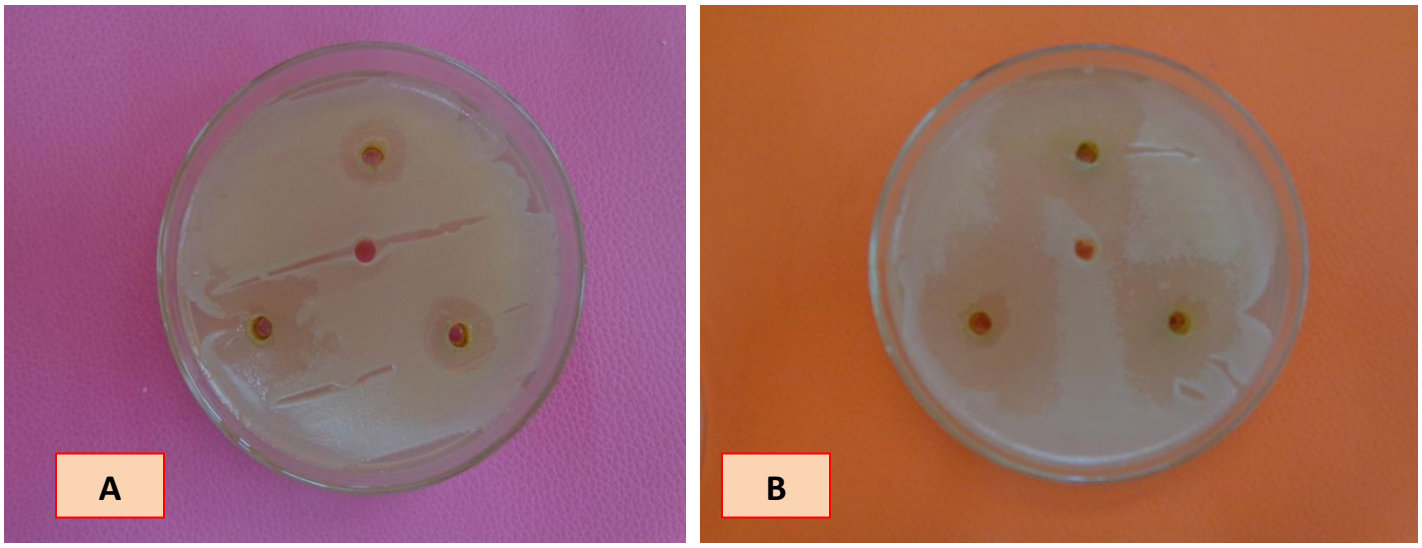

Figure ٣ : [A]: Inhibitory activity exhibited by celery seeds extract (٢٠٠ mg/ml) against *E.coli* in culture media; [B] : Inhibitory activity exhibited by peppermint leaves extract (٢٠٠ mg/ml) against *E.coli* in culture media.

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