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Synergistic Effect of Antibiotics and Plant Extract to Control Clinical Bacterial Isolates Implicated in Urinary Tract Infections

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Abstract: A total 100 urine samples were collected from patients examinated for urinary tract infection in Zagazig University hospitals, Urology Department, Egypt. A positive bacterial growth on CLED, MacConkey's agar and nutrient agar was detected. *E. coli* was the most predominant pathogen causing urinary tract infection in positive samples (40%) followed by *Proteus mirabilis*, *Klebsiella pneumoniae*, *Staphylococcus saprophyticus* and *Pseudomonas aeruginosa* were present percentage 22, 15, 13 and 10% respectively. Antibiotic sensitivity test for Gram-positive and negative bacteria showed that the antibiotic ofloxacin is more effective against clinical bacterial isolates (58%) followed by amikacin (54%), chloramphenicol (52%), norfloxacin(51%) and azithromycin (48%). The effect of medicinal plant extracts against highly resistant bacterial isolates showed that, the clove, rosemary, peppermint, hibisicus, thyme and cinnamon showed strong inhibitory action against tested isolates. The combination between ofloxacin and amikacin with clove plant extract showed that the clearly synergistic effect was obtained against tested clinical bacterial isolates. The protein band of *P. aeruginosa*, *P. mirabilis* and *K. pneumoniae* of normal growth (control) and under stress of combined of ofloxacin and clove extract showed new protein bands appeared as resistant protein to treatment stress. The active pure clove substance showed antibacterial activity was identified by ^{1}HNMR and IR as eugenol structure.

Key words: Antibiotics, plant extract clinical bacteria urinary tract Infections

INTRODUCTION

Urinary tract infection (UTI) remains the most common reason for outpatients to seek medical care and for inpatients to develop nosocomial infections. Nosocomial UTIs account for up to 40% of all hospital-acquired infections. The associated morbidity and mortality are the major drain on hospital resources[30]. Females are however believed to be more affected than males except at the extremes of life; this is a result of shorter and wider urethra[8]. The most common cause of UTI is Gram-negative bacteria that belong to the family *Enterobacteriaceae*. Members of this family include *Escherichia coli*, *Klebsiella*, *Enterobacter*, and *Proteus*. Also the Gram-positive *Staphylococcus saprophyticus* plays a role in the bacterial panorama, especially among young women. *E. coli* dominates as causative agent in all patient groups. In un complicated UTIs, 80-90% are caused by *E. coli*[20]. In complicated UTIs, *E. coli* is less prominent but still the major causative agent[25]. Conventional drugs usually provide effective antibiotic therapy for bacterial infections but there is an increasing problem of antibiotic resistance and continuing need for new solutions[19]. The etiology of UTI and the antibiotic resistance of uropathogens have been changing over the past years, both in community and nosocomial infection[24]. The use of plant extract to treat infections is an old practice in a large part of the world, especially in developing countries, where there is dependence on traditional medicine for a variety of diseases[11]. Numerous researches showed that the essential oils and plant extracts have potential in medical procedures and applications in the pharmaceutical, cosmetic and food industry[13]. Hersch *et al.*[14] found that *Cinnamomum verum*, *Origanum vulgare* and *Thymus vulgaris* extracts, showed the highest and broadest antibacterial activities against 189 Gram-negative and 135 Gram-positive pathogenic bacteria isolated from pediatric patients showed resistance to selected antibiotics. *Syzygium aromaticum* have many therapeutic uses; they relieve pain, control nausea and vomiting improve digestion, protect against internal parasities and act as antimicrobial agents against fungi and bacteria cause uterine contractions and are strongly antiseptic[6]. About four grams of *Syzygium aromaticum* are boiled in three liters of water until half the water has evaporated. This water, taken in draughts, will slow down severe symptoms of cholera[18]. In general medicine, *Syzygium aromaticum*

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is used as an agent against flatulence, stomach distension and gastro-intestinal spam[10]. Eugenol a main constituent of the essential oil obtained from commonly consumed species such as *Pimenta racemosa*, *Cinnamomum verum* and *Syzygium aromaticum* is used as antiseptic, antibacterial, analgesic agent in traditional medical practices[12]. Eugenol is a phenolic compound, which it is the main component of clove plant, with the antimicrobial data against isolated bacterial organisms. Caryophyllene has also been shown to possess antimicrobial properties, which obtained as second constituents obtained from plant[3]. The aim of the present study to evaluate the antibiotics action against bacterial pathogens to UTI by using different medicinal plant extract combined with antibiotics to minimize the dose and duration of antibiotics used. Determination the protein bands of bacterial under stress of antibiotic combined with the plant extract and identified the most active substance purified from active medicinal plant as eugenol.

MATERIALS AND METHODS

The present study is conducted on 100 urine samples were taken from patients who were examinated for UTIs in Zagazig University hospitals (inpatients) or attending Zagazig University clinics (outpatients), Urology Department, these samples were collected from April 2007 to August 2007. A positive bacterial growth on CLED, MacConkey's agar and nutrient agar, the diagnosis of UTIs in urine samples was based on the presence of $\geq 10^5$ CFU of microorganisms per ml in urine culture[7]. Also, presence of more than 5 pus cells per high power field in an unspun urine in male, and more than 10 pus cells in a female, and is defined as pyuria[26]. High number of pus cells in urine, or pyuria, usually indicates infection.

Bacterial isolates were identified according to the key of (Bergey's Manual) of Determinative Bacteriology[15]. All strains of bacteria isolated were tested for antimicrobial sensitivity by standardized disc diffusion technique, which was done as described by Bauer *et al*.[5]. Take one colony from each isolated bacterial isolates by sterile loop then inoculated into 5 ml sterile nutrient broth and incubate for 24 hours at 37 °C, then make turbidity equal 0.5 Mc ferland standard salin (0.05 ml barium chloride + 9.95 ml sulfuric acid)[4]. Each broth inocula were applied by sterile swabs on Muller-Hinton agar plates; two plates were used for each isolates in which antibiotics discs were applied to the surface of plates at constant distances. The antibiotics used in this experiment were penicillin-G (P.G) (10µg), ampicillin (AM) (10µg), azithromycin (AZM) (15µg), norfloxacin (NOR) (10µg), ofloxacin (OFX) (5µg), amikacin (AK) (30µg), chloramphenicol (C) (30µg), cefotaxime sodium (CTX) (30µg). The plates were incubated at 37 °C for 24h. The inhibition zones were measured with a millimeter ruler. The entire diameter of inhibition zone was measured including the diameter of the disc. The end point of the reading was taken as complete inhibition of the growth to the naked eye[29].

The antibiotics, ofloxacin, amikacin were selected according to sensitivity of different bacterial isolates to determine the MIC and MBC against tested bacterial isolates namely *E. coli*, *Klebseilla*, *Proteus*, *Pseudomonase* and *Staphylococcus,* according to Lowry,[22]. The medicinal plant extracts were prepared according to Huang *et al*.,[16].

Bacterial protein extraction was performed according to Saxena *et al*.,[27], where protein content were extracted and determined in treated bacteria (combination between MIC of ofloxacin for each tested organisms and extract of *Syzygium aromaticum*) and control (non-treated) bacteria.

Spectroscopic analysis of the purified antimicrobial substances obtained from *Syzygium aromaticum* by TLC method was performed by Microanalytical Center of Cairo University, Egypt. The infra-red (IR) spectrum of the active fraction was determined in potassium bromide (KBr)[21]. Infra-red spectrophotometer is model PYE Unicum SP1100 USA. Also, the active fraction was determined in Dimethyl sulfoxide (DMSO) using nuclear magnetic resonance (H-NMR)[28]. Nuclear magnetic resonance is model Gemini-200 ^{1}HNMR.

RESULTS AND DISCUSSION

The urine samples have been collected from different ages of males and females. The results in Fig. (1) indicated that the number of contaminated urine samples collected from males and females were 100 samples, 40 contaminated with *E. coli*, 22 of *Proteus mirabilis*, 15 of *Klebsiella pneomoniae*, 13 of *Staphylococcus saprophyticus* and 10 of *Pseudomonase aeruginosa*. So the highest percentage of distribution are found in *E. coli* (40.0%) followed by *Proteus mirabilis* (22.0%), *Klebsiella pneomoniae* (15.0%), *Staphylococcus saprophyticus* (13.0%) and *Pseudomonase* aeruginosa (10.0%). Out of the (87.0%) of all isolates that belong to Gram-negative organisms, *Enterobacteriaceae* constitute the main group (77.0%), while *Pseudomonas aeruginosa* was responsible for only (10.0%) of the infection. (13.0%) of the isolates belong to the Gram-positive cocci; *Staphylococcus saprophyticus*. Mady and Helmi,[23] found that 83.1% of all isolates belong to Gram-negative organisms. Out of these, *Enterobacteriaceae* constitute the main group (79.0% of all isolates), while *Pseudomonas aeruginosa* was responsible for only (4.1%) of the infection.

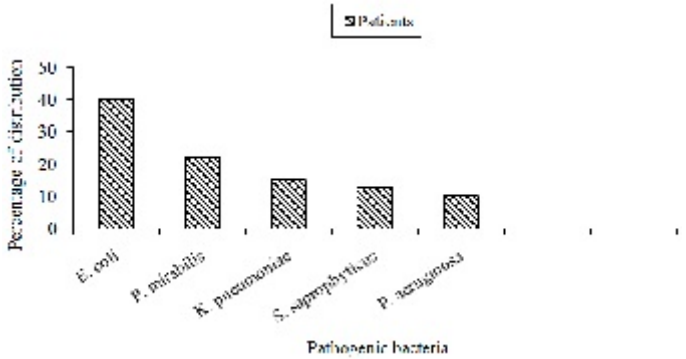


Fig. 1: The distribution number of pathogenic bacterial isolates from UTI patients.

(15.2%) of the isolates belong to the Gram-positive pathogens, and the most common was *Enterococcus* spp (9.0%). *Candida spp*. constituted only 1.7% of the isolates; these results were in agreement with our present results.

The sensitivity test results against bacterial isolates in Fig. (2) and photos No. (1) shown that the antibiotic ofloxacin is more effective against isolated pathogenic bacterial organisms, which the percentage of sensitive organism reach to 58.0% followed by amikacin 54.0%, chloramphenicol 52.0%, norfloxacin 51.0%, azithromycin 48.0%, ampicillin 11.0%, cefotaxime sodium 2.0% and penicillin-G 0.0%. The antibiotic amikacin show intermediate effect against isolated pathogenic bacterial organisms, which the percentage of organism reach to 27.0% followed by azithromycin 13.0%, cefotaxime sodium 12.0%, chloramphenicol 10.0%, ofloxacin 7.0%, norfloxacin 5.0%, ampicillin 1.0% and penicillin-G 0.0%. On the other hand, the antibiotic penicillin-G hasn't any effect against isolated pathogenic bacterial organisms, which the percentage of resistance organism reach to 100.0% followed by ampicillin 88.0%, cefotaxime sodium 86.0%, norfloxacin 44.0%, azithromycin 39.0%, chloramphenicol 38.0%, ofloxacin 35.0% and amikacin 19.0%. Adeyemo and some colleagues reported that while all urinary isolates were poorly susceptible to trimethoprim-sulphamethoxazole and ampicillin, they exhibited good susceptibility to nalidixic acid, nitrofurnation and ofloxacin[2]. The present study showed that the susceptibility rate of urinary isolates was highest for meronem (76.19%), followed by amikacin (70.27%), nitrofurantion (66.60%), norfloxacin (64.28%) and gentamicin (58.33%)[1]. These results go in line with our results, where it described that all urinary isolates were poorly susceptible to ampicillin, but exhibited good susceptibility to ofloxacin, amikacin and norfloxacin.

Fifteen bacterial isolates and The most two effective antibiotics were chosen to perform MIC and MBC test, where the results in Table (1) indicated that the maximum MBC were obtained at ofloxacin antibiotic which recorded 250 µg/ml against *P. aeruginosa* number 96 & 42, *K. pneumoniae* number 93 and *Proteus mirabilis* number 43 & 55, and the lowest MBC were obtained 31.25 µg/ml at *E. coli* number 7 and *Staphylococcus saprophyticus* number 76. MBC equal to MIC which recorded 250 µg/ml & 125 µg/ml of ofloxacin against *P. aeruginosa* number 42 & *E. coli* number 65 respectively. The results indicated that the maximum MBC were obtained at amikacin antibiotic which recorded 250 µg/ml against *E. coli* number 24, 65 & 72, *P. aeruginosa* number 42, *K. pneumoniae* number 93 and *Proteus mirabilis* number 47 & 55, and the lowest MBC were obtained 62.5 µg/ml at *E. coli* number 7 and *Staphylococcus saprophyticus* number 9 & 76. MBC equal to MIC which recorded 250 µg/ml of amikacin against *E. coli* number 24, *K. pneumoniae* number 93 followed by *P. aeruginosa* number 96, *Proteus mirabilis* number 43 & 45 and *E. coli* number 69 which recorded 125 µg/ml followed by *Staphylococcus saprophyticus* number 9 & 76 which recorded 62.5 µg/ml.

An attempt was made to test the antagonistic effect of different medicinal plant extracts (rosemary, orange peel, garlic, lemon grass, peppermint, spearmint, hibiscus, marjoram, thyme, tilia, clove, fennel, cinnamon, castor plant, ginger and chamomile), as cold water extract, boiled water extract and alcoholic extract against fifteen clinical bacterial isolates. The results in Fig. (3) and photos No. (2) illustrated that rosemary, lemon grass, peppermint, hibiscus, clove, marjoram, thyme, cinnamon, castor plant and chamomile were most effective plants extracts against selected pathogens (*Escherchia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonase aeruginosa* and *Staphyloccocus saprophyticus*). However in three cases (cold water, boiled water and alcohol extract), ginger was the lowest effective plant extracts. Orange peel and fennel were most effective only in case of alcohol extract, low effective in case of boiled water extract, and not effective in case of cold water extract. More over

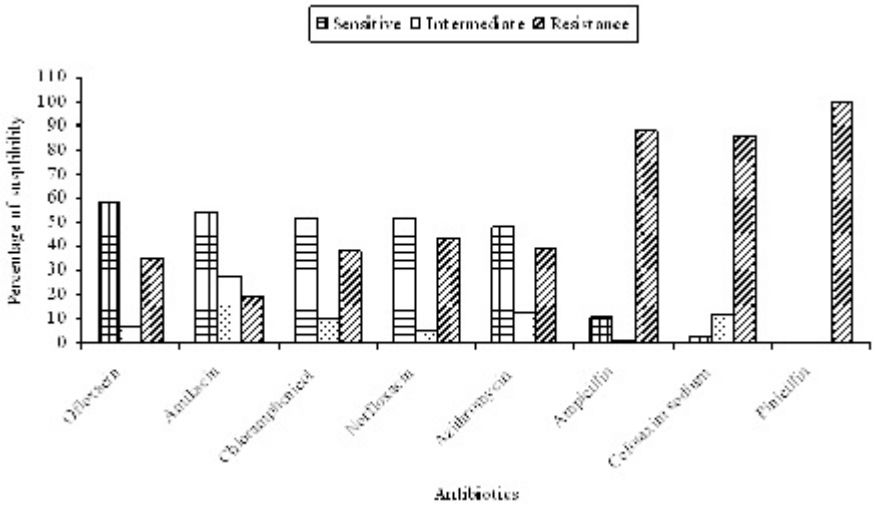


Fig. 2: The sensitivity test of pathogenic bacterial isolates against different antibiotics drugs.

Photos No. 1: Antibiotic susceptibility of bacterial isolates by disc diffusion method.
AK = amikacin; PG = penicillin-G; CTX = cefotaxime sodium; OFX = ofloxacin; NOR = norfloxacin; AM = ampicillin; AZE = azithromycin & C = chloramphenicol.

Table 1: Minimum inhibitory concentrations (MICs) (µg/ml) and minimum bactericidal concentrations (MBCs) of ofloxacin and amikacin antibiotics.

Parameters	Ofloxacin		Amikacin	
Bacterial isolates No.	(MIC) (µg/ml)	(MBC) (µg/ml)	(MIC) (µg/ml)	(MBC) (µg/ml)
E. coli 7	7.82	31.25	31.25	62.5
S. saprophyticus 9	7.82	62.5	62.5	62.5
E. coli 24	62.5	125	250	250
P. aeruginosa 42	250	250	125	250
P. mirabilis 43	125	250	125	125
P. mirabilis 45	62.5	125	125	125
P. mirabilis 47	62.5	125	125	250
P. mirabilis 55	125	250	125	250
E. coli 65	125	125	125	250
E. coli 69	62.5	125	125	125
E. coli 72	62.5	125	125	250
S. saprophyticus 76	7.82	31.25	62.5	62.5
P. mirabilis 85	15.63	62.5	62.5	125
K. pneomoniae 93	125	250	250	250
P. aeruginosa 96	125	250	125	125

garlic was the most effective in case of cold water and alcohol extract, and not effective in case of boiled water extract. Spearmint and tilia haven't any effect against bacteria. However in three cases (cold water, boiled water and alcohol extract). The present study illustrates that thyme, rosemary, caraway and dianthus are most effective plant extracts against selected pathogens (*E. coli*, *K. aerogense*, *P. aeruginosa*). In two cases, water and alcohol extract. Clove extract had potent antimicrobial activity against the entire Gram-negative

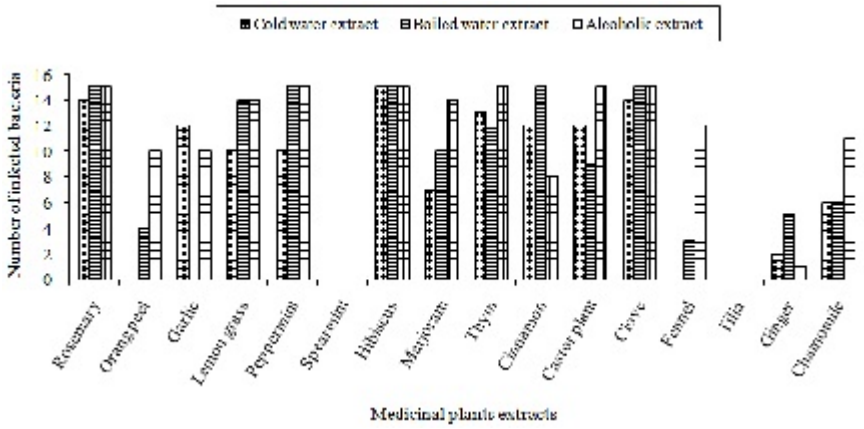
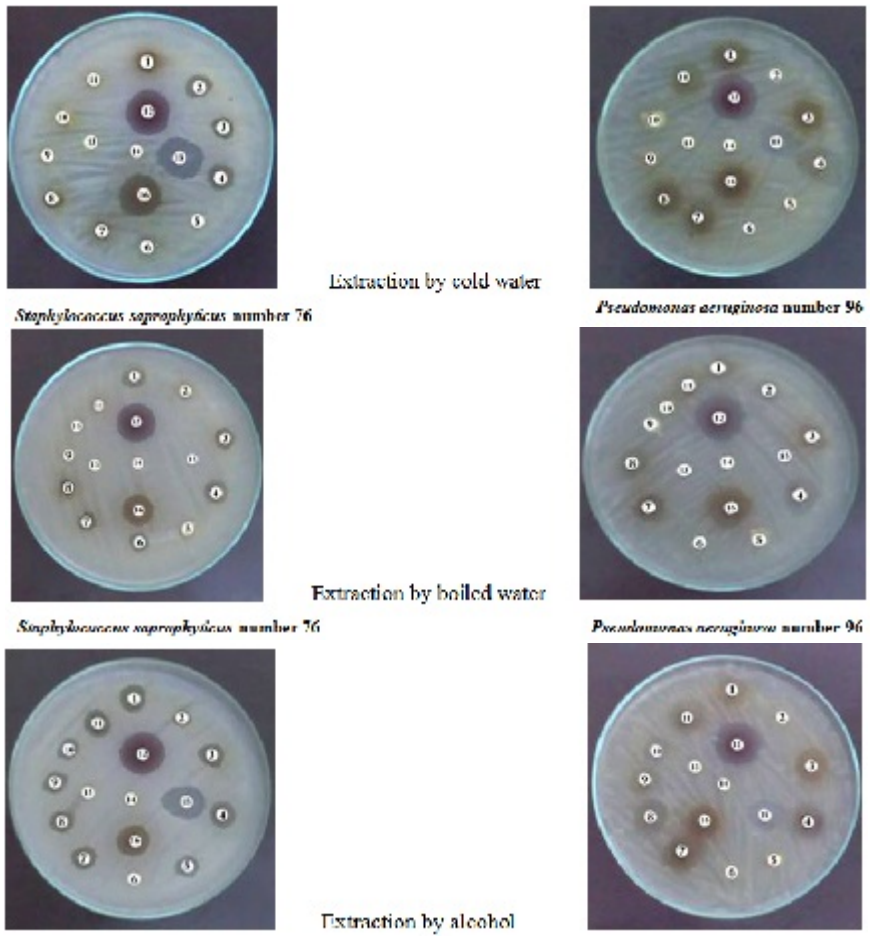


Fig. 3: Sensitivity of bacterial isolates against cold water, boiled water and alcoholic plant extracts.



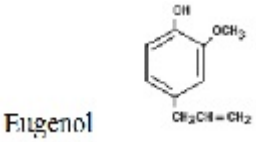
Photos No. 2: Sensitivity of bacterial isolates against cold water, boiled water and alcoholic plant extracts.
1- Rosemary, 2- Chamomile, 3- Thyme, 4- Cinnamon, 5- Ginger, 6- Orange peel, 7- Peppermint, 8- Marjoram, 9- Fennel, 10- Lemon grass, 11- Castor plant, 12- Hibiscus, 13- Tilia, 14- Spearmint, 15- Garlic, 16- Clove.

and Gram-positive organisms tested (*S. aureus*, *Enterococcus cloacae*,*Salmonella paratyphi*,*Klebsiella pneumoniae*, *E. coli*, *Citrobacter spp*, and *Candida albicans*[3].

The results in Tables (2 and 3) and photos No. (3, 4) illustrated that there is synergism between the combination of clove extract and antibiotics (ofloxacin and amikacin), but there isn't synergism between the combination of garlic extract and antibiotics (ofloxacin and amikacin). This study with agree with our study where it illustrated that clove extract show synergism with ofloxacin but no synergism between garlic extract and ofloxacin[17].

The clinical bacterial isolates *P. aeruginosa* 42, *P. mirabilis* 55, *K. pneumoniae* 93 were grow on nutrient agar as control and on nutrient agar with antibiotic ofloxacin with concentrations (125, 62.5 & 62.5) µg/ml respectively combined with clove extract (boiled water extract) to determine protein band at control and treated bacteria, the results in photo (5) indicated that there are many protein bands induced and other disappeared if compared with control.

The results obtained from spectrum analysis ^{1}HNMR of compound showed signals at 3.8(d, 2H, CH_2), 5(t, 2H, $=CH_2$), 6(m, 1H, -CH=), 6.5-6.8(m, 3H, Ar H^s) and at 8.6(S, 1H, OH); as illustrated in Fig. (4). IR of compound showed broad band at 3500 cm^{-1} corresponding to –OH group and band at 1518 cm^{-1} corresponding to C=C, in addition to O-C band absorption at 1099 cm^{-1}; as illustrated in Fig. (5). These data corresponding to eugenol structure.



Eugenol

Table 2: Combination effect between different medicinal plant extracts and MICs of ofloxacin against bacterial isolates

Bacterial isolates No.	Ofloxacin MICs		Diameter of inhibition zones (mm) of medicinal plant extracts					
	µg/ml	IZ	Rosemary IZ	Garlic IZ	Clove IZ	Castor plant IZ	Peppermint IZ	Hibiscus IZ
E. coli 7	7.8125	12	14	12	13	12	12	12
S. saprophyticus 9	7.8125	12	10	11	15	ND	ND	15
E. coli 24	62.5	12	15	ND	18	13	14	15
P. aeruginosa 42	250	15	17	15	18	11	15	17
P. mirabilis 43	125	15	15	12	23	ND	25	20
P. mirabilis 45	62.5	12	22	15	20	ND	17	20
P. mirabilis 47	62.5	12	ND	12	18	12	13	16
P. mirabilis 55	125	15	15	12	20	11	20	22
E. coli 65	125	14	17	12	15	14	13	18
E. coli 69	62.5	11	15	ND	19	ND	15	14
E. coli 72	62.5	15	ND	16	17	11	10	12
S. saprophyticus 76	7.8125	15	15	18	18	17	11	23
P. mirabilis 85	15.625	15	10	12	20	ND	10	20
K. pneomoniae 93	125	13	23	10	20	23	21	20
P. aeruginosa 96	62.5	12	14	12	18	10	15	14

Table 3: Combination effect between different medicinal plant extracts and MICs of amikacin against bacterial isolates.

Bacterial isolates No.	Amikacin MICs		Diameter of inhibition zones (mm) of medicinal plant extracts					
	µg/ml	IZ	Rosemary IZ	Garlic IZ	Clove IZ	Castor plant IZ	Peppermint IZ	Hibiscus IZ
E. coli 7	31.25	12	12	16	15	12	12	14
S. saprophyticus 9	62.5	12	15	10	16	12	14	13
E. coli 24	250	12	11	11	20	13	14	13
P. aeruginosa 42	125	10	14	12	15	11	14	18
P. mirabilis 43	125	12	15	12	19	19	14	13
P. mirabilis 45	125	10	12	14	15	13	12	15
P. mirabilis 47	125	11	12	11	18	13	14	22
P. mirabilis 55	125	13	13	10	18	22	15	12
E. coli 65	125	13	16	ND	15	17	15	15
E. coli 69	125	10	13	ND	17	10	12	20
E. coli 72	125	12	15	ND	15	ND	10	14
S. saprophyticus 76	62.5	13	22	26	25	15	20	27
P. mirabilis 85	62.5	10	14	10	18	ND	12	17
K. pneomoniae 93	250	13	15	ND	16	15	12	20
P. aeruginosa 96	125	15	18	12	19	13	12	21

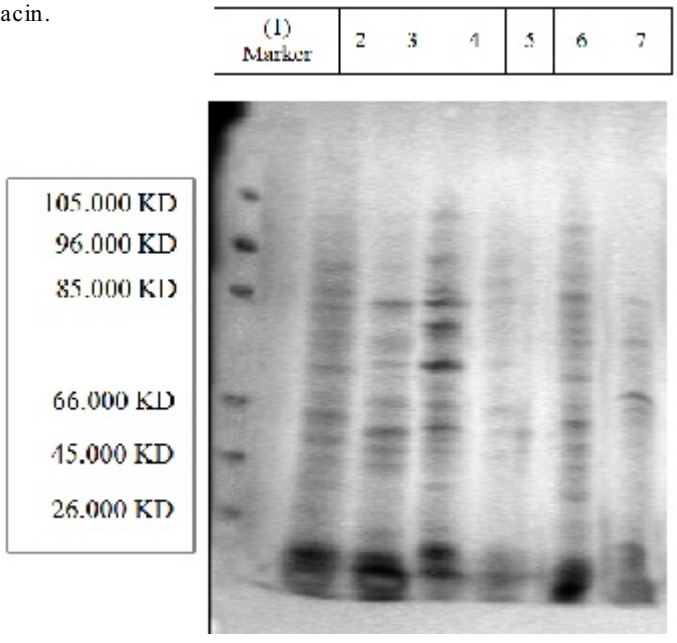
The present study illustrates that the compound responsible for the clove aroma is eugenol. It is the main component in the essential oil extracted from cloves, comprising 72-90%. Eugenol has pronounced antiseptic, antimicrobial and anaesthetic properties[9].

E. coli number 7 *Proteus mirabilis* number 85

Photos No. 3: Combination between medicinal plants extracts and ofloxacin antibiotic susceptibility of bacterial isolated by disc diffusion method.

E. coli number 7 *Proteus mirabilis* number 85

Photos No. 4: Combination between medicinal plants extracts and amikacin antibiotic susceptibility of bacterial isolated by disc diffusion method.
1- Garlic, 2- Clove, 3- Rosemary, 4- Peppermint, 5- Hibiscus, 6- Castor plant, 7- MIC of ofloxacin or amikacin.



Photos No. 5: Protein analysis for clinical bacterial isolates before and after stress by comination between ofloxacin and clove extract
1- Marker. (26.000, 45.000, 66.000, 85.000, 96.000, 105.000) KD. 2- *Pseudomonas aeruginosa* number 42 before stress. 3- *Pseudomonas aeruginosa* number 42 after stress. 4- *Proteus mirabilis* number 55 before stress. 5- *Proteus mirabilis* number 55 after stress. 6- Klebsiella pneumoniae number 93 before stress. 7- Klebsiella pneumoniae number 93 after stress.

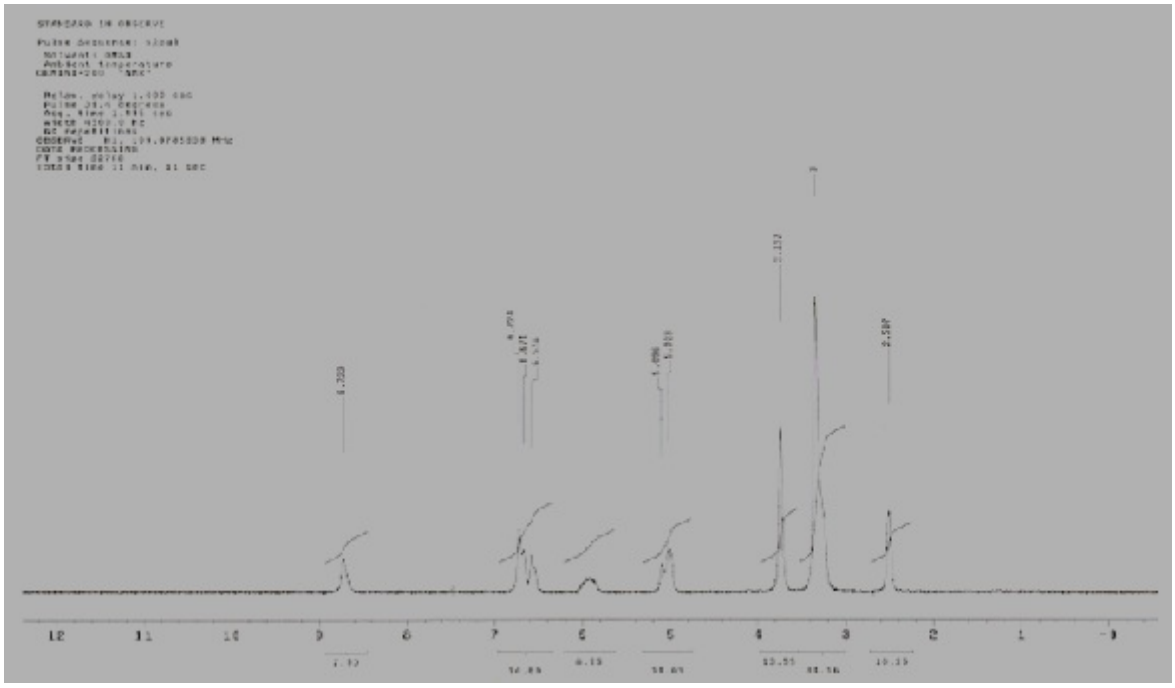
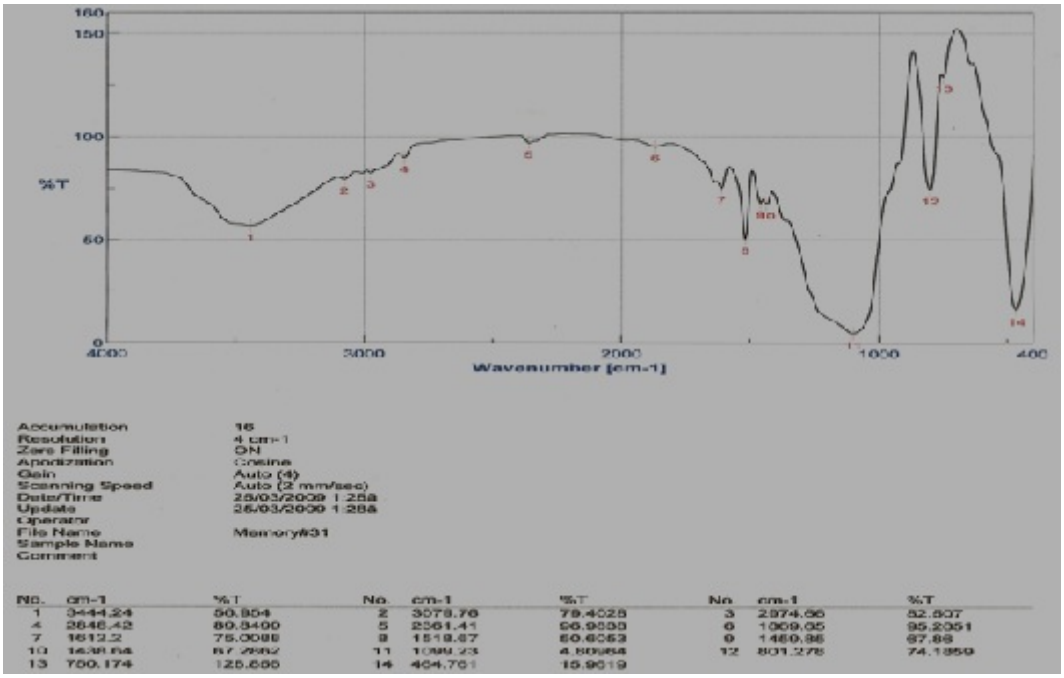


Fig. 4: [1]HNMR spectrum of the antimicrobial substances.



No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T
1	3444.24	90.804	2	3078.76	79.4028	3	2974.56	82.807
4	2846.42	80.8400	5	2361.41	96.9593	6	1609.05	95.2051
7	1612.2	75.0088	8	1518.67	60.6063	9	1460.96	87.88
10	1438.64	87.2982	11	1089.23	4.80984	12	801.278	74.1859
13	700.174	125.695	14	464.761	15.9619			

Fig. 5: IR spectrum of the antimicrobial substances.

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