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# Seeking Alternative Remedies to Treat Antibiotic-Resistant Bacteria

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**KEY WORDS**

*Klebsiella*, antibiotics, MDR, MIC, essential oils, clove oil, RAPD, resistance plasmid

**ABSTRACT**

The present study identified 63 *Klebsiella* isolates among 100 different clinical specimens collected from patients in El-Mahalla Chest Hospital (approved by Research Ethics Committee, code: 36264PR205/5/23). Their frequency varied from 53 cases (82.8% out of 64) of sputum samples, 8 cases (30.7% out of 26) of urine samples and 2 cases (20% out of 10) of stool samples. The antibiotic susceptibility test of the 63 *Klebsiella* isolates was tested against 18 commercial antibiotics, belonging to 8 groups, by the method of disk diffusion, confirming their resistance to different antibiotics between 15.8% (the least for Amikacin) and 100% (for ampicillin), with heterogeneous resistance patterns, hence a total of 16 patterns were detected among 63 tested isolates.

The genetic diversity between 20 resistant isolates that were selected by using Past program was done by Random amplification of polymorphic DNA (RAPD) finger-printing method, using 5 primers. The analysis showed that the isolate No. 40 from sputum was clearly differentiated from the remaining three different groups of other isolates. The majority of the tested Multidrug Resistant (MDR) isolates were found to have β- lactam antibiotics resistance genes that are plasmid in origin; therefore, the pathogenicity of *Klebsiella* isolates increased in the presence of virulence-associated plasmid. The Susceptibility of *Klebsiella* isolates to 5 essential oil of cinnamon, clove, orange, lemon, and lemon grass was tested. Clove essential oil showed the best effect on bacterial growth with MIC of 1/20 v/v. The GC-MS and UV analyses showed that the phenolic compound eugenol was the most dominant compound in the clove essential oil.

## Introduction

The genus *Klebsiella* of the family Enterobacteriaceae recorded an increased rate of antibiotic resistance, as an opportunistic Gram-negative pathogen most frequently implicated in community-acquired and nosocomial infections, which include septicemia, pneumonia, bacteremia, urinary, and wound infections **(Wu *et al*., 2012)**.

In developing countries, *Klebsiella* can cause severe illness and mortality **(Wang *et al*., 2020)**, and important nosocomial pathogen affecting both immunocompetent and immunocompromised and are responsible for a considerable proportion of infections in intensive care unit and pediatric patients **(Marion-Sanchez *et al*., 2019)**. A hyper-virulent strain of *Klebsiella pneumoniae*, causing fatal infections for healthy citizens, was recorded by **Shon *et al*., (2013)**.

An Egyptian survey revealed a high incidence of *Klebsiella* species in many infections with common carbapenems antibiotic resistance. Among 112 cases, about 26% of patients suffered from resistant urinary infections, followed by 18% of respiratory infections, then 8% of cases possessed resistant wound infections **(Shawky *et al*., 2019)**.

A worldwide survey for the incidence of resistant *Klebsiella pneumoniae* infection detected a critical spread of these isolates among hospitalized patients with different sources of infections. This infection constitutes 3-5% in western cultures, compared with 15% in developing countries. It possessed 8-12% of patient infections in ventilators, compared with only 7% for non-ventilating patients **(Hasan *et al*., 2021)**.

The excessive use of antibiotic therapy of humans has been led to high resistance among different micro-organisms. Drug resistant *K. pneumoniae*, such as the β-lactam family of antibiotics have limited available therapeutic options for their treatment; and could transfer the drug resistance **(Dahmen *et al*., 2013)**. *Klebsiella sp.* are among the bacteria that develop multiple resistance mechanisms; and possess the genetic ability to acquire and spread the resistance by: degradation enzymes, alteration of the drug target, reduction of membrane permeability, efflux of antibiotic molecules (**Nadeema *et al*., 2020**), due to the evolution of beta lactamases plasmid **(Tzouvelekis *et al*., 2012)** and other virulence genes associated with pathogenicity **(Aher *et al*., 2012)**. Members of this genus were

linked to epidemic diarrhea because they have acquired heat labile enterotoxin plasmids from *Escherichia coli* (**Jafari *et al*., 2012**). *K. pneumoniae* can adhere to the human intestinal cell lines due to their acquirement of non fimbrial CF29K R-plasmid; this is identical to the CS31A adhesive protein of *E. coli*, responsible for human diarrhea **(María *et al*., 2013)** and belongs to K88 adhesion family; that may indicate the transfer of CS31A genetic determinants from *E. coli* to CF29K of *K. pneumoniae* strains in the human intestine.

Infections by multidrug-resistant *K. pneumoniae* strains have become common in health care institutions. This is of concern due to increasing the incidence of drug resistant *Klebsiella pneumoniae*, the most common hazard against the treatment of microbial infections. Alternative methods of treating *Klebsiella* are required. Essential oils had an interest as natural goods throughout history. They were tested to determine whether they could be used as an alternative therapy for several infectious disorders. The antibiotic, antiviral, antifungal, and antioxidant activities of volatile oils have been demonstrated to protect the human body from infections and cellular oxidative responses. **(Shaik *et al*., 2014)**.

The variety of *Klebsiella* strains that have been examined and isolated from clinical samples utilizing molecular fingerprinting using RAPD PCR. Earlier research offered a comparative method for comprehending the genetic variety present in isolates of the species *Klebsiella.* **(Podder *et al*., 2014)**.

The aim of the present study was to survey the incidence of the causative agents of resistant lung, digestive and urinary infections. Then molecular studies track the spreading factors of drug resistance among the collected isolates. Also, antimicrobial activity of some natural products will be evaluated as a new remedy against the selected resistant pathogens.

## Materials and Methods

### Isolation and maintenance of bacteria from patients

One hundred bacterial isolates were collected mainly from male and female patients, ranged between 20-60 years old of inpatient department of El-Mahalla Chest Hospital, Egypt and other medical sources, through one year of twice weekly regular visits during the period of January 2022 to December 2022; using swabs immersed in nutrient broth medium (Oxoid, UK). The protocol of sampling and patient dealing was approved by the Research Ethics Committee, Faculty of Medicine, Tanta University, with approval code of

36264PR205/5/23. Desired isolates were differentiated and purified on McConkey agar plates (Oxoid, UK). Pure cultures of identified isolates were maintained on nutrient agar slants (Oxoid, UK), stored at 4°C for short term maintenance. For long term maintenance of pure cultures, glycerol deep-freeze storage method was used **(Somasundar *et al.*, 2011).**

**Identification of bacterial isolates by VITEK2 compact system**

The VITEK2 compact system uses a sensitive automated fluorescence-based technology, used for the identification and susceptibility testing of microorganisms in 15 hours. An ATB 1550 densitometer was used to adjust the bacterial inocula (suspended in 0.45% NaCl soln.) to a no. 2 McFarland standard (bioMérieux, Marcy- l'Etoile, France) **(Stefaniuk *et al.*, 2016).**

Each of the 64 wells on the reagent cards is filled with a different testing substrate. Various metabolic activities, including acidification, growth when an inhibitory substance is present, and alkalinization, enzyme hydrolysis, are measured by substrates. The following cards are now ready for identification of target organisms: yeasts, yeast-like organisms; Gram positive sporulating bacilli; Gram positive cocci; and Gram-negative fermenting and non-fermenting bacilli **(Crowley *et al.*, 2012).**

The VITEK2 consists of 20 tests for the assimilation of carbohydrates, including tests for adonitol (ribitol), D-trehalose, D-cellobiose and dulcitol. Palatinose, D-raffinose, D-melibiose, D-mannitol, D-melezitose, D-galactose, Salicine, D-glucose, L-sorbose, D-sorbitol, L-rhamnose, D-L-lactate, sucrose and succinate are all sugars **(Graf *et al.*, 2000).**

The six organic acid assimilation tests are mono-methyl-ester-succinate, citrate, D-gluconate, D-galacturonate and N-acetyl-glucosamine. Eight substrates: galactoside - 4MU, mannoside - 4MU, glucoside - 4MU, glucuronide - 4MU, N – acetyl – glucosaminide - 4MU, and xyloside - 4MU—are coupled with 4 - methyl umbelliferone (4MU) to detect the oxidases. Nine substrates—glycine - 7AMC, hydroxyproline - 7AMC, H – lysine – alanine - 7AMC, H – glutamyl – transferase - 7AMC, H – glycine – glycine - 7AMC, histidine - 7AMC, isoleucine - 7AMC, proline - 7AMC, and valine - 7AMC—are used to detect arylamidases. Phosphatase, urea, nitrate, and actidione are the four other tests **(Graf *et al.*, 2000*).***

The cards were filled, sealed and incubated at 35°C, using the integrated VITEK2 device; then submitted to fluorescence measurement every 15 min., automatically. The microorganism

was finally identified after 15 hours of incubation by comparison of its results to the VITEK2 database. A particular algorithm was used to interpret each profile result **(Sundaram and Navanee, 2016).**

**Antibiotic sensitivity and multi drug resistance profile of bacterial isolates from patients by agar disc diffusion method**

Agar disc diffusion method was used to examine 63 isolates of *Klebsiella* for their resistance to 18 antibiotics **(Cursino *et al.*, 2005).** Briefly, Mueller-Hinton agar plates were used to sub-culture bacterial isolates for 18 hours at 37 ºC. A few distinct colonies of each isolate were used to make a suspension in 1-2 ml of normal saline. To get a cell count of approximately $10^7$ CFU/ml using the standard turbidity (= 0.5 McFarland tube), samples were diluted with normal sterile saline. Two Mueller-Hinton agar plates that had been well dried had 100 µl of each of the fore mentioned solutions dropped in the center, evenly spread by a sterile cotton swab, and dried for 15 minutes at 37°C. Then antibiotic discs (Oxoid, UK) were placed on the prepared plates by sterile forceps, spaced 3 cm apart; and incubated for 18 h. at 37°C. The diameters of the growth inhibition zones were classified into sensitive, partially resistant, or completely resistant, according to **CLSI (2010)**. The drug resistance percentage was calculated according to the following formula:

% resistance = No of antibiotic resistance for each isolate / Total no of ab *100

**Survey for antimicrobial activity of some essential oils and their MIC against *Klebsiella* isolates**

The susceptibility of the selected MDR 20 *Klebsiella* isolates to various plant essential oils (purchased from the market, Captain oils extraction Co., Egypt) was evaluated by agar diffusion method **(Prabuseenivasan *et al*., 2006)**.

Bacterial isolates were sub-cultured for 18 hours at 37ºC on Mueller-Hinton agar (Oxoid, UK) plates. For each isolate, a suspension was made using a few distinct colonies in 1-2 ml of normal saline. To achieve a cell count of about $10^7$ CFU/ml, using spectro-photometric (Optima, SP300, Japan) turbidity (optical density 0.06 at 600 nm), each sample was diluted with sterile normal saline. Two Mueller-Hinton agar plates that had been thoroughly solidified, had 100 microliters of each of the fore mentioned solutions dropped in the center, spread evenly with a sterile cotton swab, and allowed to dry for 15 minutes at 37°C. Following the application of wells to the prepared plates using cork borer (6 mm), the essential oils (1:1 v/v) were dissolved in 10% aqueous di-methyl-sulfoxide (DMSO); and regularly

suspended by Tween 80 (0.5% v/v). Each well was filled with fifty microliters of dissolved essential oils. Inhibition zones did not overlap since there was enough room between the wells. Following a 24-hour incubation period at 37°C, then the inhibition zone diameters (cm) were measured **(Prabuseenivasan *et al*., 2006)**.

Five essential oils (Cinnamon, Clove, Orange, Lemon and Lemon grass) were screened, then the best one with strong antibacterial properties was chosen to determine its MIC. Bacterial isolates were cultivated on Mueller-Hinton agar by adding 100 µl of $10^7$ CFU/ml to the Centre of plates that had been well dried, spreading it evenly using a sterile cotton swab, and allowing it to dry for 15 minutes at 37°C.

Using a sterile cork borer, wells were made on the prepared plates and fifty microliters of the most efficient essential oil were added to each well at various concentrations (1:1, 1:5, 1:10, and 1:20 v/v) after the oil had been dissolved in aqueous 10% di-methyl-sulfoxide (DMSO) with Tween 80 (0.5% v/v). Plates were incubated at 37°C for 24 h., inhibition zone diameters (cm) were measured, in comparison with Tween 80 and DMSO individual effects; then MIC was determined **(Shadomy, *et al*., 1985)**.

**Phytochemical analysis of the clove oil**

At the Micro Analytical Centre, Faculty of Science, El Mansura University; UV spectra (Shimadzu, Japan – 240 UV-visible spectro-photometer) and gas chromatography / mass spectrometry (GC/MS) (GC-Perkin Elmer (Clarus 580/560S) GC/MS spectrophotometer, Thermoscientific, USA with Elite-5MS column) were used to analyze the clove essential oil's active components.

**UV spectra**

Using a quartz cuvette containing a diluted solution of the antimicrobial agent in dimethyl sulfoxide (DEMSO, 10% v/v aqueous solution), the UV spectra of the substance under test were measured using a spectrophotometer (UV-2101-pc), and the blank sample was made using pure DEMSO. 200 to 600 nm was the wavelength range **(Schaffer *et al*., 2009)**.

**Gas Chromatography / Mass Spectro-metry (GC/MS)**

The most potent oil was examined by FID (flame ionization detector), run in EI mode at 70 eV in a two fused GC/MS GC-quadrupole MS system QP 5000 with silica capillary columns TR-5 (30 m, 0.25 mm id, film thickness 0.25 m), where temperatures were set for the injector at 220°C and for the detector at 250°C. The column was heated up to 275°C while being increased by 3°C/min

after being kept at 50°C for two minutes. One microliter of an essential oil, dissolved in hexane, was applied. As a carrier gas, helium was used (1 ml/min). The proportion of each component of the total oil was indicated by its peak area percentage relative to total peak area. By contrast, the various constituents were qualitatively identified by comparing the relative retention durations and mass spectra of the various constituents to those of reference compounds or using retention indices (RI) **(Manzan *et al*., 2003**).

**RAPD fingerprinting of multi drug resistant *Klebsiella* isolates**

**Extraction of DNA**

Total DNA was extracted according to **Liu *et al*. (2002).** Bacterial isolates were cultured in a nutrient broth medium (15 ml) at 37ºC, shaked continuously at 250 rpm for 24 h. Cell pellets of 2 ml were centrifuged at 5000 rpm for 5 min., collected and washed by solution I (8 g NaCl; 1.21 g $K_2HPO_4$; 0.34 g $KH_2PO_4$; 1000 ml Distilled water). Pellets were frozen at -20ºC overnight and then left at room temperature until thawing. Pellets were re-suspended in 500 µl of solution II (40 Mm Tris-HCl of pH 8; 1 Mm EDTA of pH 8.0; 150 Mm NaCl), then mixed with 20 µL freshly prepared lysozyme (containing 2 mg/ml) and incubated at 37ºC for 1 h. Cell lysate was mixed and shaked with 400 µl of solution III (0.2 M NaOH; 1 % (w/v) SDS) on ice for 5 min.

The DNA was extracted in 800 µl of equal volumes of phenol- chloroform-isoamyl alcohol solution. The solution was homogenized by several times inversion and left at room temperature for a few minutes (for the phase separations). The mixture was centrifuged at 10000 rpm for 5 min. and the upper aqueous phase was transferred in clean Eppendorf and re-extracted with phenol solution from 2-3 times. The clear supernatant containing DNA was collected in clean sterilized Eppendorf and precipitated for 2 h. by mixing with 0.6 ml of isopropanol. The DNA pellet was obtained by centrifuging for 15 minutes at 13000 rpm. The pellet of DNA was washed with 70% ethanol, centrifuged at 10000 rpm for 10 minutes, then the pellet was air dried, dissolved in solution III, and then subjected to agarose gel electrophoresis analysis for an hour.

**RAPD- PCR analysis**

The RAPD-PCR analysis was carried out by using PCR reaction mixture (Promega kit, USA) of *Klebsiella* species and adding the different primers of different GC content (**Table 1**), separately. The PCR machine (Qiagen, USA) performed a program included 40 cycles of initial denaturation for 4 min. at 95ºC, denaturation for 1

min. at 95°C, annealing for 1 min. at 35°C, extension for 1 min. at 72°C and final extension at for 5 min. 72°C for each primer **(Liu *et al*., 2002)**.

**Table (1):** Primers used in RAPD-PCR analysis

| Primers | Sequences (5`- 3`) | G+C (%) |
|---|---|---|
| **RAPID Kleb** | AACGCGCAAC- | 60 |
| **IF Archi** | CACAAGTGATGACCGCTGTT- | 50 |
| **IF Actino I** | GCAGTAGGGAATTTTCCGCA- | 50 |
| **EstA R** | CGGAATTCTTACCAATCTAACGATTCAAGAAATG- | 38 |
| **AGB7** | GGTGACGCAGGGGTAACGCC- | 70 |

**Agarose gel electrophoresis**

An amount of 1.5 g agarose powder was added to 100 ml of electrophoresis buffer (1X TBE buffer) to get 1.5% agarose gel, then heated in a microwave. The melted agarose was left to cool to 55ºC and mixed with 5 µl of ethidium bromide. The agarose was poured into the mold of electrophoresis unit with comb, left to cool for an hour. After solidification, the comb was removed; the electrode chamber was filled with enough 1X TBE buffer to cover the gel. The samples were injected; electric current was run for 30 min. at 80 v/cm, then photographed under UV **(Liu *et al*., 2002)**.

**Isolation and detection of plasmid DNA of multi drug resistant *Klebsiella* isolates.**

A total of 20 *Klebsiella* isolates were treated to the alkaline lyses technique according to **Sambrook *et al*., (1989)** for the isolation of complete plasmid DNA. By shaking at 150 rpm and incubating at 37°C, overnight cultures in nutrient broth medium were created. The culture was centrifuged at for 5 minutes 10000 rpm at 4°C, the medium was removed, then the bacterial pellet was resuspended in 150 µL of ice-cold solution I by vigorous vortexing and stored for 5 min. at room temperature. Freshly prepared solution II (200 µL) was mixed with the pellet by inverting the tubes rapidly several times and stored on ice for 5 min. 150 µl of ice-cold solution III were added and mixed by vortex in an inverted position for 10 sec. Samples were centrifuged for 5 min. at 15000 rpm at 4ºC.

Equal volumes of phenol/chloroform were added to the pellet, mixed by vortex and centrifuged at 4ºC for 2 min. Two volumes of absolute ice-cold ethanol were added to precipitate the plasmid DNA and mixed by vortex. The mixture was allowed to stand at room temperature 5 min. and centrifuged at 15000 rpm for 5 min. at 4ºC. The plasmid DNA pellets were washed by two volumes of 70% ice-cold ethanol and centrifuged at 15000 rpm at 4ºC for 5 min. The plasmid DNA pellets were allowed to dry in the air for

10 min., then dissolved in 50 µL TE buffer and stored at -20ºC. The obtained plasmids were visualized by gel electrophoresis.

## Results

### Isolation and identification of bacteria from patients

One hundred bacterial isolates were collected, as mentioned previously. Sixty-three isolates were Gram negative bacilli. Isolates were identified by VITEK2 Compact System, **Table (2)** revealed the recovery of 53 (82.8%) *Klebsiella* isolates out of 64 isolates from sputum, 8 (30.7%) *Klebsiella* isolates out of 26 from urine and 2 (20%) *Klebsiella* isolates out of 10 isolates from stool, respectively.

**Table (2):** Incidence of *Klebsiella* species from different sources

| Source | Total no. Specimens | Number of *Klebsiella* spp. isolates | %*Klebsiella* spp. isolates |
|---|---|---|---|
| Sputum | 64 | 53 | (82.8%) |
| Urine | 26 | 8 | (30.7%) |
| Stool | 10 | 2 | (20%) |

% = *Klebsiella* spp. isolates from a source / no. of this source specimens X 100.

### Susceptibility of *Klebsiella* isolates to common antibiotics

The susceptibility of 63 *Klebsiella* isolates to 18 different antibiotic disks was performed using the disk diffusion method as shown in **Table (3)**. The results revealed that bacterial isolate numbered 40 showed 100% resistance to all tested antibiotics. Bacterial isolates numbered 6 and 17 recorded 94.4% resistance to the tested antibiotics. Bacterial isolates numbered 7 and 8 revealed 88.8% resistance to the antibiotics. Bacterial isolates numbered 1, 2, 3, 9, 10, 11,15, 16, 18, 20, 21, 22, 23, 24, 27, 35, 38, 42, 45, 51, 55, 56, 59 and 63 showed 55.5% to 83.3% resistance of the18 tested antibiotics. *Klebsiella* isolates numbered 12, 44, 46, 47, 49, 53, 54 and 62 revealed 50% resistance of the drugs, *Klebsiella* isolates numbered 4, 13, 29, 31, 36, 48, 57 and 61 recorded 44.4% resistance of the antibiotics. *Klebsiella* isolates number 5, 26, 30, 43, 50, 58 and 60 showed 38.8% resistance of the eighteen tested agents. *Klebsiella* isolates numbered 14, 28, 39 and 52 showed 33.3% resistance of the tested antibiotics. Bacterial isolates numbered 25, 33 and 37 showed the lowest resistance to the tested antibiotics 27.7, 22.2 and 16.6 %, respectively.

**Table (3):** Percentage of multi drug resistance for the tested *Klebsiella* isolates

| Isolate No. | % Resistance | Isolate No. | % Resistance | Isolate No. | % Resistance |
|---|---|---|---|---|---|
| K1 | 77.7 | K22 | 83.3 | K43 | 38.8 |
| K2 | 83.3 | K23 | 77.7 | K44 | 50 |
| K3 | 83.3 | K24 | 83.3 | K45 | 55.5 |
| K4 | 44.4 | K25 | 16.6 | K46 | 50 |
| K5 | 38.8 | K26 | 38.8 | K47 | 50 |
| K6 | 94.4 | K27 | 61.1 | K48 | 44.4 |
| K7 | 88.8 | K28 | 33.3 | K49 | 50 |
| K8 | 88.8 | K29 | 44.4 | K50 | 38.8 |
| K9 | 55.5 | K30 | 38.8 | K51 | 66.6 |
| K10 | 61.1 | K31 | 44.4 | K52 | 33.3 |
| K11 | 61.1 | K32 | 50 | K53 | 50 |
| K12 | 50 | K33 | 22.2 | K54 | 50 |
| K13 | 44.4 | K34 | 50 | K55 | 55.5 |
| K14 | 33.3 | K35 | 55.5 | K56 | 77.7 |
| K15 | 66.6 | K36 | 44.4 | K57 | 44.4 |
| K16 | 83.3 | K37 | 27.7 | K58 | 38.8 |
| K17 | 94.4 | K38 | 55.5 | K59 | 72.2 |
| K18 | 61.1 | K39 | 33.3 | K60 | 38.8 |
| K19 | 88.8 | K40 | 100 | K61 | 44.4 |
| K20 | 83.3 | K41 | 50 | K62 | 50 |
| K21 | 55.5 | K42 | 55.5 | K63 | 61.1 |

**Multidrug resistance profile of *Klebsiella* isolates to different commercial antibiotics**

Table (4) showed the results of screening for sensitivity to the 18 tested antibiotics of the 63 tested *Klebsiella* isolates. For β- lactam group, the resistance of all bacterial isolates to Ampicillin was 100%, amoxicillin and oxacillin were 98.4 and 95.2%. The resistance of all tested bacterial isolates for cephalosporin's group was 74.6, 73, 26.98 and 52.38% for cephradine, cefaclor, cefepime and cefuroxim respectively, in the case of the aminoglycoside drugs which include gentamicin and amikacin the incidence of resistance was 34.9 and 15.8%, respectively. On the other hand, the resistance of bacterial isolates to rifamycin, tetracyclines, macrolides and phenicol groups which include rifampicin, tetracycline, erythromycin and chloramphenicol was 73, 49.2, 95.23 and 42.85%, respectively. In the case of fluoroquinolones, the incidence of the resistance ranged between ciprofloxacin (39.85%), nalidixicacid (28.5%), ofloxacin (42.85%), pefloxacin (39.68%) and lomefloxacin (44.4%), so the incidence of resistance to different tested antibiotics ranged between 15.8 and 100%.

**Table (4):** Percentage of resistant *Klebsiella* isolates against the different tested antibiotics classes

| Antibiotic class | Antibiotic | Code | Disc concentration µg/disc | No. of resistant isolates | % of resistant isolates |
|---|---|---|---|---|---|
| Aminoglycosides | Amikacin | AK | 30 | 10 | 15.8 |
| | Gentamycin | CN | 10 | 22 | 34.9 |
| Cephalosporins | Cefepime | FEP | 30 | 17 | 26.98 |
| | Cephradine | CE | 30 | 47 | 74.6 |
| | Cefaclor | CEC | 30 | 46 | 73 |
| | Cefuroxim | CXM | 30 | 33 | 52.38 |
| Fluoroquinolones | Ciprofloxacin | CIP | 5 | 25 | 39.85 |
| | Nalidixicacid | NA | 30 | 18 | 28.5 |
| | Ofloxacin | OFX | 5 | 27 | 42.85 |
| | Pefloxacin | PE | 30 | 25 | 39.68 |
| | Lomefloxacin | PE | 10 | 28 | 44.4 |
| Macrolides | Erythromycin | E | 15 | 60 | 95.23 |
| Penicillins | Ampicillin | AMP | 10 | 63 | 100 |
| | Amoxicillin | AMX | 25 | 62 | 98.4 |
| | Oxaicillin | OXA | 1 | 60 | 95.2 |
| Phenicols | Chloramphenicol | C | 30 | 27 | 42.85 |
| Rifamycin | Rifampicin | RD | 5 | 46 | 73 |
| Tetracyclines | Tetracycline | TE | 30 | 31 | 49.2 |

The pattern of resistance of each of the *Klebsiella* isolates to the studied antimicrobial drugs is shown in **Table (5)**. As presented in this table, all tested bacterial isolates were multi-drug resistant to 3-18 antibiotics. On testing the resistance of each *Klebsiella* isolate of the total 63 cases, each isolate recorded a resistance against a certain number of the selected antibiotics (marker agents) with observable variation in types of resisted antibiotics. So the resistance patterns were grouped into 16 profiles according to the number of resisted antibiotics by each isolate, with different types of antibiotics for each one. **Table (5)** showed that the most dominant resistance pattern was P7, with 11 isolates, resisting different 9 antibiotics; followed by P6 with 8 isolates resisting 8 antibiotics; then P8 for 7 isolates resistant to 10 antibiotics and P5 for 7 isolates resisting 7 antibiotics. While P1, P2, P3, P11, and P16 were recorded only for one isolate for each pattern.

**Survey for antimicrobial activity of some essential oils against *Klebsiella* isolates**

The effect of some plant essential oils namely cinnamon, clove, orange, lemon and lemon grass were studied separately on the 20 *Klebsiella* isolates using agar well diffusion method presented in **Table (6)** and **Photo (1)**.

**Table (5):** Antimicrobial resistance patterns of tested *Klebsiella* isolates

| Pattern code | Types of antibiotics | No. of marker agents (resisted antibiotics) | No. of isolates for each pattern | % of resistant isolates for each pattern |
|---|---|---|---|---|
| P1 | AMP, CEC, RD | 3 | 1 | (1.58) |
| P2 | X, E | 4 | 1 | (1.58) |
| P3 | X, E, TE | 5 | 1 | (1.58) |
| P4 | X, Y, TE<br>X, Y, CN<br>X, E, CE, OFX<br>X, E, PE, Cip, LOM | 6 | 4 | (6.32) |
| P5 | X, CEC, Y, RD<br>X, Z, OFX, RD<br>X, Z, CE, RD<br>X, E, CE, OFX, RD<br>AMP, AMX, E, CE, NA, RD,CXM<br>X, E, PE, NA, RD<br>X, Z, TE, AK | 7 | 7 | (11.06) |
| P6 | X, Z, TE, CE<br>X, Z, CE, RD, CXM<br>X, Z, TE, CE, RD<br>X, Y, TE, CE, RD<br>X, E, CE, LOM, CN, RD,CXM<br>X, Y, PE, Cip, CN, CXM<br>X, E, TE, OFX, LOM, RD<br>X, Z, TE, Cip, CN | 8 | 8 | (12.64) |
| P7 | X, Z , CE , Cip, LOM , CXM<br>X, Y, CEC, CE, RD, CXM<br>X, Z, CE, FEP, RD, CXM<br>X, Z, CE, OFX, Cip, RD<br>X, Z, CE, Cip, LOM, RD<br>X, Z, CE, OFX, NA, CN<br>X, E, TE, AK, NA, RD , CXM<br>AMP, AMX, Z, CE, PE , LOM, CN, RD<br>X, C, E, PE, OFX, LOM , CN, CXM<br>AMP, AMX, C, CEC, CE, NA, LOM, CN , RD<br>AMP, AMX, E, TE, AK, OFX, Cip, RD, CXM | 9 | 11 | (17.38) |
| P8 | X, Z, TE30, CE, FEP, CN, RD<br>X, C, Z, CE, PE, CN, RD<br>X, Z, CE, Cip, NA, RD, CXM<br>X, Z, TE, CE, AK, PE, Cip, CXM<br>X, Z, CE, PE, OFX, LOM, RD<br>X, Z, CE, FEP, LOM, RD, CXM<br>X, Z, TE, CE, AK, LOM, RD | 10 | 7 | (11.06) |
| P9 | X, M, TE, CE, AK, FEP, RD<br>X, M, TE, CE, CN, RD, CXM<br>X, M, TE, CE, FEP, RD, CXM<br>X, Z, TE, CE, PE, Cip, LOM, CXM<br>X, M, CE, Cip, LOM, CN, CXM | 11 | 5 | (7.9) |
| P10 | X, M, CE, AK, FEP, PE, OFX, RD<br>E, OXA, CEC, CE, AK, OFX, Cip, LOM, RD, CXM | 12 | 2 | (3.16) |
| P11 | X, M, CE, OFX, Cip, NA, LOM, RD, CXM | 13 | 1 | (1.58) |
| P12 | X, Z, TE, FEP, PE, OFX, Cip, NA, LOM, RD, CXM<br>X, Z, CE, FEP, PE, OFX, Cip, NA, LOM, CN, CXM<br>X, M, TE, CE, PE, OFX, Cip, LOM, CN, CXM | 14 | 3 | (4.74) |

| Continue Table (5) | | | | |
|---|---|---|---|---|
| P13 | X, Z, TE, CE, PE, OFX, Cip, NA, LOM, CN, RD, CXM | 15 | 6 | (9.48) |
| | X, AMX, C, E, OXA, TE, CEC, CE, FEP, PE, OFX, LOM, CN, RD, CXM | | | |
| | X, Z, TE, CE, PE, OFX, Cip, NA, LOM, CN, RD, FEP | | | |
| | X, Z, TE, CE, AK, FEP, PE, OFX, Cip, RD, CXM | | | |
| | X, M, TE, CE, PE, OFX, Cip, NA, LOM, RD, CXM | | | |
| | X, M, TE, CE, PE, OFX, Cip, NA, LOM, RD, CXM | | | |
| P14 | X, M, TE, CE, FEP, PE, OFX, Cip, NA, LOM, RD, CXM | 16 | 3 | (4.74) |
| | X, Z, TE, CE, FEP, PE, OFX, Cip, NA, LOM, CN, RD, CXM | | | |
| | X,C, E, TE, CE, FEP, PE, OFX, Cip, NA, LOM, CN1, RD, CXM | | | |
| P15 | X, M, TE, CE,FEP, PE, OFX, Cip, NA, LOM, CN, RD, CXM | 17 | 2 | (3.16) |
| | X, M, TE, CE, FEP, PE, OFX, Cip, NA, LOM, CN, RD,CXM | | | |
| P16 | X,M, TE,CE,AK, FEP,PE, OFX, Cip, NA, LOM, CN,RD, CXM3 | 18 | 1 | (1.58) |

X: mix of AMP, AMX, OXA Y: mix of C, E
Z: mix of E, CEC M: mix of C, E, CEC

The preliminary test of antibacterial activity of the five tested undiluted oils revealed no inhibitory effects. Therefore, a dilution of 1:1 using DMSO solution was further tested. The obtained data showed that cinnamon oil recorded inhibition zones range between 1.36 to 2.3 cm against most tested *Klebsiella* isolates except isolates numbered 11 and 18, which were resistant to this oil. Lemon grass oil had no antibacterial activity against all tested *Klebsiella* isolates. On the other hand, lemon essential oil showed inhibition zones range from 1.1to 1.63 cm for *Klebsiella* isolates numbered 7, 10, 11, 17, 23, 33, 38, 39 and 40. It also recorded 1.26 cm inhibition zone for bacterial isolates number 23, 27 and 33. No inhibitory effect was observed against *Klebsiella* isolates number 9, 22, 28 and 41. Orange essential oil showed no antimicrobial effect on most tested bacteria except isolates 21, 22, 29, and 41. However clove essential oil revealed high antibacterial activity against *Klebsiella* isolates recording inhibition zones ranging from 1.46 to 2.56 cm for all tested *Klebsiella* isolates. Thus, the highest inhibitory effect was recorded against *Klebsiella* isolate number 40.

**MIC assay for clove oil extract**

Clove essential oil inhibited the growth of the tested *Klebsiella* isolates **(Table 7 and Photo 2).** This essential oil induced inhibition zone of 1.13 cm against the

most resistant *Klebsiella* isolate number 40 at 1: 20 v/v.

The MIC was determined by the agar well diffusion method. Dilutions (1:20, 1:10, 1:5 and 1:1 v/v) of clove essential oil were used against tested *Klebsiella* isolates. The concentration of the tested *Klebsiella* isolates was $10^7$ CFU/ml. Inoculated plates were incubated at 37ºC for 24 h. The lowest concentration of the essential oil which did not show any visible growth was considered as the MIC. Bacterial isolates No. 7, 10, 20, 21, 22, 25, 27, 29, 34, 38 and 41 showed inhibition zones at 1:5 v/v while *Klebsiella* isolates numbered 9, 11, 17, 18, 23, 33 and 39 recorded inhibition zones at 1:10 v/v finally bacterial isolates No. 36 and 40 revealed inhibition zones at 1:20 v/v.

**Phytochemical analysis of the clove oil**

**a- UV spectra**

The UV spectrum of the clove essential oil was carried out in DEMSO. The UV absorption spectrum was determined at a range of 200-600 nm. The obtained results indicated the presence of 2 peaks at 316 and 348 nm, that means the presence of an aromatic ring in the main oil component **(Fig. 1)**.

**Table (6):** Antibacterial activity of different plant essential oils against tested bacteria

| Isolate No. | Cinnamon | Lemongrass | Lemon | Orange | Clove | ANOVA | |
|---|---|---|---|---|---|---|---|
| | Inhibition zone (cm) | | | | | P. | F. value |
| **K7** | 1.96±0.36 | 0.0±0.0 | 1.43±0.85 | 0.0±0.0 | 1.46±0.50 | 1.253 | 0.058$^{ns}$ |
| **K9** | 1.63±0.52 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 1.63±0.47 | 1.417 | 0.063 $^{ns}$ |
| **K10** | 1.5±0.14 | 0.0±0.0 | 1.16±0.42 | 0.0±0.0 | 1.93±0.96 | 5.635 | 0.003* |
| **K11** | 0.0±0.0 | 0.0±0.0 | 1.33±0.62 | 0.0±0.0 | 1.8±0.24 | 6.957 | 0.001** |
| **K17** | 1.76±0.44 | 0.0±0.0 | 1.63±0.47 | 0.0±0.0 | 2.16±0.72 | 10.225 | 0.002* |
| **K18** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 1.86±0.62 | 9.255 | 0.001** |
| **K20** | 1.9±0.63 | 0.0±0.0 | 1.3±0.22 | 0.0±0.0 | 2.03±0.18 | 4.159 | 0.006* |
| **K21** | 1.76±0.85 | 0.0±0.0 | 1.53±0.24 | 1.13±0.19 | 2.43±0.63 | 0.638 | 0.198 |
| **K22** | 1.83±0.94 | 0.0±0.0 | 0.0±0.0 | 1.1±0.60 | 1.7±0.24 | 7.005 | 0.001** |
| **K23** | 2.06±0.24 | 0.0±0.0 | 1.26±0.42 | 0.0±0.0 | 2.46±0.42 | 1.253 | 0.069 $^{ns}$ |
| **K25** | 2.2±0.42 | 0.0±0.0 | 1.0±0.14 | 0.0±0.0 | 1.76±0.63 | 1.669 | 0.058 $^{ns}$ |
| **K27** | 2.1±0.24 | 0.0±0.0 | 1.26±0.63 | 0.0±0.0 | 2.46±0.24 | 7.236 | 0.001** |
| **K29** | 1.83±0.41 | 0.0±0.0 | 0.0±0.0 | 1.2±0.18 | 1.76±0.74 | 8.637 | 0.001** |
| **K33** | 1.66±0.41 | 0.0±0.0 | 1.26±0.82 | 0.0±0.0 | 1.73±0.56 | 10.152 | 0.014* |
| **K34** | 1.76±0.25 | 0.0±0.0 | 1.1±0.14 | 0.0±0.0 | 2.16±0.24 | 11.241 | 0.001** |
| **K36** | 1.36±0.20 | 0.0±0.0 | 1.26±0.63 | 0.0±0.0 | 2.1±0.62 | 15.390 | 0.001** |
| **K38** | 1.93±0.26 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 1.96±0.36 | 0.526 | 0.209 $^{ns}$ |
| **K39** | 1.93±0.63 | 0.0±0.0 | 1.36±0.62 | 0.0±0.0 | 2.33±0.42 | 6.330 | 0.001** |
| **K40** | 1.66±0.17 | 0.0±0.0 | 1.46±0.44 | 0.0±0.0 | 2.56±0.52 | 3.325 | 0.041* |
| **K41** | 2.3±0.75 | 0.0±0.0 | 0.0±0.0 | 1.2±0.22 | 2.46±0.36 | 5.325 | 0.005* |

ns = P. value > 0.05 in significant.

* = P. value < 0.05 significant. ** = P. value < 0.001 highly significant.

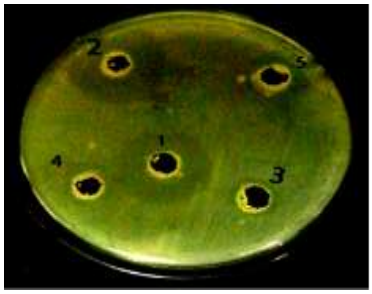


**Photo (1):** Antimicrobial activities of different plant essential oils against tested *Klebsiella* sp. isolate No. 40 (1- Cinnamon, 2- Clove, 3- Lemongrass, 4- Orange, 5- Lemon).

**b- GC/MS analysis of clove essential oil.**

The GC/MS analysis of clove essential oil is shown in **Fig. (2)** and **Table (8)**. It revealed that eugenol was the major compound (62.19%), followed by eugenol acetate (23.20%), caryophyllene (7.58%), α-humulene (4.78%) and cadinene (2.25%).

**Table (7):** Minimum inhibitory concentration (v/v) of clove oil on tested *Klebsiella* isolates

| Bacterial isolate number | Clove essential oil concentration (v/v) | | | | ANOVA | |
|---|---|---|---|---|---|---|
| | 1:1 | 1:5 | 1:10 | 1:20 | F | p. value |
| | Inhibition zone diameter (cm) | | | | | |
| K 7 | 1.46±0.50 | 1.13±0.25 | 0.0±0.0 | 0.0±0.0 | 2.325 | 0.044* |
| K 9 | 1.63±0.47 | 1.46±0.63 | 1.13±0.26 | 0.0±0.0 | 3.336 | 0.029* |
| K 10 | 1.93±0.96 | 1.33±0.41 | 0.0±0.0 | 0.0±0.0 | 6.335 | 0.001*** |
| K 11 | 1.8±0.24 | 1.33±0.41 | 1.16±0.52 | 0.0±0.0 | 2.969 | 0.017*** |
| K 17 | 2.16±0.72 | 1.56±0.52 | 1.26±0.36 | 0.0±0.0 | 0.576 | 0.201$^{ns}$ |
| K 18 | 1.86±0.62 | 1.3±0.63 | 1.13±0.44 | 0.0±0.0 | 7.250 | 0.001*** |
| K 20 | 2.03±0.18 | 1.36±0.25 | 0.0±0.0 | 0.0±0.0 | 6.025 | 0.001*** |
| K 21 | 2.43±0.63 | 1.46±0.42 | 0.0±0.0 | 0.0±0.0 | 3.669 | 0.017 *** |
| K 22 | 1.7±0.24 | 1.26±0.66 | 0.0±0.0 | 0.0±0.0 | 4.140 | 0.001*** |
| K 23 | 2.46±0.42 | 1.56±0.25 | 1.43±0.63 | 0.0±0.0 | 1.616 | 0.052* |
| K 25 | 1.76±0.63 | 1.1±0.17 | 0.0±0.0 | 0.0±0.0 | 1.845 | 0.049* |
| K 27 | 2.46±0.24 | 1.26±0.75 | 0.0±0.0 | 0.0±0.0 | 6.83 | 0.001*** |
| K 29 | 1.76±0.74 | 1.33±0.24 | 0.0±0.0 | 0.0±0.0 | 4.52 | 0.001*** |
| K 33 | 1.73±0.56 | 1.23±0.44 | 1.03±0.21 | 0.0±0.0 | 1.214 | 0.132 $^{ns}$ |
| K 34 | 2.16±0.24 | 1.36±0.51 | 0.0±0.00 | 0.0±0.0 | 6.308 | 0.001*** |
| K 36 | 2.1±0.62 | 1.43±0.24 | 1.26±0.41 | 1.03+0.20 | 8.100 | 0.001*** |
| K 38 | 1.96±0.36 | 1.3±0.36 | 0.0±0.0 | 0.0±0.0 | 0.685 | 0.303 $^{ns}$ |
| K 39 | 2.33±0.42 | 1.46±0.71 | 1.2±0.55 | 0.0±0.0 | 6.308 | 0.001*** |
| K 40 | 2.56±0.52 | 1.53±0.56 | 1.2±0.14 | 1.13±0.026 | 5.362 | 0.017* |
| K 41 | 2.46±0.36 | 1.36±0.66 | 0.0±0.0 | 0.0±0.0 | 4.226 | 0.011* |

ns = P.value > 0.05 in significant.

* = P. value < 0.05 significant ** = P.value < 0.001 highly significant


**Photo (2):** Minimum inhibitory concentration of clove oil against the tested *Klebsiella* sp. isolate No. 40 (1- 1:1, 2- 1:5, 3- 1:10, 4-1:20).

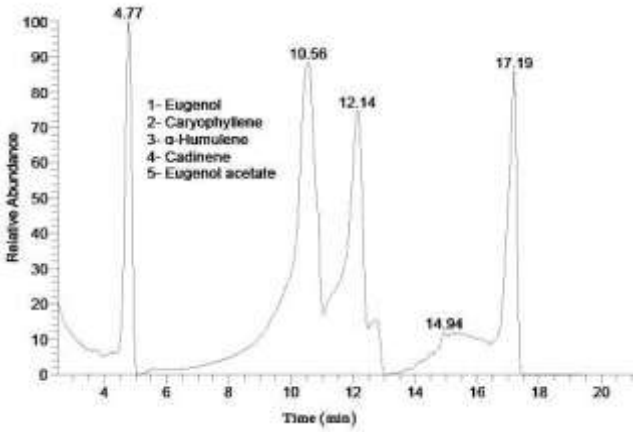


**Fig. (2):** GC/MS analysis profile of clove oil

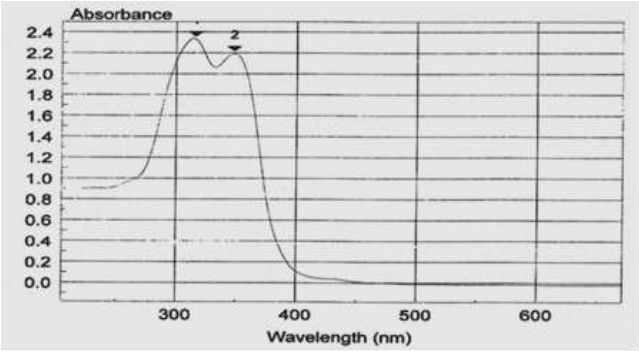


**Fig. (1):** UV- analysis profile of clove oil

**Table (8):** The major chemical compositions of clove oil

| S. No | Rt (min) | Compound | Peak Area [%] | Molecular Formula | M.wt |
|---|---|---|---|---|---|
| 1 | 4.77 | Eugenol | 62.19 | $C_{10}H_{12}O_2$ | 164 |
| 2 | 10.56 | Caryophyllene | 7.58 | $C_{15}H_{24}$ | 204 |
| 3 | 12.14 | α-Humulene | 4.78 | $C_{15}H_{24}$ | 204 |
| 4 | 14.94 | Cadinene | 2.25 | $C_{15}H_{24}$ | 204 |
| 5 | 17.9 | Eugenol acetate | 23.20 | $C_{12}H_{14}O_3$ | 206 |

S: Serial number, Rt: Retention time, M. wt: Molecular weight

**RAPD fingerprinting of multi drug resistant *Klebsiella* isolates**

Genomic DNA was isolated from 20 clinical isolates of *Klebsiella* species. The isolated DNA samples were subjected to the RAPD PCR amplification using 10–Mer operon primers of different G-C content. The products of the amplification reactions were analyzed by agarose gel electrophoresis. The electrophoretic profiles reported in **Photos (3, 4, 5, 6 and 7)** showed that all the primers gave rise to amplification products in all isolates except primer 2 which doesn't give results with isolates No. 2 and 29.

Genetic diversity between the 20 clinical isolates of *Klebsiella* sp. were assessed by converting RAPD fingerprinting into data matrix by scoring the presence of bands as 1 and the absence of bands as 0 and the analysis of the data using the Neighbor – joining method in the NTSYS-PC to produce a distance tree **(Fig. 3)**.

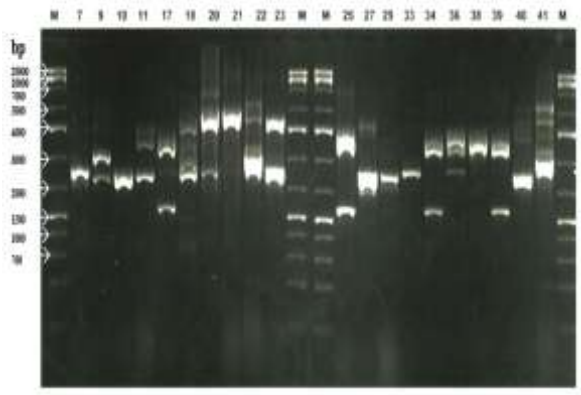

**Photo 3**: Ethidium bromide-stained agarose gel, showing RAPD fingerprinting of 20 *Klebsiella* isolates using primer 1 (M = Standard marker ladder).

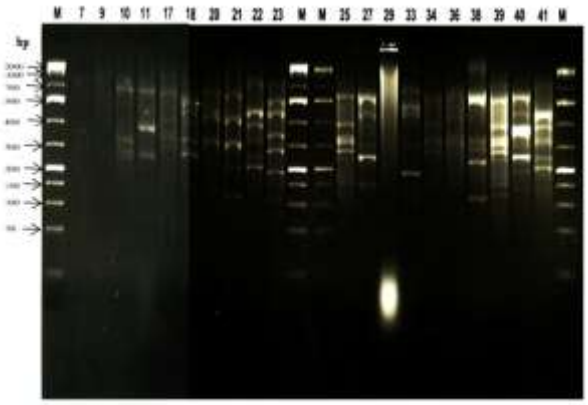

**Photo 4:** Ethidium bromide-stained agarose gel, showing RAPD fingerprinting of 20 *Klebsiella* isolates using primer 2 (M = Standard marker ladder).

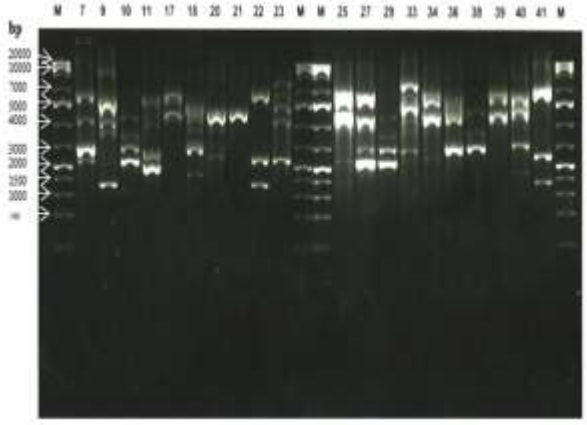

**Photo 5:** Ethidium bromide-stained agarose gel, showing RAPD fingerprinting of 20 *Klebsiella* isolates using primer 3 (M = Standard marker ladder).

However, differences were evident through the visual examination of the polymorphic bands of the DNA. The cluster analysis revealed five primary clusters. The RAPD- PCR data obtained from clinically related *Klebsiella* isolates indicated that these bacteria were not transmitted between patients in the intensive care units since the banding patterns of the isolates from different patients showed no similarity.

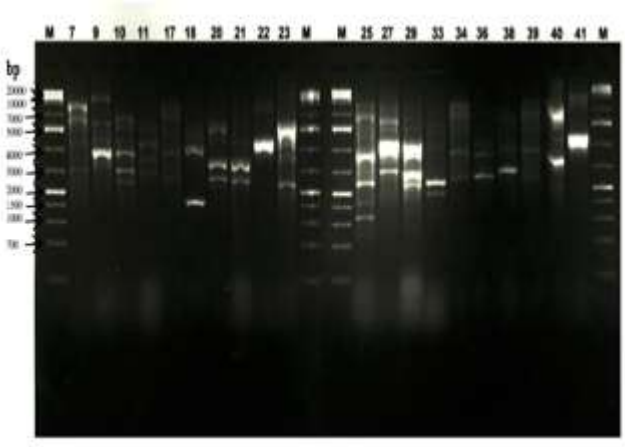

**Photo 6:** Ethidium bromide-stained agarose gel, showing RAPD fingerprinting of 20 *Klebsiella* isolates using primer 4 (M = Standard marker ladder).

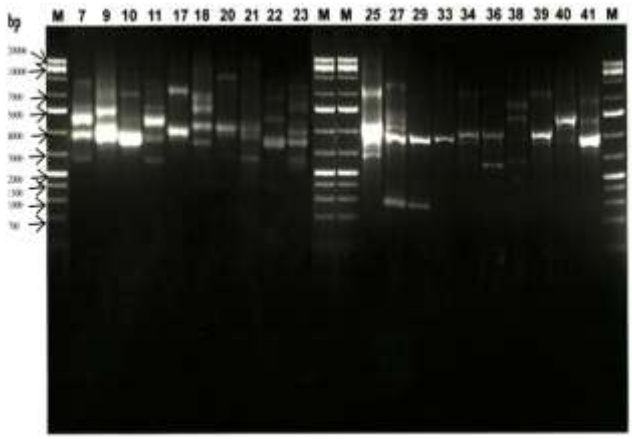


**Photo 7:** Ethidium bromide-stained agarose gel, showing RAPD fingerprinting of 20 *Klebsiella* isolates using primer 5 (M = Standard marker ladder).

The dendrogram demonstrated in **Fig. (3)**, showed that isolate 40 from sputum was clearly differentiated from all other isolates. The remaining isolates were divided into three groups, one comprises the two isolates, 29 and 33 from sputum that were close to each other.

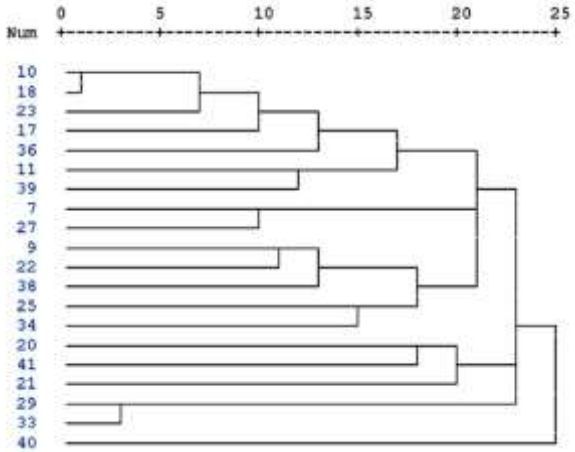


**Fig. (3):** Dendrogram of the genetic relationship for the selected *Klebsiella* isolates, based on the RAPD-PCR analysis.

The second group is comprised of the three isolates 20, 21 and 41 (also from sputum). The third group is differentiated into three clusters; one comprised of the five isolates 9, 22, 25, 34 and 38 (all from sputum except isolate 38 from urine). The second comprises isolates 7 and 27 from sputum and the third cluster is composed of isolates 10, 11, 17, 18, 23, 36 and 39 (all from sputum except isolate 23 from urine).

**Plasmid profile analysis**

The 20 bacterial isolates were tested for the presence of plasmid(s) as an attempt to know whether the β-lactam antibiotics resistance is plasmid encoded or chromosomally encoded. Plasmid(s) were found in 16 isolates (85%) of the 20 *Klebsiella* isolates from El-Mahalla Chest Hospital which contained 1 to 2 plasmids with different sizes, as revealed in **Photo (8)**.

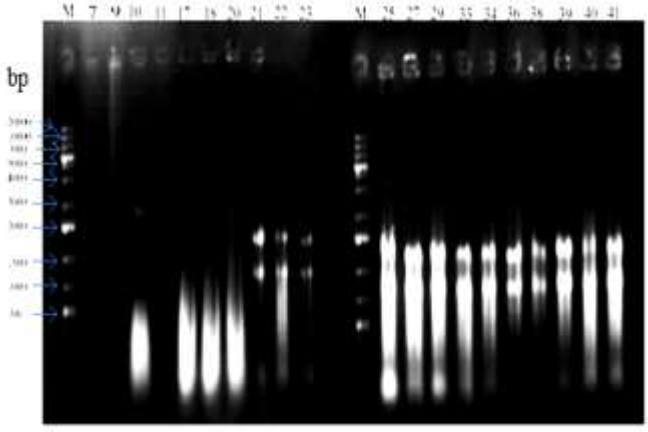


**Photo 8:** Agarose gel electrophoresis of plasmid DNA of *Klebsiella* isolates (M = Standard marker ladder).

**Discussion**

The frequency of *Klebsiella* isolates varied in different sample sources scoring the highest percentage in sputum

(82.8%) followed by urine (30.7%) samples. On the other hand, the lowest percentage of isolates was recorded in stool (20%). Comparable results were reported by **Girijan *et al.*, (2020)**. Similar results for sputum and urine infections by *Klebsiella* were reported by **Gorrie *et al.*, (2022)**, where they found that *Klebsiella* infections account for 61% of all sputum samples and account for 35 % of all urine samples. The fluctuation in the frequency of *Klebsiella* in the two sources might be due to differences in the number of the collected clinical specimens, the total bacterial isolates recovered, environmental conditions, nutrition requirements and virulence factors.

Using currently available antibiotics, MDR bacteria, causing severe nosocomial and group-acquired infections, are difficult to treat. Furthermore, widespread use of broad-spectrum antibiotics has resulted in the appearance of MDR strains, specially extended-spectrum beta lactamase resistant *K. pneumoniae* **(Kumar *et al.*, 2013)**. In this study, antibiotic sensitivity test was carried out to study the susceptibility of the 63 tested *Klebsiella* isolates collected from sputum, urine and stool of patients to 18 different antibiotics. It has been observed that *Klebsiella* isolates are resistant to more than one antibiotic. These findings are consistent with recent findings made public by other researchers **(Gorrie *et al.*, 2022)** who claimed that antibiotic treatment is typically utilized to treat infections brought on by *Klebsiella* species. However, when infections are brought on by *Klebsiella* species that are multi-antibiotic resistant, treatment options become constrained, which poses a serious problem for those who are immunocompromised. The tested isolates showed a moderate incidence of resistance to ciprofloxacin, nalidixic acid, ofloxacin, pefloxacin and lomefloxacin recording 39.85, 28.5, 42.85, 39.68 and 44.4%, respectively. Comparable results were reported by **Barguigua and Abouddihaj (2016),** where they found that ciprofloxacin resistance occurred in 31% of *Klebsiella* isolates. **Dong *et al.*, (2018)** stated that less than 20% of the *K. pneumoniae* isolates were resistance to ciprofloxacin.

The 63 tested *Klebsiella* isolates were grouped into 16 anti-biotypes (designated P1-P16) and each pattern includes sub patterns depending up on their resistance to different antimicrobial drugs. All isolates were multi resistant to 3-18 tested drugs. This phenomenon of multi resistance was previously reported **(Kumar *et al.*, 2013).** Genome plasticity plays a key role in the spread of dangerous bacteria that are resistant to antibiotics. *K. pneumoniae* and other

intestinal bacteria share many genes that cause resistance to antibiotics used in clinical settings, which is why drug resistance phenotypes are becoming more widespread in clinical settings.

The appearance of multi resistant strains of *Klebsiella* in hospitals has been clearly associated with the random use of antibiotics, resulting in a major clinical crisis **(Jikun *et al*., 2014)** and spread resistance genes such as plasmid encoded extended spectrum β-lactamases **(Jikun *et al*., 2014)**. The antibiotic action against the pathogen is considered as an external environmental pressure **(Thenmozhi *et al*., 2014).** Resistance rates varied from country to another **(Rani *et al*., 2014).**

The obtained data obviously showed statistically significant differences ($p < 0.05$) of the used essential oils against most isolates except isolates No. 7, 9, 23, 25 and 38. Similar observation for Cinnamon and Clove was reported by **EL-Farmawi *et al.*, (2014)** who mentioned that they were not harmful in food products; and also inhibited the growth of molds, yeast and bacteria. Also, the essential oils of cinnamon, and clove recorded the highest antimicrobial efficacy against some multiple antibiotic resistant *S. aureus, Streptococcus pneumoniae, P. aeruginosa, E. coli, K. pneumoniae* and *Bacillus subtilis* **(El-Shouny, 2006)**.

Clove essential oil which is the most effective in inhibiting the growth of *Klebseilla* isolates is the aromatic extract of the dried flowering buds of a family Myrtaceae tree, where it is used as an anodyne (pain killer) for dental emergencies; possessing anesthesia and antibacterial activity **(Adonu *et al*., 2013)**. Clove is also said to be a natural anti-helminthic in gastrointestinal tract **(Soni and Dahiya, 2014)**.

The obtained data revealed that the MIC values of clove oil were 1:5, 1:10 and 1:20 (v/v) against different isolates of *Klebsiella* sp. These results are in agreement with those obtained by **Adonu *et al*., (2013)** who recorded the MIC values of clove oil against *Salmonella typhi* and *Klebsiella* sp. (ranged between 1:10 to 1:20), lower than the values recorded against *E. coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (ranged between 1:20 to 1:40).

Phenyl-propanoides are the primary components of clove essential oil, including eugenol, carvacrol, thymol, and cinnamaldehyde **(Moorthy, 2016).** It was suggested that eugenol is particularly effective against the test bacteria due to its phenolic composition. 79.2% of clove oil is eugenol. Depending on the concentration utilized,

members of this class are known to be either bactericidal or bacteriostatic drugs compared to some antibiotics **(EL-Farmawi *et al.,* 2014)**. These substances were very active despite having a limited ability to dissolve in water, which is consistent with information presented by **Gong and Dai (2015).** The alkyl substitution into the phenol nucleus, which is known to increase the antibacterial activity of phenol, may further explain the high activity of phenolic compounds / fractions **(Dikpınar and Süzgeç-Selçuk, 2020)**. Furthermore, chemical composition of clove oil was also determined by GC-MS. The finding indicated that the clove oil had 62.19% eugenol, 23.20% eugenol acetate, 7.58% Caryophyllene, 4.78% α-Humulene and 2.25% Cadinene. These data support the hypothesis that eugenol present in clove essential oil is the most likely compound responsible for the strong antimicrobial activity of clove oil; that can denature proteins and interacts with cell membranes phospholipids to alter membrane permeability **(Kimura *et al.,* 2021)**.

*K. pneumoniae* possess plasmid-mediated Extended Spectrum β-lactamases (ESBLs) more frequently than in other Enterobacteriaceae species **(Azargun *et al.,* 2018)**. In the current experiment, 14 (70%) of the *Klebsiella* strains harbored large plasmids of size between 1500 and 2000 bp. The presence of plasmids in clinical isolates of *Klebsiella* as was common in several countries, frequently associated with the ESBLs production **(Zeighami *et al.,* 2015)**. This is because large plasmids carry beta-lactamases as well as genes for resistance to other antibiotics, such as tetracycline, aminoglycosides, and quinolones **(Adeyankinnu *et al.,* 2014).**

Efforts for controlling infection was at identification of the infection source and the transmission mode. Traditional techniques based on phenotypic characteristics are often insufficiently sensitive for typing all *K. pneumoniae* strains and discriminating between isolates (**Liu *et al.,* 2022**). In this study, genetic variation among 20 isolates identified as *Klebsiella* sp. was determined by RAPD-PCR finger-printing using five different primers. Differences in RAPD fingerprinting were evident by visual examination of the polymorphic bands of the DNA. These differences were sorted out by cluster analysis of the RAPD data. Analysis of the similarity among the examined isolates showed that Isolate 40 from sputum was clearly differentiated from all other isolates. The NTSYS dendrogram showed that all other isolates were clustered into two major groups from a common with further separation into subsets. Dissimilarity of

the RAPD fingerprinting of the examined *Klebsiella* isolates from various patients showed that these bacteria were not transmitted between patients in the same hospital department. These results are like those of **Munmun *et al.*, (2008)** and **Rani *et al.*, (2014)**.

## Conclusion

*Klebsiella pneumonia* was recorded as the most abundant MDR isolate among the collected samples (63 cases from sputum, stool and urine samples, out of 100 isolates of different sources), possessing 1 isolate of 100% resistance against the commercial antibiotics, 2 isolates of 94.4% and 2 isolates of 88.8% MDR. Clove oil was the most efficient essential oil against the resistant isolate with inhibition zone diameter of 1.46 to 2.56 cm and MIC of 1:20 dilution ratio. This oil activity was referred to eugenol as the main component on GC-MS analysis. Molecular studies revealed the presence of β-lactam antibiotics resistance plasmid as a major factor for spreading the multidrug resistance behavior among 85% of the tested isolates. More studies will be recommended to evaluate the histopathological effect and mechanism of action of the selected remedy.

## References

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## البحث عن علاجات بديلة للبكتيريا المقاومة للمضادات الحيوية

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تم تعريف 63 عينة كليبسيللا من ١٠٠ عينة طبية مختلفة لمرضى مستشفى الصدر بالمحلة الكبرى، محافظة الغربية، مصر. و تراوحت نسب العينات ما بين ٨٢.٨% عينات مخاطية، ٣٠.٧% عينات بولية و ٢٠% عينات براز.

كما تم إختبار حساسية العزلات ال ٦٣ للكليبسيللا ضد ١٨ مضاد حيوى تجارى، يمثلوا ٨ مجموعات، بواسطة طريقة إنتشار الأقراص. و قد تأكدت مقاومة المضادات المختلفة بينهم بنسبة ما بين ١٥.٨% (مضاد الأميكين) و ١٠٠% (مضاد الأمبيسيللين) ب ١٦ نمط مقاومة مختلف بين ال ٦٣ عزلة.

تم دراسة التنوع الوراثى للعزلات الأكثر مقاومة (٢٠ عزلة) بإستخدام ٥ بادئات بتقنية التكبير العشوائى متعدد الأوجه للحمض النووى. و قد تبين من تحليل النتائج أن العزلة رقم ٤٠ من المخاط كانت متباينة تماما عن باقى المجموعات. و قد تبين أن معظم العزلات عديدة المقاومة للمضادات تمتلك جين مقاومة مضادات البيتالاكتلام على بالزميدها، و كذلك تزداد إماضية عزلات الكليبسيللا فى وجود بلازميد الهجومية.

تم إختبار حساسية عزلات الكليبسيللا ضد ٥ زيوت عطرية (القرفة، القرنفل، البرتقال، الليمون و حشيشة الليمون). و أظهر زيت القرنفل أفضل تأثير على البكتيريا بأدنى تركيز مثبط (٢٠/١ حجم)؛ و بتحليل مكوناته بإستخدام الفصل العمودى اللونى و كشف الأشعة البنفسجية، تبين أن المكون السائد فى زيت القرنفل هو مادة الإيوجينول الفينولية.