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EVALUATION OF CITRIC ACID AND CLOVE OIL AS SAFE ANTI-VIRULENCE AND RESISTANCE-MODIFYING AGENTS AGAINST PROBLEMATIC KLEBSIELLA CLINICAL ISOLATES IN EGYPT

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ABSTRACT

Klebsiella species are regarded as terrifying pathogens that threaten different healthcare systems. These pathogens possess a variety of virulence factors which markedly account for their pathogenicity. Three virulence factors (capsule formation, hypermucoviscosity and biofilm formation) were screened, phenotypically and genotypically, among selected Egyptian *Klebsiella* clinical isolates. Two safe edible agents, clove oil and citric acid, were tested for their effect on bacterial capsules using the Anthony capsular staining method and the transmission electron microscopy. The antibiotic-resistance modifying activity of both agents was studied against selected troublesome isolates showing resistance to amikacin, meropenem and cefotaxime. Molecular docking of citric acid and eugenol with NDM-1, as well as the docking of citric acid with AAC(6′)-Ib and CTX-M-15 were carried out to explain the resistance modifying activity of the tested agents. Capsule formation was the most prominent virulence factor that was detected in all isolates. Both citric acid and clove oil could effectively reduce the size of the formed bacterial capsules. Citric acid showed promising synergistic effects when combined with the studied antibiotics against all the tested isolates. However, clove oil showed a synergistic effect only against 50% of the tested isolates when combined with meropenem. Relying on their binding efficiency mode obtained from molecular docking studies, the tested agents were recognized as promising inhibitors of the studied enzymes. In conclusion, both citric acid and clove oil are auspicious anti-virulence and resistance-modifying agents that can be combined with conventional antibiotics to combat virulent and multidrug resistant *Klebsiella* isolates.

Keywords: *Klebsiella*; bacterial capsule; clove oil; citric acid; polymerase chain reaction; transmission electron microscopy; molecular docking

INTRODUCTION

Klebsiella sp. continue to represent a real threat and fear in different healthcare systems as they frequently result in numerous nosocomial infections and are commonly associated with elevated morbidity and mortality rates **(Pereira and Vanetti, 2015)**. The most frequently encountered infections related to *Klebsiella* sp. are urinary tract infections, cholecystitis, pneumonia, catheter-associated bacteriuria, bacteremia and wound infection **(Qureshi, 2017).**
In fact, *Klebsiella* sp. possess diverse virulence factors which significantly participate in their pathogenicity **(Podschun and Ullmann, 1998).** Klebsiella can develop capsules formed from acidic polysaccharides. Such capsules can protect the bacteria from phagocytosis by polymorphonuclear granulocytes. Moreover, they provide the bacteria with a profound protection against bactericidal serum factors **(Podschun and Ullmann, 1998).** Hypermucoviscosity, characterized by the mucoid appearance of the bacterial colonies, is another prominent virulence factor of Klebsiella **(Pereira and Vanetti, 2015; Catalan-Najera *et al.*, 2017),** where the hypermucoviscous variants possess an obviously greater amount of sialic acid in their capsular extracts, and consequently have a striking phagocytosis-resistance phenotype **(Catalan-Najera *et al.*, 2017).** In addition, *Klebsiella* sp. can produce different fimbrial adhesins **(Pereira and Vanetti, 2015**) which play a leading role in adhering to host cells during infection, and also in biofilm formation **(Alcantar-Curiel *et al.*, 2013).**
Regrettably, the prevalence of antibiotic-resistant bacteria is a major problem encountered in different healthcare systems worldwide. Among the world's most dangerous multidrug resistant pathogens, *Klebsiella pneumoniae* has been recognized as a terrifying superbug **(Woldu, 2016).** In fact, developing new classes of antibiotics is too behindhand to fulfill our urgent and increasing demand for such drugs. A promising approach to overcome such problem is to focus on the pathogen's virulence factors **(Clatworthy *et al.*, 2007).** Recently, scientists have become extremely interested in studying the anti-virulence properties of natural and edible agents as novel therapeutic and anti-infective agents, instead of conventional antibiotics **(Asfour, 2017).**

Clove (*Syzygium aromaticum*) is a famous member that belongs to the family *Myrtaceae* **(Hussein *et al.*, 2014).** It is a medicinally important agent due to its powerful antiseptic and analgesic properties, especially that it is considered as generally recognized as safe (GRAS) **(Kovacs *et al.*, 2016).** The main constituents of clove oil are eugenol acetate, eugenol and caryo-phyllene, the latter two are thought to have considerable antibacterial and antifungal activities **(Hussein *et al.*, 2014).** Clove oil is characterized by a promising spectrum of activity against Gram-positive and Gram-negative bacteria **(Dorman and Deans, 2000).** Moreover, its activity against various dermatophytes has been recorded **(Park *et al.*, 2007).**
Citric acid is a natural weak organic acid which exists in different citrus fruits. It can be found in natural sources such as lemon, orange and lime. In addition, it can be obtained from synthetic sources, including chemical reactions and microbial fermentation **(Show *et al.*, 2015).** Its considerable antibacterial activity against *Staphylococcus aureus*, *Lactobacillus* sp., *Streptococcus* sp., *Klebsiella* sp. and *Corynebacterium* sp. has been reported **(Uzoh *et al.*, 2016).**
The aim of the current study was the phenotypic and genotypic characterization of selected virulence factors among selected Egyptian Klebsiella clinical isolates. This study also compared the effect of two safe edible agents: clove oil and citric acid on the bacterial capsules as a main recognized virulence factor among our isolates. Besides studying the anti-virulence properties of these agents, their antibiotic-resistance modifying activity was tested against selected problematic Klebsiella isolates which showed resistance to commonly used antibiotics. In addition, molecular docking of both citric acid and eugenol with selected enzyme(s) mediating resistance to the antibiotics under investigation was carried out.

MATERIALS AND METHODS

Isolation and Identification of Bacterial Isolates

Twenty-two Klebsiella clinical isolates were collected from Egyptian patients from two different governorates in Egypt: Alexandria and Cairo. These isolates were obtained from various clinical specimens including wound, urine, bronchoalveolar lavage, blood, pus and sputum. The collected isolates were commonly identified by classical microscopical and biochemical methods **(Bailey and Scott, 1986).** For more precise identification to the species level, the MALDI-TOF mass spectrometry **(De Carolis *et al*., 2014)** was employed using the MALDI Biotyper (Bruker Daltonik, USA).

Phenotypic Characterization of Selected Virulence Factors

Capsule detection

Bacterial capsules were detected by the Anthony capsular staining method **(Derakhshan *et al*., 2008).** Briefly, each isolate was grown to the early exponential phase (OD_{600}=0.3-0.4). Then, 20 µL of the bacterial suspension were mixed with the same volume of skimmed milk on glass slide. After the smear was air dried, it was stained with 1% aqueous crystal violet for 2 min and gently washed with copper sulphate solution (20%). The capsule was usually detected as an unstained region against a purple background; while bacterial cells were deeply stained in a purple color.

Hypermucoviscosity testing

Hypermucoviscosity of Klebsiella clinical isolates was tested using the modified string test. All the isolates were cultivated on sterile nutrient agar plates, then, a standard bacteriological loop was utilized to stretch a mucoviscous string from the bacterial colony. A positive result was described as the formation of a viscous string of a length greater than 10 mm **(Lee *et al*., 2010).**

Biofilm formation testing

All isolates were subcultured in sterile brain heart infusion (BHI) broth and then incubated at 37º C for 24 hours without shaking. Then, cultures were diluted 1:100 with sterile BHI broth. A volume of 200 µL of each diluted culture was aseptically dispensed in the wells of 96-well microtiter plates, and incubated overnight at 37º C. The planktonic cells were aspirated, and the wells were washed with phosphate buffer saline (pH= 7.4). After staining with 0.5% crystal violet for 20 minutes, excess stain was washed and the absorbance of the stained adherent bacteria was measured by microtiter plate reader at 490 nm. The OD value of the sterile medium was measured and then subtracted from the obtained values. All the isolates were tested in triplicate and the experiment was performed twice **(Hassan *et al*., 2011; Wu *et al*., 2011; Magesh *et al*., 2013; Aljanaby and Alhasani, 2016).** The interpretation of the biofilm formation results was done as follows: the isolate was considered as non-biofilm former when OD(isolate) ≤ OD(control), weak biofilm former if OD(control) < OD(isolate) ≤ 2 OD(control), moderate biofilm former when 2 OD(control) < OD(isolate) ≤ 4 OD(control) and strong biofilm former if 4 OD(control) <OD(isolate) **(Rodrigues *et al*., 2010)**.

Detection of Virulence-Associated Genes Using the Polymerase Chain Reaction (PCR)

The extraction of DNA from the tested isolates was done as follows: small part of a single colony of each isolate was suspended in 5 µL of PCR grade water in a PCR tube. The extraction step was done in the PCR thermocycler at conditions of 95°C for 10 min **(Škulj *et al*., 2008).** PCR was employed to detect six virulence-associated genes encoding: capsule synthesis (*uge* and *ycfM*) **(Candan and Aksoz, 2015),** hypermucoviscosity (*rmpA* **(Wasfi *et al.* 2016)** and *magA* **(Candan and Aksoz, 2015)**), type 1 fimbrial adhesion (*fimH*) **(Candan and Aksoz, 2015)** and type 3 fimbrial adhesion (*mrkD*) **(Candan and Aksoz, 2015)**. All primers used are listed in table 1. For the detection of the amplified bands, 1.7% agarose was prepared in Tris-Acetate-EDTA buffer (containing an approximate final concentration equivalent to 0.5 $\mu g.ml^{-1}$ ethidium bromide). Gel electrophoresis was done, using a gel electrophoresis device (Hoefer Scientific instruments USA) with its power supply, at a voltage of 120 for 25 minutes, in the presence of 100 bp DNA ladder (GeneDirex®, 100 $\mu g.mL^{-1}$, 11 fragments: 100-1500 bp) as a marker. The amplified PCR products were then visualized on a 254 nm UV transilluminator (Entela UVP Upland CA 91786 U.S.A.) and photographed by a digital camera.

Table 1 Sequence of primers used for the detection of virulence-associated genes among the studied Klebsiella clinical isolates using the polymerase chain reaction

Virulence Factor	Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product size (bp)	References
Hypermucoviscosity	*magA*	GGTGCTCTTTACATCATTGC	GCAATGGCCATTTGCGTTAG	*1282*	**Candan and Aksoz, 2015**
	rmpA	ACTGGGCTACCTCTGCTTCA	CTTGCATGAGCCATCTTTCA	535	**Wasfi *et al*., 2016**
Capsule formation	*uge*	TCTTCACGCCTTCCTTCACT	GATCATCCGGTCTCCCTGTA	534	**Candan and Aksoz, 2015**
	ycfM	ATCAGCAGTCGGGTCAGC	CTTCTCCAGCATTCAGCG	160	**Candan and Aksoz, 2015**
Fimbrial adhesion	*fimH*	TGCTGCTGGGCTGGTCGATG	GGGAGGGTGACGGTGACATC	*688*	**Candan and Aksoz, 2015**
	mrkD	TTCTGCACAGCGGTCCC	GATACCCGGCGTTTTCGTTAC	240	**Candan and Aksoz, 2015**

Testing the Effect of Two Edible Agents: Citric Acid and Clove Oil on Bacterial Capsules among Klebsiella Clinical Isolates

Agents used

Citric acid

The aqueous solution of citric acid (Oxford Lab Chem, India) was prepared in the appropriate concentration and then sterilized using Sartorius™ Minisart™ syringe filters of pore size 0.45 µm, before use.

Clove oil

For the extraction of clove oil, the dried buds of clove were ground into coarse powder using electric grinder. Batches of 200 g of the clove powder were mixed with 2 liters of distilled water and then exposed to hydro-distillation process using Clevenger apparatus for 4 hours for the isolation of oil **(Clevenger, 1928).** The oil was dried over anhydrous sodium sulfate, and then stored at 4°C in dark glass bottles for further experiments.

Gas chromatography/mass spectrometry (GC/MS) analysis of clove oil

The essential oil of clove was analyzed by GC/MS where mass spectra were recorded using Shimadzu GCMS-QP2010 (Tokyo, Japan) equipped with Rtx-5MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Restek, USA) supplied with a split–splitless injector. The capillary column was coupled to a quadrupole mass spectrometer (SSQ 7000; Thermo-Finnigan, Bremen, Germany). The initial column temperature was maintained at 45°C for 2 min (isothermal) and programmed to 300°C at a rate of 5°C/min, and kept constant at 300°C for 5 min (isothermal). The injector temperature was adjusted to 250°C. The flow rate of helium carrier gas was 1.41 $mL.min^{-1}$. Each mass spectrum was documented after adjusting the following conditions: (equipment current) filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 200°C. Diluted samples (1% v/v) were injected with split mode (split ratio 1: 15). Identification of compounds was accomplished through comparing with the NIST library and with those stated in the literature. Percentage composition was computed from GC peak areas on BD-5 ms column without applying correction factors.

Determination of the minimum inhibitory concentrations (MICs) of clove oil and citric acid against the tested Klebsiella clinical isolates using the broth microdilution method

MICs of both citric acid and clove oil against the tested Klebsiella clinical isolates were determined using the broth microdilution technique **(Fournomiti *et al.*, 2015).** Briefly, 100 µL of double strength nutrient broth were aseptically dispensed into the wells of 96-well microtiter plates. In the first column of wells, citric acid and clove oil were added at final concentrations of 80 $mg.mL^{-1}$ and 37.5 % (v/v), respectively, and then serially diluted by two-fold serial dilution. Finally, 100 µL of bacterial suspension (of an approximate count of 10^6 $CFU.mL^{-1}$) were added to every well and then the plates were incubated at 37ºC for 24 hours. All plates were read using microtiter plate reader at 630 nm. Positive and negative controls were included in the experiment. The assay for each strain was repeated twice and then the average MIC was calculated.

Examination of the effect of clove oil and citric acid on the bacterial capsules using the Anthony capsular staining method

Each strain was grown to the early exponential phase (OD_{600}=0.3-0.4), then, subcultured in sterile double strength nutrient broth containing ½ MIC of each of clove oil and citric acid, separately, to reach a final inoculum of approximately 10^6 $CFU.mL^{-1}$. Untreated cells were included in the experiment as controls. All the cultures were left for 24 hours at 37ºC in shaking incubator adjusted at 250 rpm. After incubation, control and treated cells were stained by the Anthony capsular staining method, as previously mentioned **(Derakhshan *et al.*, 2008),** and compared to each other.

Examination of the effect of clove oil and citric acid on capsule formation among selected isolates using the transmission electron microscopy (TEM)

Two isolates were selected for this experiment: *K. pneumoniae* K4 and *K. variicola* K6. Each isolate was grown to the early exponential phase (OD_{600}=0.3-0.4), then, subcultured in sterile double strength nutrient broth containing ½ MIC of each of clove oil and citric acid, separately, to reach a final inoculum of approximately10^6 $CFU.mL^{-1}$. Untreated cells of both K4 and K6 were included in the experiment as controls. All cultures were left for 48 hours at 37ºC in the shaking incubator at 250 rpm. The sample preparation and examination were done as described by **Kim *et al.* (2007),** using transmission electron microscopy (JEOL, JEM-1400 Plus Electron Microscope, Japan) at an accelerating voltage of 80 kV. Capsule size was measured using a specific software program provided by the Electron Microscope Unit. Capsule size was calculated as the difference between the diameter of the whole cell (including the capsule) and the diameter of cell body, designated by the cell wall **(Martinez *et al.*, 2010).**

Testing the Antibiotic-Resistance Modifying Activity of Clove Oil and Citric Acid against Selected Problematic Klebsiella Clinical Isolates

The antibiotic-resistance modifying activity of both clove oil and citric acid was tested against three commonly used antibiotics: amikacin (Advomikacin® 500mg/2mL, Advocure, Egypt), cefotaxime (Cefotax® 1 g, EIPICO, Egypt) and meropenem (Meronem® 1 g, Astrazenca, UK). This experiment was conducted against four selected problematic Klebsiella clinical isolates exhibiting resistance to these antibiotics. Against each isolate, the MIC of the antibiotic alone was compared to the MIC of antibiotic in presence of ½ MIC of citric acid, and the MIC of antibiotic in presence of ½ MIC of clove oil, using the broth microdilution technique. The fractional inhibitory concentration (FIC) was calculated as the ratio of $MIC_{\text{Antibiotic in combination}}/MIC_{\text{Antibiotic alone}}$. The results of this experiment were interpreted as follows: FIC index value of ≤ 0.5 indicated synergism, while an index value of > 0.5 - 4 indicated indifference, and antagonism was designated by an index value of > 4 **(Seukep *et al.*, 2016).**

Molecular Docking of Citric Acid and Eugenol with Selected Enzymes Conferring Antibiotic Resistance in Klebsiella

Depending on the results of the experiment of the antibiotic-resistance modifying activity, we studied the molecular docking of each of citric acid with NDM-1 (New Delhi metallo-beta lactamase (NDM): a metallo-β- lactamase capable of hydrolyzing all β- lactams except aztreonam) **(Khan *et al.*, 2017),** AAC(6′)-Ib (Aminoglycoside 6'-N-Acetyltransferase type Ib enzyme mediating resistance to amikacin in Klebsiella) **(Chiem *et al.*, 2015)** and CTX-M-15 (one of the most common ESBLs in Gram-negative bacteria that possesses a considerable hydrolyzing activity against cefotaxime) **(Farhadi *et al.*, 2017).** In addition, the docking of eugenol (the major compound in clove oil) with NDM-1 was studied. The X-ray crystal structures of NDM-1 in complex with hydrolyzed ampicillin (code: 3Q6X), AAC (6′)-Ib in complex with kanamycin C and acetyl coenzyme A (code: 1V0C), as well as CTX-M-15 in complex with C6S (code: 5T66) were obtained from the RCSB Protein Data Bank. Three inhibitors were used in order to benchmark the potency of citric acid and eugenol. These inhibitors were tazobactam (a well-known inhibitor of CTX-M-15) **(Poirel *et al.*, 2002),** chlorhexidine (an inhibitor of AAC (6′)-Ib) **(Chiem *et al.*, 2016)** and N-[(dimethyl BLAHyl)methyleneamino]-4-methyl-benzenesulfonamide (an inhibitor of NDM-1) **(Randhawa and Jamwal, 2011).** Their three dimensional structures, along with those of citric acid and eugenol, were derived from ZINC Database **(https://zinc.docking.org/)**. The accurate docking was performed using the docking tool iGEMDOCK v2.0. on the basis of the binding energy in kcal/mol. The numbers of runs were 70 and the max interactions were 2000 with a population size of 200 and an energy threshold of 100. In addition, at every step, the least 'min' torsions/translations/ rotations were investigated and the one showing the lowest energy was selected. The hydrophobic preference and electrostatic preference were adjusted to 1.00. The binding site of each target was recognized at a distance of 8 Å.

RESULTS AND DISCUSSION

RESULTS

Identification of Clinical Isolates

All the collected isolates were Gram-negative scattered rods which grew on MacConkey's agar. All the isolates showed acid slant and acid butt with the triple sugar iron test and a delayed positive result (pink color after 6 hours) with the urease test. All the isolates were negative for both the indole production test and the methyl red test, while showed positive results with both the Voges-Proskauer test and the citrate utilization test. Thus, all isolates were identified as *Klebsiella* sp. The MALDI-TOF mass spectrometry showed that 13 isolates were *K. pneumoniae*: K1, K2, K4, K7, K8, K9, K12, K13, K15, K17, K19, K20 and K21. On the other hand, 9 isolates were recognized as *K. variicola*: K3, K5, K6, K10, K11, K14, K16, K18 and K22.

Phenotypic Characterization of Virulence Factors

Capsule formation was detected in 22 isolates (100% of the tested isolates) when tested by the Anthony capsular staining method. Only one isolate (K4) (4.55%) showed a positive hypermucoviscous phenotype. Regarding biofilm formation, eighteen isolates (81.82%) were classified as weak biofilm formers, while four isolates (18.18%) were non-biofilm formers.

PCR Detection of Virulence-Associated Genes

Out of the six tested virulence-associated genes, four genes were detected among our isolates. Concerning the hypermucoviscosity-associated genes, only one isolate (4.55%) was positive for *magA*, while two isolates (9.09%) were positive for *rpmA* (Fig. 1). Regarding the capsule-associated genes, 18 isolates (81.82%) were positive for *uge*, while 22 isolates (100%) were positive for *ycfM* (Fig. 2 & 3). Type 1 fimbrial adhesion (*fimH*) gene and type 3 fimbrial adhesion (*mrkD*) gene were not detected among any of the tested isolates.

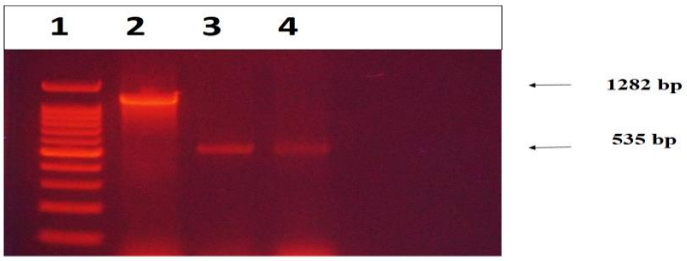

Figure 1 PCR amplification of the genes encoding for hypermucoviscosity (*magA and rmpA*) in selected Klebsiella clinical isolates. Lanes: 1: 100 bp DNA ladder *; 2: K4 (*magA*) (1282 bp); 3: K4 (r*mpA*) (535 bp); and 4: K10 (r*mpA*)) (535 bp).

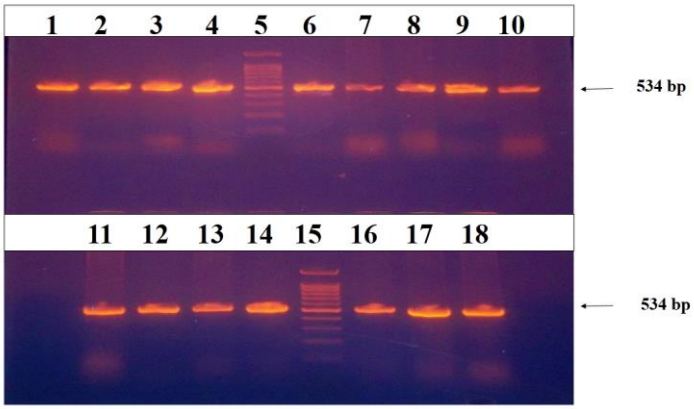

Figure 2 PCR amplification of the capsule-associated gene (*uge*) (534 bp) in selected Klebsiella clinical isolates. Lanes: 1: K3; 2: K4; 3: K6; 4: K7; 5: 100 bp DNA ladder; 6: K8; 7: K10; 8: K11; 9: K12; 10: K13; 11: K14; 12: K15; 13: K16; 14: K17; 15: 100 bp DNA ladder; 16: K18; 17: K20 and 18: K 22

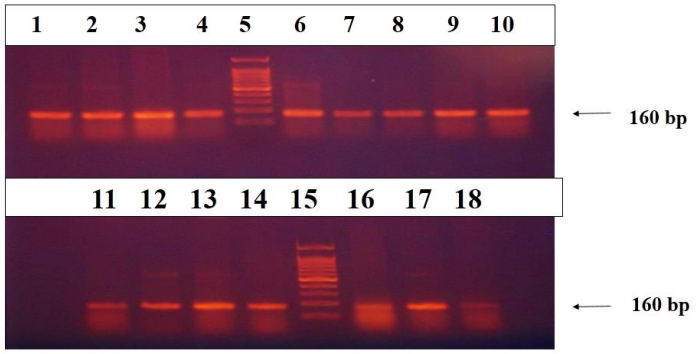

Figure 3 PCR amplification of the capsule-associated gene (*ycfM*) (160 bp) in selected Klebsiella clinical isolates. Lanes: 1: K17; 2: K18; 3: K19; 4: K20; 5: 100 bp DNA ladder; 6: K21; 7: K22; 8: K1; 9: K2; 10: K5; 11: K6; 12: K7; 13: K9; 14: K10; 15: 100 bp DNA ladder; 16: K11; 17: K14 and 18: K16

Testing the Effect of Two Edible Agents: Clove Oil and Citric Acid on Bacterial Capsules among Klebsiella Clinical Isolates

GC/MS analysis of clove oil

The major constituent of clove oil was eugenol (96.49%). In addition, four other peaks: β-caryophyllene (1.89%), eugenyl acetate (1.45%), humulene (0.09%) and copaene (0.01%) were detected.

MICs of clove oil and citric acid

The average MICs of citric acid against the tested isolates ranged between 2.5 and 5 mg.ml^{-1}, while the average MICs of clove oil ranged between 0.00018 and 0.11 (%) (v/v).

Examination of the bacterial capsules using the Anthony capsular staining method

In case of citric acid-treated isolates, an obvious effect was detected in 21 (out of 22 tested) isolates (95.45%). The most noticeable and common effect of citric acid on capsule formation, observed among 20 (out of 22 tested) isolates (90.9%), was the thinning of the bacterial capsules to the extent that, in some cases, capsules were difficult to be detected and almost rods only could be seen (Fig. 4). Another effect of citric acid on the bacterial capsules was the elongation of the formed capsules. This effect was noticed in case of 9 isolates (40.9%). Concerning clove oil-treated isolates, only a considerable effect could be detected in case of 4 (out of 22 tested) isolates (18.18%). In all these four isolates, the capsules were extremely thinned after being treated with clove oil, and were sometimes difficult to be detected. Obvious elongation of the formed capsules was detected in two of these four affected isolates (Fig. 5).

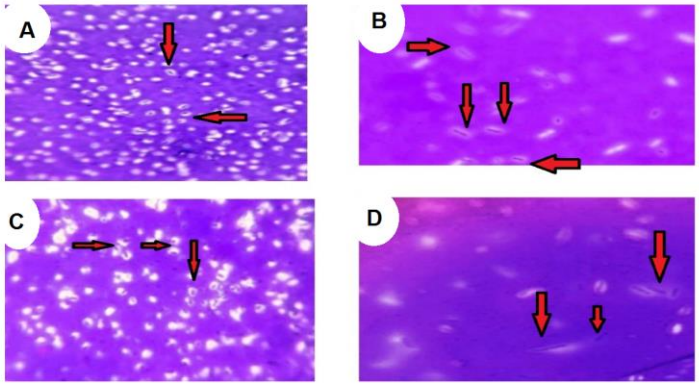

Figure 4 The effect of citric acid ($^1/_2$ MIC) on the capsules of treated bacterial isolates as revealed by the Anthony capsular staining method under light microscopy: (A) Control *K. pneumoniae* K1, (B) Citric acid-treated K1, (C) Control *K. pneumoniae* K17 and (D) Citric acid-treated K17.

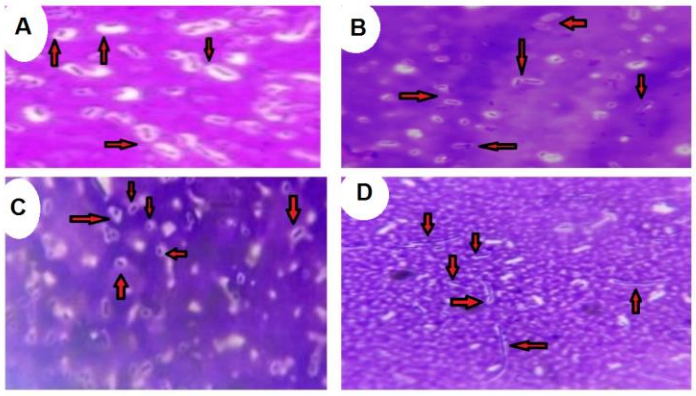

Figure 5 The effect of clove oil acid ($^1/_2$ MIC) on the capsules of treated bacterial isolates as revealed by the Anthony capsular staining method under light microscopy: (A) Control *K. pneumoniae* K4 , (B) Clove oil-treated K4, (C) Control *K. pneumoniae* K8 and (D) Clove oil-treated K8.

Transmission electron microscopy (TEM)

TEM was used for the examination of the effect of both citric acid and clove oil on bacterial capsules in both isolates: K4 and K6. For each isolate, the capsule size of each of the control and treated cells was calculated as an average of three measurements to minimize any error. Regarding K4, noticeable thinning of the bacterial capsules was detected in case of both citric acid- treated cells and clove oil-treated cells, when compared to control cells. The average capsule size of control cells was 149.65 nm (Fig. 6). On the other hand, the average values of capsule size of citric acid- treated cells and clove oil-treated cells were 65.54 and 62.53 nm, respectively. The sizes of the capsules of citric acid- treated cells and clove oil-treated cells were approximately 0.44 X and 0.42 X the capsule size of control cells, respectively (Fig. 7 & 8).

Similarly, in case of K6, both citric acid and clove oil showed a prominent effect in reducing capsule formation. In case of control untreated cells, the average capsule size was 141.94 nm (Fig. 9). On the contrary, the average values of capsule size of citric acid- treated cells and clove oil-treated cells were 37.35 and 30.81 nm, respectively. The sizes of the capsules of citric acid- treated cells and clove oil-treated cells were approximately 0.26 X and 0.22 X the capsule size of control cells, respectively (Fig. 10 & 11).

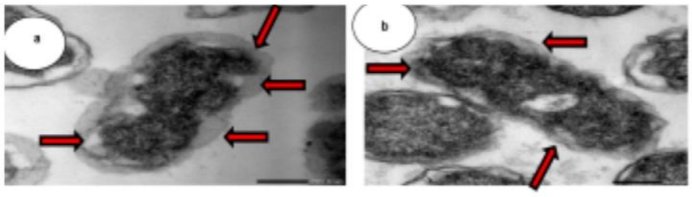

Figure 6 TEM demonstrating cells of the control (untreated) *K. pneumoniae* clinical isolate (K4) showing obvious formation of capsules of considerable thickness in different cells (a & b)

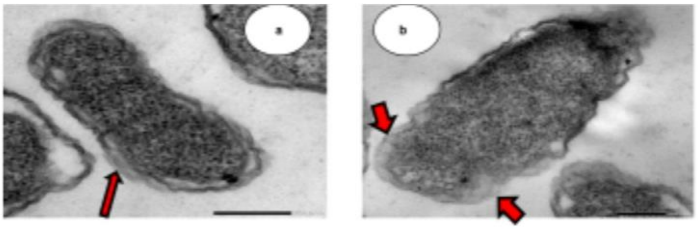

Figure 7 TEM demonstrating cells of the citric acid-treated *K. pneumoniae* clinical isolate (K4) showing obvious thinning of capsules in different cells (a & b)

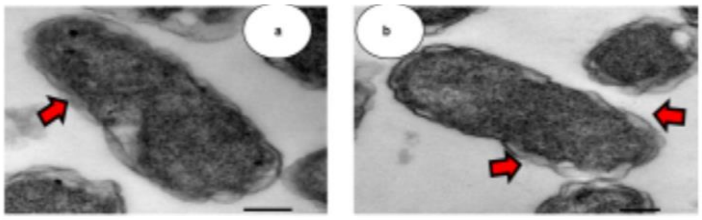

Figure 8 TEM demonstrating cells of the clove oil-treated *K. pneumoniae* clinical isolate (K4) showing obvious thinning of capsules in different cells (a & b)

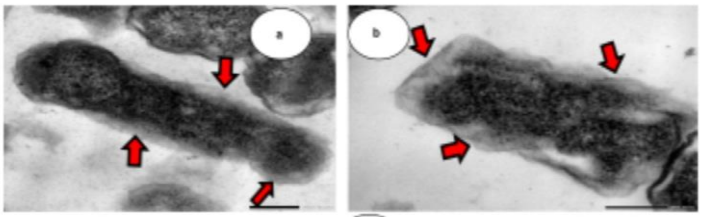

Figure 9 TEM demonstrating cells of the control (untreated) *K. variicola* clinical isolate (K6) showing obvious formation of capsules of considerable thickness in different cells (a & b)

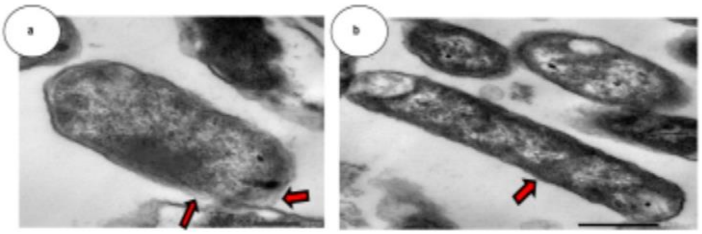

Figure 10 TEM demonstrating cells of the citric acid-treated *K. variicola* clinical isolate (K6) showing obvious thinning of capsules in different cells (a & b). Also, cell elongation was clearly shown in (b)

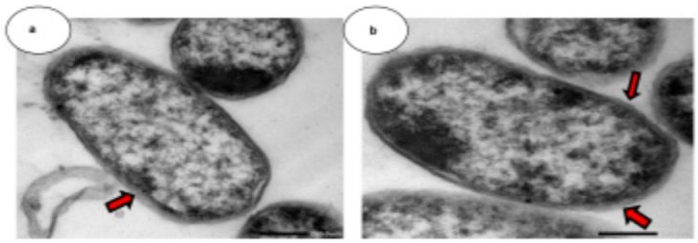

Figure 11 TEM demonstrating cells of the clove oil-treated *K. variicola* clinical isolate (K6) showing obvious thinning of capsules in different cells (a & b)

Antibiotic-Resistance Modifying Activity of Clove Oil and Citric Acid

Three antibiotics (amikacin (AK), meropenem (MEM) and cefotaxime (CTX)) were combined with citric acid and clove oil at their corresponding ½ MICs against four selected problematic Klebsiella clinical isolates. At ½ MIC of citric acid, it showed promising synergistic effects with each of AK, MEM and CTX against the four tested isolates (Tab 2). Regarding clove oil (when tested at a concentration equivalent to ½ MIC), it showed a synergistic effect only against two isolates (K9 and K12) when tested in combination with MEM, however, this combination showed indifference against both K5 and K7. On the other hand, the combinations of clove oil (½ MIC) with each of AK and CTX showed indifference against all isolates (Tab 2).

Table 2 MICs of antibiotics alone and in combination of citric acid and clove oil at MIC/2 against selected problematic *Klebsiella* clinical isolates

Antibiotic[a]	Agent and concentration	Bacterial strains, MIC (µg.ml^{-1}) of antibiotics in the absence and presence of agent and FIC[b] (in brackets)			
		K5	K7	K9	K12
AK	0	8192	>16384	8192	16384
	Citric acid	1024 (0.125) (S)	4096 (0.25) (S)	1024 (0.125) (S)	1024 (0.0625) (S)
	Clove oil	8192 (1) (I)	16384 (1) (I)	8192 (1) (I)	16384 (1) (I)
MEM	0	4	16	8	4
	Citric acid	<0.5 (0.125) (S)	<0.5 (0.03) (S)	<0.5 (0.0625) (S)	<0.5 (0.125) (S)
	Clove oil	4 (1) (I)	64 (4) (I)	4 (0.5) (S)	2 (0.5) (S)
CTX	0	>1024	>1024	>1024	>1024
	Citric acid	2 (0.00195) (S)	8 (0.0078) (S)	2 (0.00195) (S)	<0.5 (0.000488) (S)
	Clove oil	>1024 (1) (I)	>1024 (1) (I)	>1024 (1) (I)	>1024 (1) (I)

[a]: Antibiotics: (AK: amikacin, MEM: meropenem, CTX: cefotaxime).
[b]: Fractional inhibitory concentration (FIC) was calculated as the ratio of MIC $_{\text{antibiotic in combination}}$/ MIC $_{\text{antibiotic alone}}$. S: synergism, I: indifference.

Molecular Docking

Concerning NDM-1, N-[(dimethyl BLAHyl) methyleneamino]-4-methyl-benzenesulfonamide showed a binding energy of (-99.06) most of which were Van der Waals forces (-86.3) and hydrogen bond forces (-12.7). Citric acid showed a slightly higher binding energy of (-125.92) most of which were hydrogen bond forces (-67.4), Van der Waals forces (-23.5) and electrostatic attraction (-35). On the other hand, eugenol showed a binding energy of (-63.7) most of which were Van der Waals forces (-45) and hydrogen bond forces (-18.5). It was noticed that citric acid could bind via hydrogen bonds with HIS 122,189, ASP 124, LYS 211 and ASN 220, in addition to Van der Waals forces with TRP 93, ASN 220, ASP 223 and HIS 250. The inhibitor could bind via hydrogen bonds with HIS 122, GLN 123 and ASP124, in addition to Van der Waals forces with TRP 93, HIS 122, GLU 152 and ASP 223, as well as weak forces with ASN 220. Eugenol showed hydrogen bonds with HIS 122, 189, ASP 124 and ASN 220, in addition to Van der Waals forces with TRP 93, ASP 124 and HIS 250.

Regarding the enzyme AAC (6′)-Ib, citric acid showed a binding energy of (-83.3) and that of chlorhexidine was (-134.4). Nearly half of the binding energy of citric acid was due to hydrogen bonds (-45.9), Van der Waals forces (-40.5) and only (-3) as electrostatic forces. On the other side, chlorhexidine showed most of its binding energy as Van der Waals forces (-95.2) and only (-39.2) as hydrogen bonds. The binding sites that formed hydrogen bonds with citric acid were ASP 115, SER 154, ASN 157, TRP 48, 49 and GLY 50, while weak Van der Waals

forces were detected with ASP 115,179. On the other hand, chlorhexidine showed hydrogen bonds with GLY 50, TYR 93 and ASP 115,152,179, as well as Van der Waals forces with TRP 49, GLY 50 and TRP 102.

As for CTX-M-15, tazobactam showed a binding energy of (-111.12) most of which were Van der Waals forces (-62.9) and hydrogen bonds (-50.1), while citric acid showed a comparable binding energy of (-91.2) where most of which were also Van der Waals forces (-46.7) and hydrogen bonds (-42.6). Tazobactam showed hydrogen bonds with SER 73, 133, 240, ASN 107,135, 173, TYR 108, LYS 237, THR 238 and GLY 239, as well as Van der Waals forces with TYR 108, SER 133, 240 and THR 219. On the other hand, citric acid showed strong hydrogen bonds with SER 73,133 and ASN 107, 135, 173, and also Van der Waals forces with ASN 107, TYR 108 and SER 133, 135,240.

DISCUSSION

Klebsiella sp. have been recognized as horrible and scary superbugs in different hospital settings resulting in numerous nosocomial infections with outbreaks of 20% **(Woldu, 2016).** Commonly, the pathogenicity of such species is owing to an assortment of virulence factors which are capable to result in various diseases through invading the human's immune system **(Gharrah *et al.*, 2017).** Our results illustrated that capsule formation was detected in 100% of the tested isolates. Virtually, the existence of such capsules is regarded as a significant virulence factor that commonly could be interconnected to the graveness of an infection **(Pereira and Vanetti, 2015).** In our study, *uge* and *ycfM* were detected in 81.82 and 100% of the isolates, respectively. Such results were closely similar to those obtained by **Aljanaby and Alhasani (2016)** who detected the capsule associated genes, *uge* and *ycfM*, in 93.75 and 100% of the tested *K. pneumoniae* strains, respectively, in Iraq **(Aljanaby and Alhasani, 2016). Candan and Aksöz (2015)** postulated that these genes are regarded to be the principal element of the pathogenicity of such isolates.

In this study, only one isolate (K4) showed a positive result when tested for hypermucoviscosity. In this isolate, both genes *rmpA* and *magA* were detected. In fact, the low prevalence of hypermucoviscous phenotype among Klebsiella isolates, as in this study, has been noticed before. For example, **Vernet *et al.* (1992)** reported that only 7% of the studied *K. pneumoniae* strains possessed this hypermucoviscous phenotype. In addition, **Pereira and Vanetti (2015)** reported that only 6.7% of the studied Klebsiella isolates showed hypermucoviscosity. Inspite of showing negative hypermucoviscous phenotype, the isolate K10 possessed the *rmpA* gene. Similar to our case, and according to **Yu *et al*. (2006)** they noticed that out of 93 isolates of negative hypermucoviscous phenotype, fourteen isolates possessed *rmpA* gene. They attributed this to the fact that such isolates showing negative hypermucoviscous phenotype might be deficient in an additional positive regulator for *rmpA*, as for instance the *rmpB* gene, that is in charge of the complete expression of *rmpA* **(Yu *et al*., 2006).**

Actually, the absence of genes encoding for fimbrial adhesion in our isolates was matching with the results obtained from the biofilm assay which showed that 81.82% of the isolates were considered as weak biofilm formers, while the rest were non-biofilm formers. **Barati *et al.* (2016)** had previously reported the absence of the virulence gene *fimH* in two aquatic-borne *K. pneumoniae* isolates, in Malaysia, that were recognized to be weak biofilm formers. In addition, **Bellifa *et al.* (2013)** studied biofilm formation among 24 *K. pneumoniae* isolates collected from medical devices. They found that 22 out of 24 strains harbored type 3 fimbriae-encoding gene *mrkD*, and were biofilm formers. On the other hand, the remaining two strains were biofilm non-producers. Thus, they postulated that the presence of *mrkD* gene was correlated with high adhesion indexes (**Bellifa *et al*., 2013).** An important suggestion has been made by **Krapp *et al.* (2017)** concerning the virulence of *K. pneumoniae* strains. They postulated that some hypervirulent strains might possess traits which help them to result in serious invasive infections even if they lack many of the genes which are often interconnected with the hypervirulence phenotype. Although some studies considered the genes encoding for type 1 and type 3 fimbrial adhesion as ubiquitous among different clinical *K. pneumoniae* isolates, with currency of 95-100%, other studies have illustrated that the widespread presence of such genes might be commonly influenced and affected by various factors, including source of infection, hypermucoviscous phenotype and capsule genotype **(Krapp *et al.*, 2017).**

In this study, the antimicrobial activity of two edible and safe agents, clove oil and citric acid, had been studied. Both agents possessed a noticeable activity against the tested Klebsiella isolates. Regarding clove oil, and as illustrated in the results obtained from GC/MS analysis, its powerful antimicrobial activity could be attributed to the elevated level of eugenol **(Nunez and Aquino, 2012).** In fact, eugenol, as well as the phenolic compounds existing in clove oil, are capable of denaturing proteins. They can also react efficiently with phospholipids of bacterial cell membrane, thus, altering the permeability of bacterial cell **(Nunez and Aquino, 2012).** On the other hand, the considerable antimicrobial activity of citric acid can be attributed to two major mechanisms. First, citric acid is able to efficiently lower the pH. Second, it is a powerful chelating agent that can bind to metal ions, which consequently inhibits the bacterial growth **(Søltoft-Jensen and Hansen, 2005).**

Striking thinning of bacterial capsules was observed upon treating the bacterial cells with either clove oil or citric acid. However, it was noticed that citric acid was capable of exerting such effect in a higher number of isolates, compared with clove oil. In addition, for selected isolates, the transmission electron microscopy revealed that the size of the capsules of treated cells, by either agents, was profoundly diminished when compared with that of control untreated cells. On the whole, it has been documented that plant-derived compounds might be regarded as a promising resource that could assist in developing novel therapeutic approaches that target the production of bacterial capsules **(Upadhyay *et al*., 2014).** Also, **Lin *et al.* (2013)** had reported that citric acid was the main organic acid in the conventional Chinese medicine *Fructus mume* extract and was believed to be responsible for the extract's profound effect in inhibiting the biosynthesis of bacterial capsular polysaccharides among virulent *K. pneumoniae* isolates.

Concerning the antibiotic-resistance modifying activity of the studied agents, both clove oil and citric acid could modify the resistance of Klebsiella isolates to frequently used antibiotic(s). However, citric acid was a more powerful resistance-modifying agent. Regarding this issue, **Dharmik *et al.* (2012)** had previously mentioned that concentrations of citric acid equivalent to 0.05% and 0.1% were capable to improve the potency of different tested antibiotics against enteropathogenic *Escherichia coli*. They also found that the combination of antibiotics with citric acid at a concentration above 0.1% had completely inhibited the bacterial growth **(Dharmik *et al*., 2012).** In addition, it has been believed that the combination therapy of traditional antibiotics with essential oils, as potential resistance-modifying agents, is regarded nowadays as a promising issue for future studies, as this may lead to discovering advanced pharmacological regimes for combating multidrug resistant bacteria **(Yap *et al*., 2014).** Regarding this issue, **Atteia and Hussein (2014)** had previously reported a promising synergistic effect obtained when combining the clove ethanolic extract with diverse antibiotics against *K. pneumoniae* clinical isolates.

Regarding the obtained results from molecular docking, and for the enzyme AAC(6′)-Ib, previously published data suggested that amino acids ASP115, 152 are critical acidic residues that form ionic interactions. Also, three tryptophan residues TRP 49,102,103 and the backbone amide ASN 115 can form hydrogen bonds **(Vetting *et al*., 2008).** This can be considered as closely matching with the results obtained from the binding sites of citric acid with AAC(6′)-Ib, in our study. **Cahill *et al.* (2017)** mentioned the amino acids in the active site of CTX-M-15 to be ASN 107,135, 173, SER 73, 133, TYR 108 and THR 238, which were in agreement with our results of citric acid docking. The active sites of NDM-1 are characterized to be flexible, among which are HIS 120,122, 189, ASP 124, CYS 208, Lys 211, ASN 220 and HIS 250. It could be easily noticed that the obtained binding sites with NDM-1, in this study, can be regarded to be very close to those stated in the literature **(Green *et al*., 2011; Zhang and Hao, 2011; Wang *et al*., 2015).** Upon comparing the total binding energy released due to the association of the tested agents, citric acid or eugenol, with the target enzymes with that released upon the association of the standard inhibitors with the same enzymes, it was found that the obtained values were comparable to a great extent which indicated that both citric acid and eugenol can act as potential inhibitors of the studied enzymes. In fact, the widespread of multidrug resistant pathogens possessing NDM-1 necessitates developing novel NDM-1 inhibitors. As no *inhibitor* for medical treatment has yet been approved for *NDM-1* or other classes of metallo-β-lactamases, we hereby, present citric acid and eugenol as potential inhibitors and we recommend further in vitro, as well as structural modifications studies.

CONCLUSION

In conclusion, capsule formation was the most prevalent virulence factor among Klebsiella isolates. Both clove oil and citric acid could noticeably reduce capsule formation among the studied isolates. Also, both agents sensitized problematic isolates to commonly used antibiotic(s) in Egypt. However, citric acid has been considered as a more powerful anti-virulence and antibiotic-resistance modifying agent, when compared to clove oil. Citric acid and eugenol acted as promising inhibitors of selected enzyme(s) mediating antibiotic resistance in *Klebsiella* sp. Such encouraging properties of both agents represent a valuable invest in other future *in vitro* and *in vivo* studies aiming at making use of such edible and safe agents and utilizing them as a promising part of the therapeutic regimen targeting the virulent and multidrug resistant microorganisms, rather than focusing on traditional antibiotics.

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