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The Effect of Oak, Cinnamon, Oregano and Thyme Extracts on Biofilm producing ESBL *Klebsiella pneumoniae*

Ali Hassan Ahmed Al-Shammary* and Moutaz A.W. Abdul Mounam

Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad, Iraq.

* Corresponding author: Ali Hassan Ahmed Al-Shammary, e-mail: alideerwoeesh76@gmail.com

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ABSTRACT

Pluripotent medicinal plants and their derivatives were famous spices and flavoring ingredients widely used in cooking and can be incorporated into different varieties of lifestyle as antioxidants and antimicrobial agents. The purpose of this project was to investigate the inhibitory properties of oak, cinnamon, oregano and thyme extracts on multidrug resistant and biofilm producing Klebsiella pneumoniae strains isolated from clinical mastitis cases in Baghdad. The statistical and scientific designing of this project depends on detection of ESBL producing K. pneumoniae strains from mastitis cases in regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya; via culturing and biochemical series confirmation tests. Extract disc sensitivity test, Kirby-Bauer technique using Carbapenems (Imipenem and Meropenem) with Oxoid Cefpodoxime Combination double diffusion inhibition kit using Muller-Hinton agar and McFarland turbidity tubes for checking antibiotic susceptibility pattern of isolates depending on instructions of USFDA Clinical Laboratory Standards Institute (CLSI). Preparation of crude concentrated and diluted, double distillated or purified extracts of locally collected and imported oak, cinnamon, oregano and thyme barks, leaves and nuts from different regions in Baghdad as watery and oily or alcoholic extracts according to recommendations of Codex international standards of Association of Official Agricultural Chemists (AOAC). Totally collected milk samples from mastitic Cows were thirty: ten samples from each region from January to April (2017), in which they processed according to modified food microbiological methods. Serum bactericidal power, minimum inhibitory concentration and minimum bactericidal concentration (MIC & MBC), Haemolysis pattern and Siderophore activity were checked using modified procedures. Chi-square analysis via SPSS software was applied for checking significant differences. The results revived detection of five (16.66%) isolates of K. pneumoniae out of thirty samples: three (10%) from Abu-Ghraib region and one (3.33%) from each Al-Fudhaliyah and Al-Sadrya regions, in which ESBL producers were only detected from Abu-Ghraib cases as totally three (10%) isolates themselves. Pluripotent Oak (Baloot), Cinnamon, Oregano and Thyme Extracts exhibit inhibitory effects on isolated strains of K. pneumoniae compared to selected antibiotics. These selective toxicity properties reflex their active ingredients that interacts directly and indirectly in different mechanisms with electromagnetic cloud (biofilm complex layers) of these multidrug resistant clinical isolates.

Keywords: Oak, Cinnamon, Oregano, Thyme, Klebsiella pneumoniae, Biofilm, ESBL, Mastitis.

1. INTRODUCTION

Herbs and spices have been used since ancient times, not only as antioxidants and flavoring agents but also for their antimicrobial properties increasing the safety and shelf life of food products by acting against degradation induced by foodborne and spoilage microbes. Plants and their phytochemical constituents have historically been used in traditional medicine as sources of natural antimicrobial substances for the treatment of infectious disease. Therefore, much attention has been paid to medicinal plants as a source of alternative antimicrobial strategies. Many plants used in traditional medicine represent rich sources of natural bioactive substances with health-promoting effects and no side effects [1-7]. During the last two decades, growing evidence shows that plants are rich sources of different classes of antimicrobial substances acting as defense systems to protect them against biotic (living) and abiotic (non-living) stressors [1 and 8], among these secondary metabolites, polyphenols, terpenoids, alkaloids, lectins, polypeptides and

polyacetylenes that approved as generally recognized as safe materials for food products (*GRAS*), showing minor side effects. These properties give them special economic importance [1 and 9]. There are many edible and medicinal plants with high antimicrobial effects, such as thyme (*Thymus vulgaris* L.), tea (*Camellia sinensis* L.), garlic (*Allium sativum* L.), turmeric (*Curcuma longa* L.), berries belonging to *Rosaceae* family, and cinnamon (species belonging to *Cinnamomun* genus) (8 & 10). Multidrug growing repeated cycles of biofilm producing microbial threats led to discovery and development of new antimicrobial candidate agents that might act against these developing resistance problems [8, 11 and 12].

Cinnamon, the eternal tree of tropical medicine, belongs to the *Lauraceae* family. Cinnamon is one of the most important spices used daily by people all over the world. Cinnamon primarily contains vital oils and other derivatives, such as cinnamaldehyde, cinnamic acid and cinnamate. In addition to being an antioxidant, anti-inflammatory, antidiabetic, antimicrobial, anticancer, lipid-lowering, and cardiovascular-disease-lowering compound, cinnamon has also been reported to have activities against neurological disorders, such as Parkinson's and Alzheimer's diseases [13]. To date, several antimicrobial activities of cinnamon and its oils have been reported in various studies, such as study a combination of cinnamon and clove oils against Gram-positive organisms *(Listeria monocytogenes, Enterococcus faecalis, Staphylococcus aureus,* and *Bacillus cereus*), as well as against Gram-negative bacteria (*Salmonella choleraesuis, Escherichia coli, Pseudomonas aeruginosa,* and *Yersinia enterocolitica)* [14]. Another study indicated that cinnamon oils have potential action against various bacteria (*Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia coli*) and yeast (*Torulopsis utilis, Schizosaccharomyces pombe, Candida albicans,* and *Saccharomyces cerevisiae*) [15]. A recent study reported the activity of the aqueous extract of cinnamon and other plants against oral microflora. Cinnamon has been used as a spice in daily life without any side effects. Several reports have dealt with the numerous properties of cinnamon in the forms of bark, essential oils, bark powder, phenolic compounds, flavonoids, and isolated components. Each of these properties plays a key role in the advancement of human health. The antioxidant and antimicrobial activities may occur through the direct action on oxidants or microbes, whereas the anti-inflammatory, anticancer, and antidiabetic activities occur indirectly via receptor-mediated mechanisms [13].

Thymus species have a very rich flora and are used in herbal tea, condiments and medicine. Their essential oils contain a high concentration of the isomeric phenolic monoterpenes thymol and carvacrol [2 and 16]. Oregano is prevalent herbaceous and perennial plant growing mainly in calcareous and non-calcareous rocks, slopes, and screes. Different literatures concerning the determination of chemical compositions and antimicrobial properties of the essential oils of various *Origanum* and *Thymus* species, as well as their applications in various commercial preparations, mainly as antimicrobial and antioxidant agents [2, 4 and 17]. Previous studies demonstrated that the extract or essential oil of *Origanum acutidens* [5] had inhibitory effect against foodborne pathogens. *Quercus infectoria* is a small tree native of Greece, Asia Minor and Iran. Quercus is a plant genus in the family of Fagaceae. This species is generally known under the name ''baloot" in Iran, Iraq, Turkey and Syria and are commonly used as medicinal plant. In traditional medicine from Quercus infectoria is used in the treatment of intertrigo, impetigo, eczema, hemorrhages, chronic diarrhea and dysentery [18 and 19].

Klebsiella pneumoniae was an opportunistic pathogens isolated from various infections in animals and humans. Increasing proportion of multidrug resistant and extended-spectrum beta lactamases producing strains (*ESBLs*): enzymes that confer resistance to penicillins like ampicillin or amoxicillin, to first and new generations of cephalosporins like cefotaxime, ceftazidime, cefoxitin and ceftiofur, and to aztreonam (20). *Klebsiella pneumoniae* is an important nosocomial pathogen causing urinary tract infections and infections of the respiratory tract predominate. Infections are frequently preceded by gastrointestinal colonization that believed to be the most important reservoir for transmission of the bacteria. Several studies have described the pathogenic potential of *Klebsiella pneumoniae* isolated from environmental origin to be nearly identical to clinical isolates with respect to several phenotypic properties. The ubiquity general ability of *K. pneumoniae* strains to infect susceptible hosts might explain the high frequency of opportunistic infections caused by this species [21].

Escherichia coli and *Klebsiella* spp., followed by *Serratia* spp. and *Enterobacter* spp., are the most frequent Gram-negative pathogens isolated from bovine clinical mastitis (22-25). The incidence of mastitis due to such bacteria has increased in recent years [26]. *Klebsiella* is usually referred as particularly aggressive and prone to cause severe clinical mastitis, which responds poorly to treatment and is likely to have a fatal outcome [27 and 28]. Klebsiellae are defined as germs that easily produce enzymes like extended-spectrum β-lactamases (*ESBL*), thanks to their ability to survive longer than other Gram-negative rods in the environment and on the skin and, in particular, to allow ESBL genes to evolve [29]. At the onset of clinical mastitis, it is impossible to identify the causative agent; thus, it is necessary to use broad-spectrum intramammary preparations which contain narrow- to extended-spectrum cephalosporins, alone or in combination with other antibiotics [30]. The broad-spectrum cephalosporin ceftiofur is used as well in supportive systemic therapy [27]. Few recent works have studied *Klebsiella* isolation and ESBL detection in veterinary medicine [20 and 31], and no data are available describing *ESBL* mediated resistance in *Klebsiella* spp. isolated from bovine mastitis. Worldwide, mastitis is

one of the most important and costly infectious diseases of the dairy industry, affecting animal welfare and having potential public health implications if untreated or if inadequately treated milk is consumed. In the etiology of bovine mastitis, *Escherichia coli* and *Klebsiella pneumoniae* are regarded as significant agents of environment associated bovine mastitis [32]. Extended spectrum beta lactamases (*ESBLs*) have become widespread enzymes in food producing and companion animals worldwide. However, in cattle mastitis, a major cause of economic loss in the dairy industry, conferring high levels of resistance to last generations of beta lactams in *Enterobacteriaceae*, including ceftiofur and cefquinome (33). The spread of multidrug resistant microorganisms from nosocomial to community and livestock will rise infection rate and cost treatment strategies [34 and 35].

The aim of this project was evaluation of inhibitory effect of Oak, Cinnamon, Oregano and Thyme Extracts on Biofilm Producing *ESBL Klebsiella pneumoniae* compared to selected antibiotics with the determination of breakpoints titers of these extracts.

2. MATERIALS AND METHODS

2.1 Samples Processing

Totally collected milk samples from mastitic Cows were thirty: ten samples from each region during January till April (2017), in which they processed according to modified food microbiological methods inside food laboratory in Veterinary Public Health department as soon as possible (**36** & **37**). Samples were collected from acute and per-acute mastitic Cows via clean containers and handled correctly until conveyance to work lab by icebox.

2.2 Isolation & Confirmation

Coagulated, clotty and watery mastitic milk samples will mixed, homogenized and refrigerated at 4 ©C overnight for resuscitation of *K. pneumonia*e, then inoculated in tryptone soya yeast extract broth (one part sample (15 ml) to nine part diluent (135 ml) and incubated at 37 ©C overnight to boost bracing *K. pneumonia*e with capsule and biofilm creation, then cultured in McConkey agar and incubated at 37 ©C for 48 hours (36 & 37). Pure mucoid glistening and large pink colonies with viscous threads were picked up and recultured in tryptone soya yeast extract broth, then cultured in tryptone soya yeast extract agar for additional identification. Gram stain, catalase, oxidase and negative staining technique with nigrosine and carbol fuchsin for capsule detection were proceeded. Biochemical confirmation by Indole, Methyl red, Voges–Proskauer and Citrate series tests (*IMViC*). Modified Congo red tryptone soya yeast extract agar was used for detection of biofilm producing isolates depending on formation of black or dark colonies with a dry crystalline consistency as positive slime producers while, pink colonies were weak slime producers (38).

2.3 Antibiotics Susceptibility & *ESBL* Profile

Kirby-Bauer technique using Carbapenems (Imipenem and Meropenem) with Oxoid Cefpodoxime Combination double diffusion inhibition kit (39-43) using Muller-Hinton agar and McFarland turbidity tubes for checking antibiotic susceptibility pattern of isolates depending on instructions of *USDA* Clinical Laboratory Standards Institute (*CLSI*). The zones of growth inhibition around each of the antibiotic disks measured to the nearest millimeter. The diameter of the zone related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. Combination discs were a mix of cephalosporin and clavulanic acid on a single disc, which are, used in conjugation with a plain cephalosporin for *in vitro* detection of *ESBLs* strains. The presence of Clavulanate enlarged the zones for all of *ESBL*-producing *klebsiellae* by ≥ 5 mm, whereas zones for Cefpodoxime-susceptible isolates and Cefpodoxime-resistant isolates with *AmpC* and *K1* β-lactamases enlarged by ≤ 1 mm.

2.4 Haemolysis Pattern, Siderophore Activity and Serum Power

Modified Human type O blood agar (Glychophorin phenomenon) was prepared, in which 10 ml blood added to 100 ml autoclaved warmed tryptone soya yeast extract agar, mixed gently and poured into plates carefully to prevent formation of bubbles (36 & 37). Non-iron restricted method (without chrome azurol sulfate agar) was used for detection of siderophore activity (44 & 45). *K. pneumonia*e was generally non-haemolytic, but haemolytic and siderophore activity was developed and noticed in virulent strains especially those isolated from clinical cases and owned specific sets of enzymes that modulate their multi-resistance activity. Siderophore activity was measured depending on calibration of zone size of haemolytic strain after incubation at 37 ©C for 48 hrs. and after 72 hrs. at 4 ©C, in which enlarged zone indicate much siderophore activity. Serum bactericidal or static (inhibitory) power (serum resistance) was checked using freshly prepared sera from Human type O blood by sterile tubes, in which they processed in to two units firstly sera inactivated at 56 ©C for 30 min for destroying pseuoglobulins (Complements activity) and secondly other units non water bath treated as whole serum power. Freshly prepared culture of *K. pneumoniae* on tryptone soya yeast extract broth was adjusted to match turbidity of the McFarland opacity tube 0.5 (25-30) * 10^5 cfu/ml or approximately (6.397-6.477 log) (**36**), then 0.1 ml of it was added to concentrated non-diluted 1 ml whole and inactivated parts of sera in separated series tubes, mixed well by vortex and incubated inside shaker water bath at 37 ©C for series time intervals one, two and three hours and checked for development of turbidity and counted *K. pneumoniae* for each hour by droplet or Miles-Misra method (46) for noticing any increase or decrease in bacterial count load during these periods as an indicator of serum power or strain resistance.

2.5 Plants Extraction

Preparation of crude concentrated and diluted, double distillated or purified extracts of locally collected and imported oak, cinnamon, oregano and thyme barks, leaves and nuts from different regions in Baghdad as watery and oily or alcoholic extracts according to recommendations of Codex international standards of Association of Official Agricultural Chemists (*AOAC*) (47). Since 1884, *AOAC* International has ensured the ability of analytical scientists to have confidence in their results through the adoption of methods as *AOAC*®Official Methods℠. It is an international source of methods, in which scientists worldwide contribute their expertise to standards development, method development, and the systematic evaluation and review of methods. It is the most comprehensive collection of chemical and microbiological methods available in the world, and many methods within the compendium have notation indicating their adoption as harmonized international reference methods by the International Organization for Standardization (*ISO*), the International Dairy Federation (*IDF*), the International Union of Pure and Applied Chemistry (*IUPAC*), and the Codex Alimentarius Commission (*CAC*). The barks, leaves and nuts of medicinal plants were cleaned and dried in shadow and powdered using a mechanical grinder. For the aqueous extract, 100 gm powder for each plant was added to liter of hot water and boiled for 15 minutes, then filtered through a cloth. The filtrate was evaporated to dryness under reduced pressure to obtain a viscous residue. The residue was suspended in 100 ml normal saline. For the ethanolic extract, 100 gm powder for each plant was defatted with petroleum ether (40–60) ©C using the Soxhlet apparatus. Then, the powder was soaked in 800 ml ethanol (80%, *v/v*) for 72 hours, and the mixture was subsequently filtered and concentrated at 40 ©C. The residue was suspended in 100 ml normal saline. All crud extracts were centrifuged at 3000 rpm for 15 minutes and filtered in three sequential times for purification and sieving, then stored in dark bottles in refrigerator until use (48).

2.6 Extract Disc Sensitivity Test

Preparation of extract impregnated discs with comparison to Kirby antibiotic and Cefpodoxime combination discs assays was carried out. Clean thick Whatman Cellulose filter papers were cut it as standard circular antibiotic like disc by appropriate applicator and distributed in sterile plates for autoclaving before treatment with extracts. Sterile cooled filter papers were impregnated properly by each concentrated non-diluted extract in each plate separately, then left to absorb and adsorb the crude active ingredients in each extract for twenty minutes. Preparation of freshly 0.5 McFarland culture broth of *K. pneumoniae* (10^4-10^5 cfu/ml) and distributed thoroughly on Muller-Hinton agars as Kirby technique, then transferring each impregnated extracts discs by sterile applicator and distributed evenly in inoculated plates (4-5 discs/plate) and incubated at 37 ©C for 24 hrs. Depending on diffusion rate for each impregnated disc, measuring size of inhibition zones to nearest millimeter for each isolate and compared to inhibition zones sizes of antibiotics as standards, then determine minimum inhibitory and bactericidal concentration for each extract.

2.7 MIC & MBC Threshold Breakpoints

Prediction and titration of the lowest concentration or threshold breakpoint for each extract was proceeded (49 & 50). Vitek™ and E-tests now available for these purposes. Cut-Off values of Minimum inhibitory concentrations (*MICs*) are defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a *K. pneumoniae* after overnight incubation and minimum bactericidal concentrations (*MBCs*) the lowest concentration of antimicrobial that will prevent the growth of *K. pneumoniae* after subculture on to antibiotic free media, or kill at least 99.9 % (3 log) of tested bacteria. *MICs* used by diagnostic laboratories, mainly to confirm resistance, but most often as a research tool to determine the *in-vitro* activity of new antimicrobials, and data from such studies have been used to determine *MIC* breakpoints. Two fold dilution series tubes for each extract with appropriate diluent performed test procedure (PBS & ethanol) with standard 0.5 McFarland fresh tryptone soya yeast extract broth for each isolate was prepared. 0.1ml for each standard inoculum for each isolate was distributed in sterile clean 5 ml tubes containing two fold diluted extracts for each type and diluent, mixed well by vortex, then incubated in shaker water bath at 37 ©C for 24 hrs. The cut-off values of *MIC* measured as the lowest concentration or reciprocal titer for each extract that inhibits the visible of growth of *K. pneumoniae* (absence of turbidity). *MBC* was measured by culturing the titer tube and above with no visible growth on tryptone yeast extract agar, so that absence of any colony indicate bactericidal cut-off value of that extract for each isolate. Extract was considered bactericidal if the *MBC* is within two dilutions of the *MIC*, $MBC/MIC \leq 4$ log or fold, or considered bacteriostatic if the *MBC* is more than two dilutions higher than the *MIC*, $MBC/MIC > 4$ log or fold. *MBC* was the lowest concentration of antibiotic that caused at least a 99.9% fall in viable count during overnight incubation, i.e. presence of one or few colonies in agar cultured from non-turbid broth (49 & 50).

2.8 Statistical Analysis

Chi-square (æ ²) analysis via statistical package for social sciences software for checking significances among project variables done.

3. RESULTS AND DISCUSSION

3.1 Isolation & Confirmation

The results revived detection of five (16.66%) isolates of *K. pneumoniae* out of thirty samples: three (10%) from Abu-Ghraib region and one (3.33%) from each Al-Fudhaliyah and Al-Sadrya regions, in which *ESBL* producers were only detected from Abu-Ghraib cases as totally three (10%) isolates themselves. Colonies were large, mucoid, glistening with thread texture on

McConkey agar. Cells under microscope appears red coccobacilli with clear hue around them as an indicator of capsule formation. Biochemically, isolates were Indole and Methyl red negative and Voges–Proskauer and Citrate positive. All isolates were good producers of biofilm as noticed large black colonies on modified Congo red medium. Table (1) reveal isolation ratio:

Table 1: Isolation of *K. pneumoniae* & *ESBL* producers from mastitic Cow's milk in Baghdad.

Region	Samples	Isolation % (30)	*ESBL* % (30)
Abu-Ghraib	10	3 (10) ^{A*}	3 (10) ^{A*}
Al-Fudhaliyah	10	1 (3.33) ^{B}	None (0) ^{B}
Al-Sadrya	10	1 (3.33) ^{B}	None (0) ^{B}
Total	30	5 (16.66)	3 (10)

*: Indicate highest isolation percentages from Abu-Ghraib, especially *ESBL* producers.
A,B: Indicate significant differences ($æ^2$) vertically at level (P≤0.05).

Mastitis cases were problematic in developing countries due to multiple factors related to triangle of animal, environment and causative agent. Milk producing animals especially Cows in regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya, were subjected to many stressors like poor nutrition and careless monitoring, as well as climatic conditions with dirty environment and subclinical carrier handlers with unclear epidemiological pattern of mastitis predisposing factors as infection comes from environment, vectors, utensils, equipment, milk and cheese cans, active and passive carriers, infected animals or diseased persons. Detection of *ESBL* producers from Abu-Ghraib region may indicate development of resistance problems. Infectious foci may present in these areas and that with other factors may encourage infection with opportunistic pathogens like *K. pneumoniae*. Presence of capsulated and biofilm producing strains might worse the case to acute and per-acute due to poor responsiveness to therapy, as well as sensitivity of mammary tissue to infection. *Escherichia coli* and *E. coli O157* were noticed on McConkey and Sorbitol-McConkey agars as small pink to pale-pink colonies with *Pseudomonas aeruginosa* on Cetirmide-Naldixic acid agar as polymorphic blue-green colonies, and this may complicate the case. Isolation ratio during cold months from small sample size might reflex dangerous situation during processing of milk from these animals.

3.2 Antibiotics Susceptibility & *ESBL* Profile

All isolates showed resistance to imipenem and meropenem, but only those isolated from Abu-Ghraib region were show positivity in Cefpodoxime/Clavulanic acid test as noticed differences in inhibition zone size larger than five millimeter when compared. *ESBL* producers were only detected from Abu-Ghraib cases as totally three (10%) isolates themselves in spite of isolates from Al-Fudhaliyah and Al-Sadrya that showed resistance to Carbapenems alone. This indicates phenotypic and genotypic differences in resistance pattern among these variants as noticed in table (2):

Table 2: Resistance Profile of *K. pneumoniae* from mastitic milk in Baghdad.

Antibiotics	Concentration	Resistance %	*ESBL* %
Imipenem (IMI) Meropenem (MEM)	10 µg	5 (100) ^{A*}	3 (60) ^{B**}

A,B: Indicate significant differences ($æ^2$) horizontally at level (P≤0.05).
*: Indicate Carbapenems resistance pattern.
**: Indicate *ESBL* producers from Abu-Ghraib.

Genetic makeup variation in each isolate as expressed phenotypically in capsule, biofilm formation might partially interpretate ability to resist carbapenems, and cephalosporin but not clavulanic acid as revealed in table (2). This might indicate presence of extra set of resistance enzymes in Abu-Ghraib isolates. Multidrug resistance profile linked to biofilm formation was noticed also on *Pseudomonas aeruginosa* isolated from raw milk and soft cheese from same regions in Iraq as an indicator of symposium of these pathogens noticed in this subject (*K. pneumoniae*, *E. coli O157* & *P. aeruginosa*) in life forming agents (biofilm, capsule, siderophores, haemolysins, multidrug resistance pattern, etc.) in mammary tissue, environment and handlers. This might partially give data about epidemiological nature of mastitis syndrome in regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya. This complicated scenario might the sequel of acute and per-acute cases of mastitis noticed from this study through case history, clinical signs with fever, California mastitis test puddle and isolation scheme.

3.3 Haemolysis Pattern, Siderophore Activity and Serum Power

Diverse factors are encountered in the pathogenicity of *Klebsiella*, such as their serum resistance, siderophore production and acquired haemolysis capacity (45), as *Klebsiella* are commonly described as non-hemolytic microorganisms, so that the detection and determination of the haemolytic profile was critical due

to this might determine the degree of virulent strains especially those isolated from clinical cases from those resident in nature. Serum resistance in *Klebsiella* has been attributed to their surface components, such as capsule, lipopolysaccharide and outer membrane proteins. There appears to be a strong correlation between serum resistance and the ability of *Klebsiella* to invade and survive in the human blood stream. *Klebsiella* need iron, which is an essential element for life and plays an important role in host-bacterial interactions, as iron is a cofactor for several enzymes and acts in transport processes and redox reactions. *Klebsiella* have evolved iron chelators called siderophores, which under low iron stress produce them, but the size of haemolytic zone might reflex how much this isolate need iron and how much they equipped with virulence factors, that need activated or triggering by iron (45) as noticed from table (3) & (4).

Table 3: Hemolysis Pattern and Siderophore activity on modified Human tryptone soya yeast extract blood agar after 48 hrs. at 37 ©C and after 72 hrs. at 4 ©C.

Isolate	Region	Hemolysis Pattern and Siderophore zone (mm)	
		48 hrs. at 37 ©C	72 hrs. at 4 ©C
K1	Abu-Ghraib	α & β (7) ^{Aa}	α & β (10) ^{Ab}
K2		α & β (8) ^{Aa}	α & β (12) ^{Ab}
K3		α & β (9) ^{Aa}	α & β (13) ^{Ab}
F1	Al-Fudhaliyah	α & β (15) ^{Ba}	α & β (21) ^{Bb}
S1	Al-Sadrya	α & β (7) ^{Aa}	α & β (12) ^{Ab}

A,B: Indicate significant differences (æ²) vertically at level (P≤0.05).
a,b: Indicate significant differences (æ²) horizontally at level (P≤0.05).
α = Incomplete hemolysis with green hazy zone.
β = Complete clear hemolysis zone.

Table 4: *K. pneumonia* log count (cfu/ml) after incubation at 37 ©C with Human type O Serum*.

Isolate	Region	*K. pneumonia* log count (cfu/ml) according to Miles-Misra					
		Whole Serum			Complement Inactivated Serum		
		One Hr.	Two Hr.	Three Hr.	One Hr.	Two Hr.	Three Hr.
K1	Abu-Ghraib	6.518	6.146	5.954	6.568	6.230	6.176
K2		6.602	6.264	6.113	6.672	6.544	6.643
K3		6.698	6.672	6.591	6.707	6.602	6.612
F1	Al-Fudhaliyah	7.068	6.889	6.872	7	7	7
S1	Al-Sadrya	7	6.845	6.724	7	7	7

*: Indicate none significant differences (æ²) vertically & horizontally at level (P≤0.05).

All isolates showed modified haemolytic capacity with alpha and beta type's haemolysins as well as, the hot-cold phenomenon of haemolysis like *Staphylococcus aureus* as in table (**3**). These were wonderful and dangerous notification as they reflex phenotypically the genetic makeup of these isolates of animal origin with their ability to lyse human red blood cells for iron acquisition especially those isolated from Al-Fudhaliyah region, and that might indicate partially the origin of these ancestor isolate might be from handlers or environment or zoonotic, or they acquired these criteria due to symposium with other Eco-biota noticed in this subject like *E. coli O157* & *P. aeruginosa*. The presence of dual phase of haemolysis in these clinical isolate indicate intelligent strategy for switching from phase to phase inside mammary tissues as clever mechanism to resist phagocytosis and other defense mechanisms that need more detailed study. Checking zone size of haemolysis especial inside refrigerator as a chelator power might indicate the thermo-modulating or buffering capacity of these developed virulence factors inside isolated biofilm and *ESBL* producing *K. pneumoniae* from mastitis cases. Human type O blood type was choosed in this study depending on Glychophorin theory, as this isotype of blood might not interfere or affect the biochemical reactive ability of *K. pneumoniae* to reveal their haemolysis pattern, siderophore activity and or serum resistance.

All isolates showed resistance profile to human whole and complement inactivated sera as in table (**4**). This might correlate its biofilm electro-magnetic cloud to resist serum true and pseudo globulins especially those isolated from Al-Fudhaliyah and Al-Sadrya regions. The ability of clinical isolates from acute and per-acute cases of Cows mastitis to resist serum bactericidal power from human type O sera might indicate the real origin and tolerance nature of these isolates and their relationship to handlers and environment. As noticed in this subject that incubation of these clinical isolates with non-diluted sera for three consecutive hours might somewhat decrease their load o count, and that might reveal why the disease developed clinically in dangerous manner in Cows mammary tissues and noticed obviously during testing milk samples from these infected animals via puddle or California mastitis tests from all infected quarters to cheek their healthy status, but missing depending on their somatic cell count as an indicator and prediction tool for healthy status of udder or subclinical mastitis guide for me. As

we know, that complements, antibodies and other defense plasma and serum factors might protect or decrease microbial load directly and indirectly with polymorphonuclear leukocytes or phagocytic cells any foreign invaders, but in these situations as we noticed in this search that these defense barriers has little o no effects on biofilm and *ESBL* producing *K. pneumoniae* isolated from mastitis cases, might partially as we think due to their developed genetic makeup in Iraqi environment or due to symposium with other pathogens.

3.4 Extract Disc Sensitivity Profile with MIC & MBC Cut-Off Values

Development of biofilm producing and multidrug resistance microbes over a years with coasty treatments with new generations of antibiotics regimes and their fluctuated selective toxicity, all these and others led us for searching for another cheap and effective microbicidal strategies to evades these clever adapted microbes. Most extracts especially ethanolic types showed inhibitory activities on clinical isolates as in table (**5**) & (**6**) compared and linked to their resistance pattern for selected antibiotics noticed in Antibiotics Susceptibility & *ESBL* Profile paragraph above. Watery extracts showed some inhibitory effects on some isolates might partially due to active ingredients concentrated or filtered through oily extracts. Cinnamon showed most effects followed by Thyme, Oregano an Oak respectively. Ethanolic extracts showed the powerful inhibitory power on *K. pneumoniae*, thus we designing the determination of the lowest titer that inhibit or kill these developed strains. Oak or Baloot oily extract showed the powerful effect followed by Cinnamon, Thyme and Oregano. This might partially ensure our hypothesis compared to imipenem, meropenem, cephalosporin and clavulanic acid used in this study. As we noticed above that all isolates were sensitive for clavulanic acid and to ethanolic extracts of selected plants her, thus we recommend using of these plants during treatment with antibiotics as combination strategy to overcome these sophisticated resistance thresholds.

Minimum inhibitory and maximum bactericidal concentrations for ethanolic extracts were proceeded in order to determination of the threshold interface or cut-off breakpoints values that inhibit or kill these developed strains of *K. pneumoniae*. As we noticed from table (**7**) that two fold or log diluted extracts showed inhibition titers ranged from tubes 4-16, i.e. 2-6 log fluctuations in log count of tested 5 log of standard inoculum of *K. pneumoniae* isolates. Baloot and Cinnamon showed inhibitory to bactericidal effects rather than Thyme and Oregano, and that might partially indicate the synergistic effects of the active ingredients of these extracts on study isolates. The real problem of these clinical isolates was the development of barrier coat biofilm or interconnected slime layers that prevent antibiotics from work on them, but our study indicate the ability of these extract to penetrate the biofilm clouds of developed strain of *K. pneumoniae*, or might indirectly and partially block active and passive transporter pores in cell membrane of these pathogens, or might acts in a manners (directly *vis* indirectly) that needs more studies in this field from specialists.

Table 5: Size of Inhibition Zone (mm) after treatment with Watery Extracts of selected plants.

Isolate	Size of Inhibition Zone / mm after treatment with Watery Extract			
	Cinnamon	**Thyme**	**Oregano**	**Oak**
K1	7 ^{Aa}	6 ^{Aa}	7 ^{Aa}	7 ^{Aa}
K2	5 ^{Aa}	None ^{Bb}	None ^{Bb}	None ^{Bb}
K3	None ^{Ba}	None ^{Ba}	None ^{Ba}	None ^{Ba}
F1	6 ^{Aa}	6 ^{Aa}	7 ^{Aa}	7 ^{Aa}
S1	5 ^{Aa}	8 ^{Ab}	5 ^{Aa}	None ^{Bc}

A,B: Indicate significant differences ($æ^2$) vertically at level (P≤0.05).
a,b,c: Indicate significant differences ($æ^2$) horizontally at level (P≤0.05).

Table 6: Size of Inhibition Zone (mm) after treatment with Ethanolic Extracts of selected plants.

Isolate	Size of Inhibition Zone / mm after treatment with Ethanolic Extract			
	Cinnamon	Thyme	Oregano	Oak
K1	13 ^{Aa}	13 ^{Aa}	12 ^{Aa}	15 ^{Ab}
K2	10 ^{Ba}	10 ^{Ba}	9 ^{Ba}	11 ^{Ba}
K3	7 ^{Ca}	7 ^{Ca}	7 ^{Ba}	8 ^{Ca}
F1	10 ^{Ba}	11 ^{Aa}	11 ^{Aa}	12 ^{Ba}
S1	12 ^{Aa}	10 ^{Bab}	9 ^{Bb}	12 ^{Ba}

A,B,C: Indicate significant differences ($æ^2$) vertically at level (P≤0.05).
a,b,ab: Indicate significant differences ($æ^2$) horizontally at level (P≤0.05).

Table 7: MIC & MBC (titer or log or fold) Cut-Off Values for Ethanolic Extracts of selected plants.

Isolate	Cinnamon		Thyme		Oregano		Oak	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
K1	Tubes 8-16 (3-4) log	5 log (1-3) cfu	Tubes 8-16 (3-4) log	5 log (1-3) cfu	Tubes 8-16 (3-4) log	5 log (1-3) cfu	Tubes 16 4 log	6 log (1-3) cfu
K2	Tube 8 3 log	4 log 3 cfu	Tube 8 3 log	4 log 3 cfu	Tube 8 3 log	4 log 3 cfu	Tube 8 3 log	4 log 3 cfu
K3	Tubes 4-8 (2-3) log	3 log 3 cfu	Tubes 4-8 (2-3) log	3 log 3 cfu	Tubes 4-8 (2-3) log	3 log 3 cfu	Tubes 4-8 (2-3) log	3 log 3 cfu
F1	Tube 8 3 log	4 log 3 cfu	Tube 8 3 log	4 log 3 cfu	Tube 8 3 log	4 log 3 cfu	Tubes 8-16 (3-4) log	5 log (1-3) cfu
S1	Tubes 8-16 (3-4) log	5 log (1-3) cfu	Tube 8 3 log	4 log 3 cfu	Tube 8 3 log	4 log 3 cfu	Tubes 8-16 (3-4) log	5 log (1-3) cfu

4. CONCLUSION

Pluripotent Oak (Baloot), Cinnamon, Oregano and Thyme Extracts exhibit inhibitory effects on strains of *K. pneumoniae* isolated from mastitic milk samples of infected Cows from regions of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya compared to selected antibiotics. These selective toxicity properties reflex their active ingredients that interacts directly and indirectly in different mechanisms with electromagnetic cloud (biofilm complex layers) of these multidrug resistant clinical isolates. Thus, we recommend monitoring these *ESBL* producers in environment of those regions, in which accurate diagnoses of cases especially subclinical mastitis before development to acute and per-acute with early treatments profile must operate and coordinated with combination strategies of hygienic therapy and controlling regimes (test & therapy) in these areas to prevent spreading of risk, as well as monitoring handlers and processors as a target host of *K. pneumoniae*.

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