STUDY OF THE INHIBITORY EFFECT OF THE ETHANOLIC EXTRACT OF *CORIANDRUM SATIVUM, VITIS VINIFERA*, AND *ZINGIBER OFFICINALE* ON THE GROWTH OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM MILK OF COWS INFECTED WITH CLINICAL MASTITIS

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ABSTRACT

The present study had thrown the light on the *in vitro* antimicrobial potential of the ethanolic extract of three local medicinal plants; *Coriandrum sativum* (Coriander), *Vitis vinifera* (Grape seeds), and *Zingiber officinale* (Ginger) against the growth of pathogenic *Staphylococcus aureus* isolated from milk of some local cows infected with clinical mastitis. The antibacterial activity was carried out by using agar well diffusion technique in Mueller-Hinton agar. Four concentrations could be prepared from each plant extract, these concentrations were 50, 100, 200, and 400 mg/ml.

The results were obtained by measured the zone of inhibition around the well that could be exhibited by each plant concentration that followed incubation of bacterial plates and expressed as mean±Standard error (SE). Ethanolic extract of *Coriandrum sativum* was possessed the strongest antibacterial effect among the tested plants, the results were: 29.44±1.17, 29.22±0.32, 27.77±0.99, and 26.11±1.27 mm at a concentration of 50, 100, 200, and 400 mg/ml respectively, Followed by *Vitis vinifera* extract which showed moderate values recorded as 20.88±0.77, 20.11±0.58, 18.22±0.36, and 20.88±0.35 mm at a concentration of 50, 100, 200, and 400 mg/ml respectively. The least antibacterial activity was exhibited by the extract of *Zingiber officinale* that produced the following inhibition zones; 15.11±0.80, 15.77±1.12, 17.66±0.33, and 17.55±0.44 mm at a concentration of 50, 100, 200, and 400 mg/ml respectively. On the other hand, *S.aureus* was variably susceptible to five of the used standard antibiotics; Lomefloxacin, Erythromycin, Amoxicillin, Ciprofloxacin, and Rifampin. Means of their inhibitory zones were; 29.44±0.41,

23.22±0.46, 21.77±0.36, 19.88±0.42, and 11.11±0.26 mm respectively. Whereas Cefprozil showed no effect against the growth of the tested organism.

INTRODUCTION

The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization (1). *Staphylococcus* spp. is the main causative agent of bovine mastitis, with higher prevalence in cases of clinical and subclinical manifestations (2). Despite the general practice to treat bacterial infections including mastitis with antibiotics, Salasia *et al*. (3) suggested that treating mastitis incidences amongst the dairy cows with antibiotics was no longer effective. This was based upon their research findings regarding 32 *S. aureus* isolates that were obtained from mastitic milk samples collected from Kaliurang, Boyolali, Baturaden, and Bantul in Yogyakarta and Central Java provinces. Their findings demonstrated that the *S. aureus* isolates were resistant to several commonly used antibiotics, such as Ampicillin, Erythromycin, Gentamycin, Oxacillin, and Tetracycline (3). According to Gur *et al*. (4) microorganisms had developed resistance against many antibiotics due to the indiscriminate use of antimicrobial drugs. This created problems in the treatment of infectious diseases. Antibiotic resistance had become a global concern (5). Since bacterial resistance to antibiotics was at an increasing rate, interest in discovering new natural antimicrobials was rising.

Alternative treatments to bovine mastitis with bacteriocins (6) and plant derived compounds (7,8). Finding plant products with antimicrobial properties for a possible application in food production as well as in human and animal health care to prevent the bacterial and fungal growth was emphasized (9,10,11,12,13). Moreover, there were some advantages of using antimicrobial compounds of medicinal plants, such as fewer side effects, better patient tolerance, less expensive, acceptance due to long history of use, and being renewable in nature (4). Parekh and Chanda (14) further elaborated that higher plants represented a potential source of novel antibiotic prototypes. Numerous studies had identified compounds within herbal plants that were effective as antibacterial agents. Traditional healing systems around the world that utilized herbal remedies were an important source for the discovery of new antibiotics (15). Besides, some traditional remedies had already produced compounds that were effective against antibiotic-resistant strains of bacteria. Research showed that the antimicrobial activity of such plants was due to specific phytochemicals or essential oils (16,17,18). The aim of this study was to screen and

evaluate the antimicrobial activity of the ethanolic extract of three local plants; *Coriandrum sativum* (Coriander), *Vitis vinifera* (Grape seeds), and *Zingiber officinale* (Ginger) against the growth of *S. aureus* isolated from milk samples of cows infected with clinical mastitis. An *in vitro* agar well diffusion methodology was employed for this study.

MATERIALS AND METHODS

Plant materials: Fruits of *Coriandrum sativum* (19), seeds of *Vitis vinifera* (20), and rhizomes of *Zingiber officinale* (21) had been dried and well grinded to be used in this study. All these plant materials were purchased from the local market, at Al-Qadisiya province.

Preparation of ethanolic extracts: Ethanolic extracts were accomplished according to the method of Le Grand (22). Briefly 50 gm of each powdered plant sample was mixed with 250 ml of 96% ethanol. The mixture was kept for 2-5 days in tightly sealed containers at room temperature and shaked several times daily. This mixture was filtered through filter paper to remove the coarse plant materials. Further extraction of the residue was repeated 3-5 times until a clear supernatant extraction liquid was obtained. The filtrates of each tested plant were evaporated to dryness using a rotary evaporator at 40ºC. The final dried samples were weighed and stored at -20ºC until use.

Antibiotics: Six standard antibiotics had been chosen according to their broad-spectrum activity used as positive control against the test microorganism (*Staphylococcus aureus)*, they include: LOM 10 (Lomefloxacin-10 mcg), E 15 (Erythromycin-15 mcg), Ax 25 (Amoxicillin-25 mcg), CIP 5 (Ciprofloxacin-5 mcg), RA 5 (Rifampin-5 mcg), and CPR 30 (Cefprozil 30 mcg) (Bioanalyse)®.

***Staphylococcus aureus* isolates:**

A-Sample collection:

Milk samples were collected in sterile tubes (2 tubes) for each sample one for California mastitis test (CMT) and another for bacteriological tests and a septic technique used for milk samples collection according to (23).

B-Bacterial culture and identification:

All milk samples from subclinical mastitis cases which gave a positive reaction with (CMT) were submitted to centrifugation at 3000 rpm / 15 minute, and the precipitate was cultured on: Blood Agar, Nutrient Agar and MacConky Agar, and were incubated at 37 ºC for 24 - 48hrs. Diagnosis depend on morphological character & cultural character (24) , then followed by examination with Gram's stain, after that, the colonies were subcultured on selective media and

differential media according to the type of isolated bacteria, then incubated at 37 ºC for 24 - 48 hrs. The biochemical tests used for diagnosis of *staphylococcus aureus* were include:

- Catalase test , Oxidase test , Coagulase test , Urease test , Heamolysis on blood agar, Gelatin Liquefaction test (Gelatinase), Voges-Proskauer test, Nitrate reduction test , Sugar Fermentation test (Mannitol, Lactose, Mannose, Xylose, Trehalose , Sucrose , Maltose) according to the method of (23, 24, 25).

Production of pigment in Mannitol salt agar and in (Staph 110 media) (LAB-U.K)

MAST STAPH™: (Mast Group Ltd, USA)

API Staph (biomerioux, France).

Sensitivity test:

Inhibition of bacterial growth was tested by using the agar well diffusion method (26). A serial dilution of each extract was prepared for studying of their antibacterial activity at different concentrations. It was done by diluting 2 gm of each dry extract with 5 ml of 96% ethanol to obtain stock solution at a concentration of 400 mg/ml. From this stock solution various concentrations were made including: 200 mg/ml (consist of 2 ml of 96% ethanol and 2 ml of the stock solution at 400 mg/ml concentration), 100 mg/ml (it was made by adding 1 ml of 96% ethanol to 1 ml of the extract solution at a concentration of 200 mg/ml), and 50 mg/ml (prepared by drawing 1 ml of the extract solution at a concentration of 100 mg/ml and adding to 1 ml of 96% ethanol) (27). On the other hand, *S. aureus* isolate was subcultured in nutrient broth (HIMEDIA Laboratories, Mumbai-India) that was prepared according to the instructions given by the manufacturing company. After that, several colonies of *S. aureus* were suspended by using sterile cotton swab in sterile tube containing 10 ml of nutrient broth mixed, and incubated at 37ºC for 24 hours to produce bacterial suspension revealed by the presence of turbidity. The turbidity of the culture was compared with 0.5 McFarland Nephelometer standard to get 150 x 106 CFU/ml (28). The standardized inoculum suspension was inoculated within 15-20 minutes. Mueller-Hinton Agar (HIMEDIA Laboratories, Mumbai-India) which is a growth media used for testing antibiotics and the chosen plant extracts susceptibility of the tested microorganism was prepared also according to the manufacturer guide. This media was poured aseptically at 45 ºC into sterilized Petri plates by using sterile pipette (20 ml capacity) on the flat horizontal surface to a depth of 20 mm. After complete solidification, a standard cork borer of 5 mm diameter was used to cut 5 uniform wells on the surface of each agar plate aseptically (with exception of those plates used for antibiotic study). A sterile cotton swab was dipped into the bacterial suspension produced by *S. aureus* to be inoculated on the Mueller-Hinton agar surface by streaking of the swab over its. Finally and after the inoculums were dried,

0.1 ml of each concentration of each plant extract was dropped into the wells of its inoculated plates ie., each plate contained 4 different concentrations of each plant extract (50, 100, 200, and 400 mg/ml) besides 0.1 ml of 96% ethanol which considered as a negative control was dropped in one well on the same extract plate. As well as one disc of each antibiotic control was placed with a sterile forceps over the surface of its own plate (so that 3 different discs of antibiotics were applied over each plate). All plates were incubated at 37 °C for 24 hours. Zone of inhibition around each well measured in mm with the ruler (29). The values were given as mean ± SE and the data were analyzed by Anova test with least significant differences (LSD) at significant level of $P<0.05$ by using SPSS (Version 10).

RESULTS AND DISCUSSION

Research results about the antibacterial effect of 4 concentrations of ethanolic extract of 3 local plants and 6 standard antibiotics in inhibiting the growth of *S. aureus* isolated from milk specimens were shown by the size of each bacterial growth inhibition zone as summarized in tables (1, 2) and figures (1,2,3,4) which was varied according to the type of plant and the used concentration. Among the tested plant extracts, the most active one was that obtained from Coriander which gave highest zone of inhibition; 29.44±1.17, 29.22±0.32, 27.77±0.99, and 26.11±1.27 mm at the concentrations 50, 100, 200, and 400 mg/ml respectively (Figure 2). These values indicated that the sensitivity of this strain of *S. aureus* toward the extract of Coriander could be increased gradually by decreasing the concentration. Coriander efficacy was followed by those of Grape seeds extract which showed moderate antibacterial activity revealed from measuring of the zone of inhibition and recorded as 20.88±0.77, 20.11±0.58, 18.22±0.36, and 20.88±0.35 mm at the concentrations 50, 100, 200, and 400 mg/ml respectively (Figure 3), also the values were decreased by increased concentration, but return to increase at 400 mg/ml concentration. Finally, the average of growth inhibition zone of *S. aureus* exhibited by ginger extract was the lowest and showed as 15.11±0.80, 15.77±1.12, 17.66±0.33, and 17.55±0.44 mm at a concentration of 50, 100, 200, and 400 mg/ml respectively (Figure 4). It is interesting that these differences in the antibacterial effects of plant extracts are due to the phytochemical differences between species and collection site (16,17). *S.aureus* was variably susceptible to five of the used standard antibiotics; Lomefloxacin, Erythromycin, Amoxicillin, Ciprofloxacin, and Rifampin. Means of their inhibitory zones were; 29.44±0.41, 23.22±0.46, 21.77±0.36, 19.88±0.42, and 11.11±0.26 mm respectively. Whereas Cefprozil showed no effect against the growth of the tested organism (Table 2).

Table (1): Inhibition zones (mm) of *Staphylococcus aureus* growth produced by plant extracts in culture media.

Plant extracts	Zone of growth inhibition (mm)/Concentration (mg/ml)			
	50	100	200	400
Coriandrum sativum	29.44±1.17 aA	29.22±0.32 aA	27.77±0.99 aA	26.11±1.27 aA
Vitis vinifera	20.88±0.77 aB	20.11±0.58 aB	18.22±0.36 aB	20.88±0.35 bB
Zingiber officinale	15.11±0.80 aC	15.77±1.12 aC	17.66±0.33 aB	17.55±0.44 aC

* Different small letters mean significant changes for horizontal values at level ($p<0.05$)

* Different capital letters mean significant changes for vertical values at level ($p<0.05$).

* Results were expressed as mean ± SE.

Table (2): Inhibition zones (mm) of *Staphylococcus aureus* growth produced by antibiotic drugs in culture media when used as positive control.

Antibiotics	Zone of growth inhibition (mm)
LOM	29.44±0.41 A
E	23.22±0.46 B
AX	21.77±0.36 C
CIP	19.88±0.42 D
RA	11.11±0.26 E
CPR	0±0 F

* Different capital letters mean significant changes for vertical values at level ($p<0.05$).

* Results were expressed as mean ± SE.

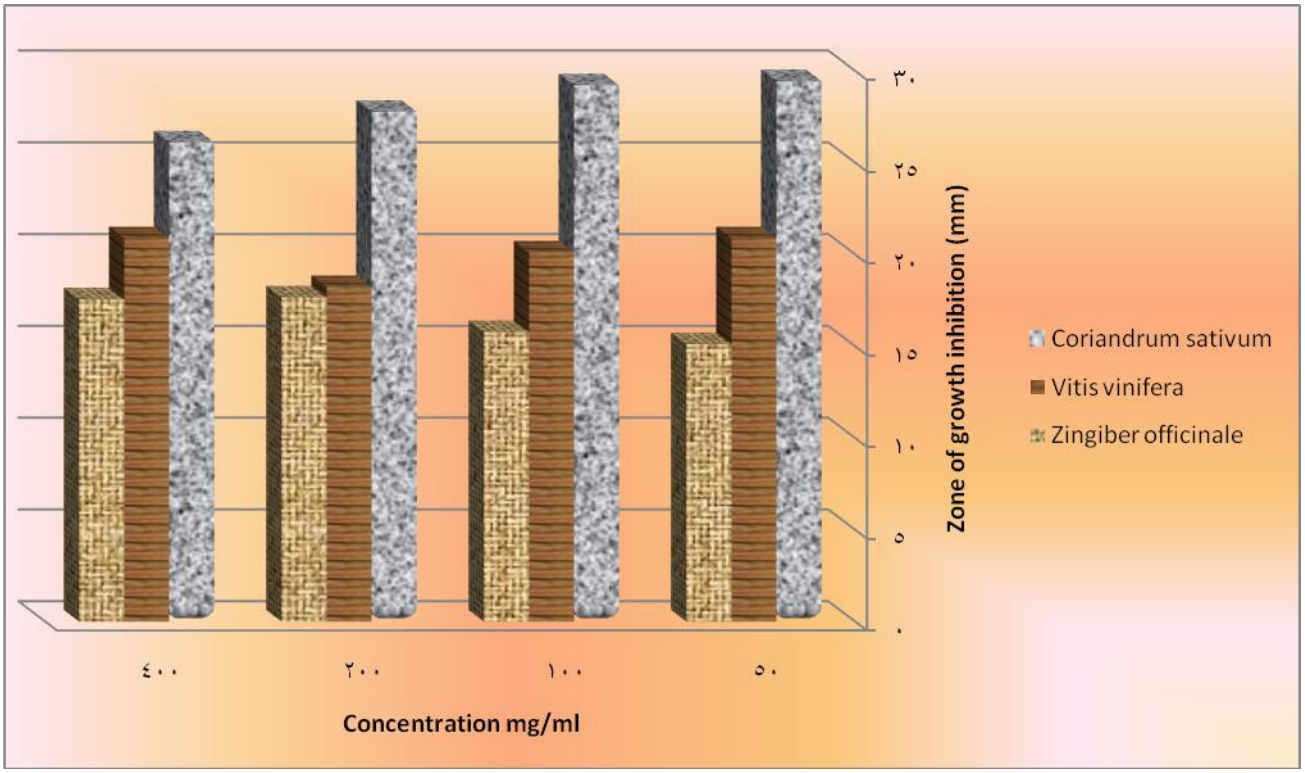

Figure (1): Inhibition zones of *Staphylococcus aureus* exhibited by plants ethanolic extracts.

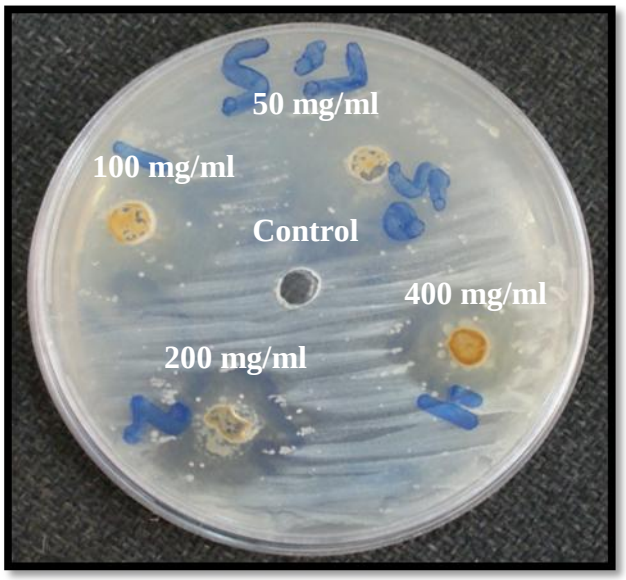

Figure (2): Inhibition zones of *Staphylococcus aureus* growth on Mueller-Hinton agar produced by ethanolic extract of *Coriandrum sativum*, the peripheral four wells contained extract concentrations (50, 100, 200, and 400 mg/ml), where as the central well contained 0.1 ml of 96% ethanol.

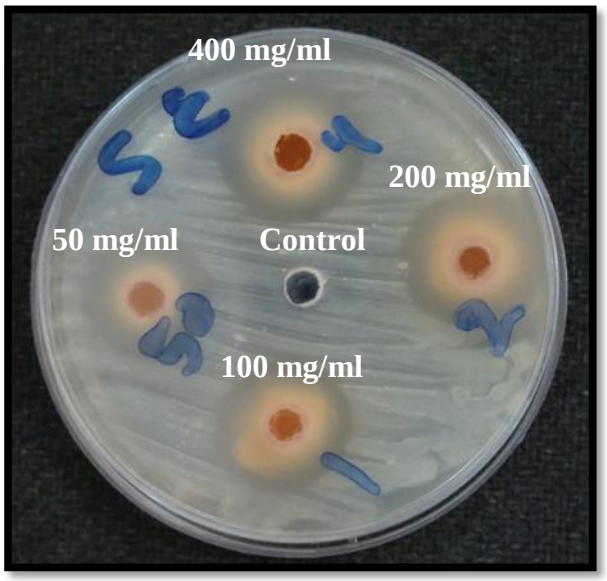

Figure (3): Inhibition zones of *Staphylococcus aureus* growth on Mueller-Hinton agar produced by ethanolic extract of *Vitis vinifera*, the peripheral four wells contained extract concentrations (50, 100, 200, and 400 mg/ml), where as the central well contained 0.1 ml of 96% ethanol.

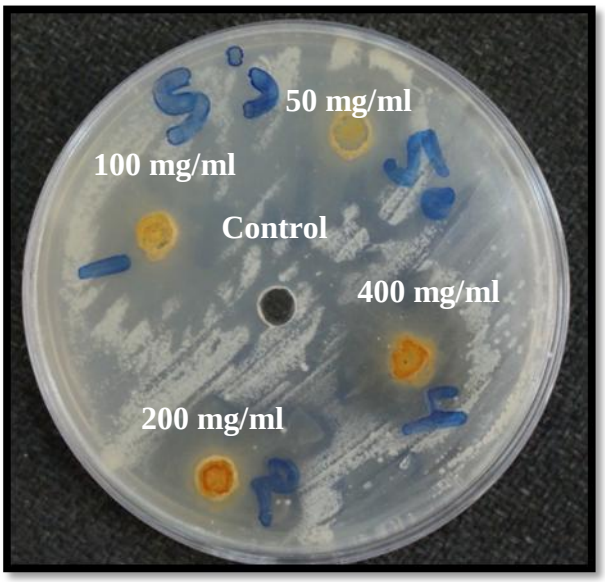

Figure (4): Inhibition zones of *Staphylococcus aureus* growth on Mueller-Hinton agar produced by ethanolic extract of *Zingiber officinale*, the peripheral four wells contained extract concentrations (50, 100, 200, and 400 mg/ml), where as the central well contained 0.1 ml of 96% ethanol.

Many researchers investigated the effect of Plant extracts and mode of action of their chemical constituants that can be explained as follows.

1- *Coriandrum sativum* is considered both as an herb and a spice. Coriander seeds have health-supporting reputation that is high on the list of healing spices. It has traditionally been referred to as antidiabetic (30). It is also used as carminative, diuretic, stimulant, stomachic, refrigerent, aphrodisiac, analgestic (31), antihelmintic (32) and hypoglycemic (33). The seeds of *Coriandrum sativum* contain 0.5-1 % essential oil and are rich in beneficial phytonutrients including carvone, geraniol, limonene, borneol, camphor, elemol and linalool. Coriander's flavonoides include quercitin, kaempferol, rhamnetin and epigenin. It also contains active phenolic acid compounds including caffeic and chlorogenic acid (34, 35).

According to a study done by Keskin and Toroglu (36), the methanol extract and the ethyl acetate extract of coriander seeds showed no inhibition zone to *S. aureus*. Saeed and Tariq (32) reported that the aqueous infusion and decoction of coriander did not show any antimicrobial activity against *S. aureus*.

In contrast, some workers have found that the essential oil of Coriander fruits have high inhibitory efficacy against *S. aureus* (19, 37). Similarly, some workers have found that *Coriandrum sativum* has strong antibacterial activity against both Gram-negative and Gram-positive bacteria (38).

The reason of the different results was plant collection site and bacterial strains that used in this study.

2- Grape seeds extract was also effective as antibacterial agent. It contains vitamin E, flavonoids, linoleic acid, polyphenol, and oligomeric proanthocyanidin complexes are highly concentrated in grape seeds (39).

Only a few studies have been conducted to determine the antimicrobial activity of the ethanolic extract of grape seeds against *S. aureus*.

Findings of the present study are in fair correlation with the studies carried out by Jayaprakasha *et al*. (40), Baydar *et al*. (41), and Peng *et al*. (42), they reported that grape seed extracts and root extract inhibited G +ve bacteria, especially *S. aureus*.

Al-Habib *et al*. (20) found that the Antibacterial activity against *S. aureus* was bactericidal as shown by a disruption of the bacterial cell wall in scanning and transmission electron microscopy. Grape seed extract is known to be rich in potent antioxidant polyphenolics that could show antibacterial activity.

3- Ginger is a promising plant material with numerous biological activities. Various solvents were used for extraction of bioactive compound from ginger and the extract yields were measured (43).

Evidence found through research show that the ginger active ingredients that contributed to its antimicrobial properties were likely resided in its volatile oils, which comprised of approximately 1 to 3% of its weight. Oonmetta-aree et al. (44) listed essential oils (bisabolene, phellandrene, citral, borneol, citonellol, etc.), oleoresin (gingerol, shogaol), phenol, vitamins and minerals as the ginger ingredients.

Then, Omoya and Akharaiyi (21) described that the ethanolic extract of ginger was positive for saponin, alkaloides, flavonoids and cardiac glycosides.

Poeloengan (45) investigated the antibacterial activity of the methanolic extract of ginger rhizomes against the growth of *S. aureus* isolated from milk of mastitic cows. Whereas Omoya and Akharaiyi (21) can firmed our findings, they demonstrated that the activity of the ethanolic extract of ginger at the concentration of 100 mg/ml against the tested organism.

Betoni *et al*. (46) and Sebiomo *et al*. (43) found the synergistic activity between the ginger ethanolic extract and antimicrobial drugs on *S. aureus* isolate.

Since large number of different chemical compounds presented in the ginger crude extract, therefore, its mechanism of action could affect multiple target sites against the bacterial cells. In this case, Oonmetta-aree *et al*. (44) mentioned that terpenes and other phenolic compounds found in this crude extract could be involved in disruption of the cytoplasmic membrane and coagulation of the cell contents. On the other hand, Volk and Wheeler (47) explained that the phenolic compound and the proteolytic enzyme of the ginger extract – Zingibain – precipitated the outer protein membranes, ruptured the cell wall, coagulated and caused loss of the cell contents and energy through cell wall leakages of *S. aureus*.

In conclusions, The results of this study shed light into the antimicrobial abilities of tested substances, potentially providing ground for natural alternatives to pharmaceutical antibiotics medication. This study has consistently demonstrated the effectiveness of coriander, grape seeds, and ginger as an *in vitro* antibacterial agents against *S. aureus*, besides 5 of the antibiotics tested (Lomefloxacin, Erythromycin, Amoxicillin, Ciprofloxacin, Rifampin) showed also their ability to inhibit the growth of *S. aureus* while Cefprozil showed no effect against the growth of the tested organism.

دراسة التأثير المثبط للمستخلص الايثانولي لنباتات الكزبرة وبذور العنب والزنجبيل على نمو المكورات العنقودية الذهبية المعزولة من حليب الأبقار المصابة بالتهاب الضرع ألسريري

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الخلاصة

إن الدراسة الحالية التي أجريت في المختبر قد سلطت الضوء على الكفاءة الضد – بكتيرية للمستخلصات الايثانولية المحضرة من ثلاث نباتات محلية شملت الكزبرة وبذور العنب والزنجبيل ضد نمو جرثومة المكورات العنقودية الذهبية المرضية والمعزولة من عينات الحليب لعدد من الأبقار المحلية المصابة بالتهاب الضرع ألسريري. ضوقد أنجزت الفعالية الضد – بكتيرية بواسطة استخدام تقنية الانتشار في حفر أكار المولر- هنتون. لقد أمكن تحضير أربع تراكيز لكل مستخلص ايثانولي: 50، 100، 200، 400 ملغم/مل. تم الحصول على النتائج من خلال قياس نطاق التثبيط الذي أظهره كل تركيز حول الحفر وذلك بعد حضن الأطباق البكتيرية، هذه النتائج تم التعبير عنها بهيئة المتوسط الحسابي ±الخطأ القياسي. إن المستخلص الايثانولي للكزبرة امتلك التأثير الأقوى كمضاد للبكتريا من بين النباتات المختبرة حيث كانت النتائج 1,17 ± 29,44 و 0,32± 29,22 و 0,99±27,77 و 1,27± 26,11 ملم عند تركيز 50، 100، 200، 400 ملغم/مل على التوالي. يليه مستخلص بذور العنب الذي أظهر قيم معتدلة سجلت كالأتي: 0,77± 20,88 و 0,58 ± 20,11 و 0,36 ± 18,22 و 0,30 ± 20,88 ملم عند تركيز 50، 100، 200، 400 ملغم/مل على التوالي. في حين كان الزنجبيل الأقل تأثيرا على نمو البكتريا من خلال ملاحظة النتائج التي سجلها وهي: 0,80± 15,11 و 1,12± و 0,3315,77± 17,66 و 0,44± 17,55 ملم عند تركيز 50، 100، 200، 400 ملغم/مل على التوالي. من جانب آخر فان المكورات العنقودية الذهبية أظهرت حساسية متفاوتة ضد خمسة من المضادات الحياتية القياسية المستخدمة في الدراسة والتي شملت: لوموفلوكساسين، أريثرومايسين، أموكسيسيلين، سايبروفلوكساسين، ريفامبين مع معدل نطاق تثبيط: 0,41 ± 29,44 و 0,46± 23,22 و 0,36± 21,77 و 0,42± 19,88 و 0,26± 11,11 ملم على التوالي. بينما لم يظهر السيفبروزول أي تأثير ضد البكتريا المختبرة.

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