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Potential Inhibition of Methanolic Khella Seed Extract against Milk Borne Pathogenic Bacteria *in Vitro*

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ABSTRACT

Dairy products and milk are spoiled and refers to contain microbe or poison substances that make them unfit for consumption. The aim of the work extracted and identified an anti phyto chemical of *Ammi visnaga* as a novel antimicrobial activity against milk borne bacteria. **Materials:** The pathogenic bacteria were isolated from row milk and cottage cheeses on selective media. The isolated bacteria were identified by morphological, cultural characteristics, antibiotics sensitivity and biochemical tests according to (Bergey's manual, 2009). Methanolic Khella extract was derived from seeds (MKSE) and tested for its antimicrobial activity against isolated bacteria. The antimicrobial activity was determined by well diffusion assay and broth microdilution method using ELISA reader. **Results** : The analysis of Methanolic extract by GC-MS showed eight active gradient compounds, belonging to the fused hetero-cyclic , which contended pyrimidine or purine , The isolated bacteria were identified *Bacillus cerise, Escherichia coli, Listeria sp., Pseudomonas aeruginosa, Salmonella sp.* and *Staphylococcus aureus*. The isolated bacteria showed different antibiotic susceptibilities with range to resistant, moderate and sensitivity. The methanolic extract (MKSE) showed high antimicrobial activity against *B. cerise, E. coli, Listeria sp., P. aeruginosa, Salmonella sp. and Staph. aureus*. Antivirulence activity of MKSE was reduced Biofilm formation of *E. coli, Ps. aeruginosa*, and *Staph. aureus*. The dialyzed MKSE had ability as microbialicidal agent against milk borne pathogens, *Ps. aeruginosa* and *Staph. aureus* due to 0-12 h time kill. SEM images observations confirmed the physical damage and considerable morphological alteration, empty, flaccid, stuck together and melted, giant cells, appendages on cell surface, Pseudomycellium like structures of *Ps. aeruginosa* and *Staph. aureus* treated with MKSE. **Conclusions:** Methanolic Khella seed extract to be one of the novel agents in anti- microbes and could be potential as bactericidal to be used food drug therapeutically.

Keywords: *Ammi visnaga*, methanolic Khella extract, Food borne pathogens, Antivirulence activity.

1. Introduction

Milk and dairy products borne microbes tainted, whose composition depends microbes, survive and interact in the milk and dairy products over time. The originated microbes present from the natural micro flora of the raw milk and those microbes introduced in the course of harvesting milking, slaughter, processing, storage and distribution (Barnhart *et al.,* 2002). As well as, milk considered as rich medium for the growth of different microbes, among these, the most important group are bacteria (Beck, 2000). Okpalugo *et al.* (2008) and Rosengren (2012) examined the microbiological quality of some milk products. Thirty three and twelve fungal isolates belonging to 12 genera were identified from the yoghurt samples. Presence of yeast was found to increase the microbial load of bacterial groups, 19 bacterial isolates belonging to 6 genera were identified from the pasteurized milk samples.

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Singh and Prakash (2008) and Ordiales *et al.* (2013) were isolated *E. coli, Staph.aureus* and *L. monocytogenes* from milk products i.e. curd and cottage cheese. Gram negative bacteria known to be more resistant to antibiotics than Gram positive bacteria due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism (Ceylan, and Fung, 2004).

A. visnaga is considered to have antimicrobial activities which were associated with khellin and visnagin. Both these constituents were considered to have antifungal, antibacterial, and antiviral activities. It has photosensitizing ability and considered useful as a photosensitizer in patients with psoriasis (Abdelfattah *et al.,* 1983). The most active extract against Gram-positive bacteria was ethanol extract with minimal inhibitory concentration value (5 mg/ml) against *Enterococcus faecalis., E. coli, Staph aureus and Ps aeruginosa S. mutans, S. salivarius and S. sangguis* (Khalfallah *et al.,* 2011) and (Semyaril *et al.,* 2011). Twenty one compounds seeds of A. visnaga by GC/MS analysis and characterized representing essential oil 2-dimethylbutaoic acid), isobutyrate, croweacin, linalool, bornyl acetate, and thymol by GC/MS analysis (Satrani *et al.,* 2004 and Khalfallah *et al.,* 2011).
This antimicrobial activity attributed to similarity in the mechanism of action plant extract and antibiotics refer to the presence of alkaloids, flavonoids and poly phenol fractions (Okorondu *et al.,* 2010 and Bhalodia *et al.,* 2011). The Khella Seeds extract showed greater antimicrobial activity due to the presence of active compounds including alkaloid, quinines, tannins, flavonoides, saponins and iridoids (Nilforoushzadeha *et al.,* 2008). The main target for these compounds might be some enzymes, cell wall or membrane of bacteria.It has been shown that, alkaloids are able to intercalate DNA, lipophilic compounds that might bind within or internal to the cytoplasmic membrane (Moreno *et al.,* 2006 and Raybaudi-Massilia *et al.,* 2009). As well as they cause disruption of the cellular membrane, inhibition of ATPase activity, and release of intracellular ATP of *E. coli, E. coli* O157:H7, *L. monocytogenes, Lactob. sakei, Ps. aeruginosa, S. enteritidis, and Staph. aureus* (Moreno *et al.,* 2006 and Raybaudi-Massilia *et al.,* 2009). Minimum inhibitory concentrations (MICs) is defined as the lowest concentration of a drug/compound that will inhibit the visible growth of an organism in vitro after overnight incubation., considered the "gold standard" for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing (European Comm. Antimicrobial, 2000 and Barros *et al.,* 2007). In recent years, (MICs) have been used in Phytotherapy, the use of plants for medical purposes, which is one of the oldest practices in the world (Watase-Renee and Reppun-Thomas, 2011).

The aim of the work is extracted and identifed an anti phyto chemical of *Ammi visnaga,* present & novel antimicrobial activity against milk borne bacteria.

2. Materials and Methods

2.1. Collection and preparation of dairy samples:

Total of 100 crud Bovine and cow milk of animals at age 2 to 3 years and cottage cheeses were randomly collected from the local areas of the province Qalubia governorate in Egypt during 2019 and 2020.They were transported to the laboratory under refrigeration (4°C). Aseptically (25g) of each sample was mixed with 225(ml) of sterile buffer peptone water and incubated at 35°C for 24h. One to ten (ml) mixture was transferred to selenite cysteine broth and incubated at 35°C for 72h (FDA, 2002).

2.2. Aerobic agar plate count.

The total mesophilic bacterial count was carried out using plate count nutrient agar medium (Oxiod; CM325) according to (FDA, 2002). Ten-fold dilutions of homogenate samples were prepared. The medium was mixed with one ml homogenate /9 ml medium and poured in plates then left to solidify. The inoculated plates were incubated at 37°C for 48 h. The number of colony forming units were counted and calculated per gm. or ml of sample as follows:
Total mesophilic count / g = No. of colonies x dilution factor.

2.3. Isolation of *Salmonella*.

Salmonella and Shigella (SS) agar (Oxoid; CM0099) and xylose lysinedeoxycholate agar (XLD), (Oxoid; CM0469) plates were inoculated by diluted homogenate samples then incubated at 37°C for

24h. Suspected colonies were creamy with or without black centers on SS agar and red in color with without black centers on (XLD) agar.

2.4. Isolation of *Ps. aeruginosa*.

M-PA-C agar medium poured in plates. Spread plating 0.1(ml) diluted homogenate sample onto the medium and incubated at 37^{o}C /72h. Typically, *Ps. aeruginosa* colonies were confirmed by streaking on cetrimide agar plates (Oxoid; CM559), a selective medium which inhibits bacterial growth except *Ps. aeruginosa* which enhances fluorescein and pyocyanin"blue green" pigment production (AOAC, 2000).

2.5. Isolation of *B. cereus.*

Bacillus cereus agar medium (Oxoid; CM0617), supplemented with polymyxin B and egg yolk poured in plates. Spread plating 0.1(ml) diluted homogenate sample onto the medium and incubated at 37^{o}C /72h. The suspected colonies peacock blue-colored and surrounded by precipitation zone were counted and tested (AOAC, 2000).

2.6. Isolation and enumeration of *E. coli*.

Spread plating 0.1(ml) diluted homogenate sample was streaked on plates of Eosin Methylene blue (EMB) (Oxoid; CM0069) and incubated at 37°C for 24h. Typical colonies (greenish metallic with dark purple center) were picked up and transferred to nutrient agar slants and incubated at 37°C for 24h for further identification (FDA, 2002).

2.7. Isolation of *Listeria monocytogenes*.

Twenty-five grams of the sample were added to 225 (ml) Listeria Primary Enrichment medium (UVM1) in stomacher bag and homogenated for 2min. The stomacher bag were incubated at 30°C for 24h. 0.1(ml) from the inoculated UVM1 was transferred to 14(ml) of Listeria Secondary Enrichment medium (UVM2) and incubated at 30°C. After 24h, incubation one loop was streaked onto PALCAM agar plates (Oxoid; CM0877). The plates were incubated at 37°C for 48h suspected colonies (2mm in diameter, grey-green in color with a black sunken center and a black halo.) were picked up and plated onto trypticase soy agar with 0.6% yeast extract and incubated at 37°C for 24h then put in refrigerator for further identification (AOAC, 2000).

2.8. Isolation of *Staphlylococcus aureus* was carried out by spreading 0.1(ml) of each diluted homogenate sample was streaked on Baird parker media (Oxoid; CM0275) supplemented with egg yolk and potassium tellurite solution. Plates were incubated at 37°C for 48h.The presence of typical colonies, which appears gray-black, shiny and convex with a narrow white margin surrounded by a clearing zone were isolated by culturing on brain heart infusion agar(Oxoid;CM225) slants medium for further identification(FDA, 2002).

2.9. Identification of bacterial isolates.

Bacterial colonies obtained from all previously mentioned media were chosen and picked up according to variation in culture characteristics and colony formation then purified by streak-plate method on Nutrient agar medium. Almost all microscopically examinations and biochemical testing used for identification were carried out according to Bergey's manual, (2009), Collins and Lyne (2004) and Cheesbrough (2006).

2.10. Detection of free coagulase.

The tested isolated bacteria brain heart broth culture at 24hr, 100 uL was transferred in tube and added 0.5 ml of diluted rabbit plasma in citrated buffer. The tubes were incubated at 370c and examined for clotting area for 24hr. The result was recorded as follows: score of (+2) =small formed clot; (+3) = large formed clot and (+4) = entire content of the tube coagulates and is not displayed when the tube is inverted. The results were considered positive evidence of coagulate production.

2.11. Preparation of Methanolic khella Seeds extract (MSKE).

Ammi visnaga (bisnaga, toothpick weed or khella) belongs to the family Apiaceae and it is a herbaceous medicinal plant was obtained from National Research Center (NRC). Fifty grams of the khella seeds powder was extracted with half liter methanol with HCl 3% until pH=1.5. The methanolic extract was concentrated under reduced pressure until dryness at a temperature not exceeding 60°C. The aqueous extracts were sterilized by (0.22µm) Millipore filter. The residues were stored at 4°C for further use, (Woo *et al.,* 1977).

2.12. Gas chromatography–mass spectrometry (GC-MS) analysis:

The chemical composition of Methanolic extraction were performed using Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral database (The Regional Center of Mycology and Biotechnology, Cairo, Egypt).

2.13. Bioactivity of methanol extract

2.13.1. Well diffusion assay.

The nutrient agar plates were inoculated with tested bacteria and incubated for overnight at 37°C. The agar wells were filled with 100uL methanol extract (50 mg/ml concentration). The Petri dishes were kept at 1h in the refrigerator to allow for the diffusion of the active compounds. Negative controls were done using saline buffer. The plates were incubated at 37°C for 24h. The susceptibility of the bacteria to methanol extract was estimated by the diameter of the inhibition zones and recorded values as the average of three replicates (NARMS, 2002).

2.13.2 Tissue culture plate method.

The isolated bacterial were tested for their ability to form biofilm was done by Christensen *et al.,* (1995) using Tissue culture plate method. The each of isolated Bacteria were inoculated in 10 (ml) of Mueller Hinton Broth (MHB) and incubated at 37ºC for 24h. The cultures were diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture (Sigma-Aldrich, Costar, USA) were filled with (200µL) of the diluted cultures with 0.5 McFarland standard. Negative control wells inoculated with 200µL sterile broth medium. The plates were incubated at 37ºC for 24h. After incubation, the free floating bacteria of each well were removed by gentle tapping. The wells were washed with 0.2(ml) of phosphate buffer saline (pH=7.2) four times. Biofilm formed by bacterial adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (O.D.) of stained adherent biofilm was obtained by using ELISA microtiter-plate reader (Sun Rise–TECAN, Inc. ®, USA) at wavelength (570 nm). The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done according to the criteria of Stepanovic *et al.* (2007).

2.13.3. Determination of Minimum Inhibitory Concentration (MIC) of methanol khella Seeds extract (MKSE) was determined by the broth microdilution method as approved by (NCCL/CLSI, 2007). The MKSE were serially diluted to (50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, 0.195 and 0.097mg/ml) concentrations. Mueller Hinton Broth (MHB) for isolated bacteria, (100µL) and MKSE (100µL) were added in microtitre plates. Twenty microliters (20µL) of bacterial suspension (0.5 McFarland standards), were added to each of the wells except the control wells (control wells contained broth only and distilled water only). The plates were incubated at 37 °C for 24h. The absorbance bacteria of the plates was measured before and after incubation 24h ELISA microplate reader (Sun Rise–TECAN, Inc. ®, USA) at (600nm). The lowest concentration of the tested plant extract resulting in inhibition of bacterial was recorded as the MIC**.**

2.13.4. Antibiofilm activity of methanol extract:

The methanol extract was tested for has potential to inhibition biofilm formation of bacterial isolates using tissue culture plate method. The concentrations of methanol extract were prepared in the 96-well microtiter plate containing Mueller Hinton Broth (MHB) for bacteria to obtain (5, 2.5, 1.25,

0.65 0.35 and 0.15 mg/ml) in 100 µL. Bacterial suspensions 50 µL (5×10^5cfu/ml, final concentration) were transferred into the plate. Mueller Hinton Broth (MHB), containing saline buffer was employed as a (negative control). Inoculated Mueller Hinton Broth (MHB) for bacteria was used as the (positive control). Following incubation at 37°Cfor 24h the effect of the extracts on the bacterial growth was evaluated using the ELISA microplate reader at optical density of (570nm) according to Lin *et al.* (2011).

2.13.5. Time kill assays.

The methanol extract at 6.25 mg/ml (MICs) against isolated pathogenic bacterial were mixed with bacteria harvested at late log. phase and diluted to approximately 4.0 (log cfu /ml). A total volume of 25 (ml) in 50 mL flask included of 10 (ml) broth medium, 10 (ml) of filtered methanol extract at 2.5(mg/ml MICs) and inoculum of isolated bacteria diluted to approximately 4.0 - 5.0 (log cfu /ml) at late log. phase. The flasks were incubated at 35-37ºC, at regular intervals (0, 3, 6, 12 and 24h). One mL of each bacterial suspension was evaluated at of each previous times using the spectrophotometric assay at optical density of (OD 620 nm). All experiments were duplicated and average values were reported (Souza *et al.,* 2006).

2.13.6. Scanning Electron Microscope (SEM).

Ps. aeruginosa and *Staph. aureus* were mixed with 4.0-5.0 (log cfu /ml) at late log. phase respectively with methanol extract at (MICs) concentrations at 6.25 (mg/ml). The treated and Non treated bacteria cultures were incubated for 24 h for 37^0C. The samples were fixated by glutheraldhyde 2.5% and dehydrated by serial dilution of ethanol using automatic tissue processor (Leica EM TP).The The treated and Non treated bacteria were dried using CO2 critical point drier (Tousimis Audosamdri-815) and coated by gold sputter coater (SPI-Module).The samples were exanimated by scanning electron microscopy (JEOL-JSM-5500LV) by using high vaccum mode at the Regional Center of Mycology and Biotechnology, Cairo, Egypt.

3. Results

One hundred and fifty included, 50 bovine, 50 cow milk and 50 cottage cheese samples were investigated for their load of pathogenic microbes.

3.1. Total aerobic bacterial count:

The inoculated nutrient agar plates with 150 bovine, cow milk and cottage cheese showed different morphological colonies (Table,1) showed that, The contaminated positive samples were 12 (8, -vG & 4, +vG), 8 (5, -vG & 3, +vG) and 21 (16,-v G & 5 +vG) with incidence percentages 24, 16 and 42 out of 150 samples. The average log no., of total bacterial count were 2.25, 1.16 and 4.15 cfu/g of bovine, cow milk and cottage cheese respectively.

Table 1: Assessment of total aerobic bacterial count of dairy products

		Dairy samples		
Total bacterial count		**Bovine milk (n=50)**	**Cow milk (n=50)**	**Cottage cheese (n=50)**
Positive samples	**-vG**	8	5	16
	+vG	4	3	5
Incidence percentage		24	16	42
Average log no.		2.25	1.16	4.15

3.2. Contaminant Gram negative bacteria:

The results exhibited that, Gram negative dairy bacteria were incidence included *Eshershia sp.* with log no. 0.45 0.67, 0.65, *Salmonella sp.* with log no. 0.05, 0, 0.06 and *Pseudomonas sp.* : with log no. 0.35 0.12, 0.75 for the bovine milk, cow milk and cottage cheese respectively.

3.3. Contaminant Gram positive bacteria:

The obtained results in Table (3) emphasized the incidence of *Bacillus sp*, with the average log no. 0.52, 0.25, 1.25 cfu/g, *Listeria sp* with log no. 0.45, 0.32, 0.75 cfu/g and *Staphylococcus* sp. with

the average log no. 0.20, 0.31 and 0.43 cfu/g in Bovine milk, Cow milk and cottage cheese. respectively.

Table 2: Incidence of some Gram negative bacteria in dairy products

Dairy products / **Contaminant bacteria**		**Bovine milk (n=50)**	**Cow milk (n=50)**	**Cottage cheese (n=50)**
Eshershia sp.	**Positive samples**	3	2	3
	% Incidence	6	4	6
	Log number	0.45	0.67	0.65
Salmonella sp.	**Positive samples**	1	ND	1
	% Incidence	2	ND	2
	Log number	0.05	ND	0.06
Pseudomonas sp.	**Positive samples**	1	1	2
	% Incidence	2	2	4
	Log number	0.35	0.12	0.75

Table 3: Incidence of Gram positive bacteria in dairy products

Dairy products / **Contaminant bacteria**		Bovine milk (n=50)	Cow milk (n=50)	cottage cheese (n=50)
Bacillus sp	**Positive samples**	2	1	2
	% Incidence	4	2	4
	Log number	0.52	0.25	1.25
Listeria sp	**Positive samples**	1	1	1
	% Incidence	2	2	2
	Log number	0.45	0.32	0.75
Staphylococcus sp.	**Positive samples**	1	1	2
	% Incidence	2	2	4
	Log number	0.20	0.31	0.43

The contaminated aerobic bacterial of dairy products due to their containing of all nutrient acquirements and rich medium for the growth of different microbes, among these, the most important group are bacteria (Beck, 2000 and Barnhart *et al.,* 2002). The our results similar to obtained by many reachers, Leriche *et al*. (2004) reported that, thirty *Pseudomonas* spp. strains isolated from milk, Torkar and Teger, 2006, Varga 2007, Rosengren (2012) reported that, the presence of pathogenic *E. coli, Salmonela* and *L. monocytogenes* in and some indicator microorganisms were studied in 40 samples of cheese. Singh and Prakash (2008 Khakpoor and Safarmashaei, 2011) were isolated *E. coli, Staph.aureus* and *L. monocytogenes* from milk products i.e. curd and cottage cheese. (Ordiales *et al*. 2013).

3.4. Identification of bacterial isolates

The isolated bacteria were identified according to their morphological, cultural characteristics, antibiotics resistance and consumption of broth manual some biochemical tests according to (Bergey's manual, 2009).

3.5. Morphological and biochemical identification:

The results of morphological and biochemical characteristics of bacterial isolates were given in Table (4). The identified bacterial isolates from all were belonging to *B.cereus, E.coli, L. monocytogenes, Ps. aeruginosa, Staph.aureus* and *S. typhi*.

Table 4: Morphological characteristics, Microscopic examination Biochemical characteristics (Fermentation of sugar, enzymes and tests) of identification of bacterial isolates.

Isolates / Tests	*B. cereus*	*E. coli*	*L. monocytogenes*	*Ps. aeruginosa*	*Staph aureus*	*S. typhi*
Morphological characteristics						
Shape of colony	Flat	Low convex, entire	Round, translucent	Flat	Raised, circular, entire	Low convex, entire
Texture	Rigid	smooth	smooth	smooth	smooth	smooth
Pigmentation	+	-	-	Blue-green	Golden yellow	-
Golden yellow	-	+	+	+	-	+
O_2 requirements	Aerobic	F. anaerobic	F.anaerobic	F.anaerobic	F.anaerobic	F.anaerobic
Microscopic examination						
Gram stain	+	-	+	-	+	-
Cell shape	**Rods in pairs**	**Rods singly**	**Short rods**	**Straight rods**	**Cocci in clusters**	**Rods singly**
Sporulation	+	-	-	-	-	-
Capsule	-	-	-	-	-	-
Biochemical characteristics (Fermentation of sugar)						
D-Glucose	**A/-**	**A/G**	**A/-**	**A/-**	**A/-**	**A/-**
D-Mannitol	+	-	**A/-**	+	**A/-**	**A/-**
Mannose	-	-	**A/-**	-	**A/-**	**A/-**
Lactose	**-/-**	**A/G**	**A/-**	**-/-**	**A/-**	**-/-**
L-Rhamnose	+	-	-	-	+	
D-Sucrose	**A/-**	**A/-**	**-/-**	**-/-**	**A/-**	**-/-**
D-Melibiose	**A/-**	**-/-**	**-/-**	**-/-**	**A/-**	**-/-**
L-Arabinose	**-/-**	**-/-**	**-/-**	**-/-**	**-/-**	**-/-**
Biochemical characteristics (Enzymes)						
Catalase	+	+	+	+	+	+
Coagulase	+2	--	+3	-	+3	+3
Oxidase	+	-	-	+	-	-
Urease	-	-	-	-	+	-
Gelatin liquefaction	+	-	-	-	+	+
Starch hydrolysis	+	-	-	-	-	+
Phenyl alanine deaminase	-	-	-	-	-	
Biochemical (Tests)						
H_2S production	+	-	-	-	+	+
Hemolysis on blood agar	Beta	Gamma	Beta	Beta	Beta	Alpha
Nitrate reduction	+	+	-	-	+	+
Indole formation		+	-	-	-	-
Methyl red	-	+	+	-	+	+
Voges-Proskauer	+	-	-	-	-	-
Citrate utilization	+	-	-	+	+	-

A/G= acid/gas; F=facultative; (+) = positive; (-) = negative ; F= facultative

3.6. Antibiotics resistance:

The results indicated that, the antibiotic sensitivity of isolated bacteria showed different susceptibilities ranging from sensitive, intermediate and resistant as shown in Table (5). *B.cereus* and *E. coli* were exhibited sensitive 11*; L.monocytogenes* and *Staph. aureus* exhibited intermediate 8, *Ps. aeruginosa* and *S. typhi* exhibited resistant 11 out of 18 antibiotics.

In the present work, the bacterial isolates from Bovine milk, Cow milk and Karsh cheese were identified according to their antibiotics resistance, morphological, cultural characteristics and consumption of broth manual some biochemical tests according to (Bergey's manual,2009). The results obtained from morphological and biochemical characteristics indicated that bacteria isolates from dairy and Karsh cheese products were belonging to five main bacterial families (Bacillaceae, Enterobactericeae, Listeriaceae, Pseudomonadaceae and Staphylococcaceae). Identification of isolates which belonging to, *B. cereus, E.coli, L. monocytogenes, Ps. aeruginosa, Staph. aureus* and *S. typhi*. These results in agreement with those obtained by Hosny *et al.,* 2011) and Beck, (2000) identified six

hundred and seventeen bacterial isolates (617) were obtained from various food samples by morphological and biochemical tests and the identification confirmed by API and biolog systems.

Table 5: Antibiotics sensitivity profiles of dairy bacterial isolates.

Isolates Antibiotics	*B.cereus*	*E. coli*	*L. monocytogenes*	*Ps. aeruginosa*	*Staph. aureus*	*S. typhi*
Clindamycin	R	I	R	R	I	R
Levofloxacin	S	S	S	I	S	S
Kanamycin	S	S	R	R	I	I
Tobramycin	I	S	S	S	R	R
Flucloxacillin	S	R	R	R	I	R
Ofloxacin	S	S	S	R	R	S
Rifamycin	S	R	R	I	I	R
Ampicillin	R	R	R	R	R	I
Vancomycin	S	S	I	R	S	R
Aztreonam	R	R	R	S	R	R
Gentamicin	S	S	R	S	R	I
Norfloxacin	I	S	S	R	I	R
Gatifloxacin	S	I	I	I	S	S
Cephradine	R	S	S	R	R	S
Oxacillin	S	R	R	R	S	R
Tetracycline	S	S	I	S	R	R
Ciprofloxacin	S	S	I	R	I	I
Erythromycin	I	S	S	R	R	R

R; Resistant, I; Intermediate sensitive, S; Sensitivity.

3.7. Active gradient compounds of Methanolic khella Seed extract:

Methanolic khella Seeds extract (MKSE), was analyzed by Gas chromatography–mass spectrometry (GC-MS) analysis. The analysis of Methanolic extract by GC-MS showed 8 active gradient compounds, Visnagin, Benzopyran, P Camphorene, Khellin, Ditriethyelsilyl ether, Z-Cnidimine, Benzenepropanoic acid and Proscillaridie, which contended pyrimidine and /or purine cycles related to inhibit pathogenic bacteria (table 6).

The analysis of active gradient compounds of Methanolic khella Seeds extract by (GC-MS) showed 8 active gradient compounds, which contended pyrimidine and /or purine cycles. The phytochemistry of *A. visnaga* has revealed the presence of diverse groups of chemical constituents such as pyrones, saponins, flavonoids and essential oils (Talaat *et al.,* 2013; Sellami *et al.,* 2013). GC/MS analysis of *A. visnaga* revealed the presence of twenty one compounds seeds and characterized representing, essential oil with 2,2-dimethylbutaoic acid, isobutyrate, croweacin, linalool, bornyl acetate and thymol (Satrani *et al.,*,2004, Abdul-Jalil *et al.,* 2010, Bencheraiet *et al.,* 2011 and Khalfallah *et al.,* (2011). Antibacterial activity of khella Seeds extracts : The results of the inhibition zones diameter (IZD) of Aqueous Khella Seeds extract (AKSE) and Methanolic khella Seeds extract (MKSE) and mean growth inhibition percentages (MGI) against isolated bacteria (table, 7 & fig.,1). It was found that, MKSE more effect antibacterial than AKSE, where as IZD *of* AKSE *was* 13.6, 15.3, 12.32, 9.0, 11.5, 8.35 and % MGI *was* 71.8, 76.4, 64.93, 56.76, 70.2, 46.6. while IZD *of* MKSE *was* 42.2, 34.5, 49.8, 36.0, 45.8, 45.3, and % MGI *was* 100, 96.4, 92.2, 94.0, 100, 100 for *B. cereus E. coli*, *L. monocytogenes*, *Ps. aeruginosa*,. *Staph. aureus* and. *S. typhi* respectively.

3.8. Minimum Inhibitory Concentrations (MICs):

The growth of bacteria isolates were inhibited in the presence of aqueous and methanolic Seed extracts of Khella and potency of methanolic was higher than aqueous Seed extracts. MICs of aqueous Seed extract was 25 (mg/ml) for *B.cereus* ; *E.coli ; L. monocytogenes ; Ps.aeruginosa* and 50 (mg/ml) for *Staph.aureus and S.typhi.* The methanolic extracts were inhibited at 6.25 ; 12.5 ; 6.25 ; 12.5 ; 6.25 ; 6.25 (mg/ml) for *B.cereus* ; *E.coli ; L. monocytogenes; Ps.*aeruginosa ;*Staph.aureus and S.typhi*(Table, 8 & Fig., 2).

Table 6: Quantitative and Qualitative Active gradient compounds of Methanolic khella Seeds extract (MKSE) by GC mass.

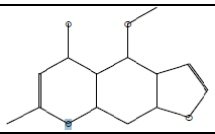
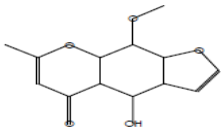
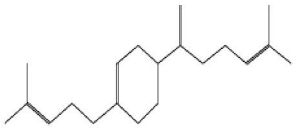
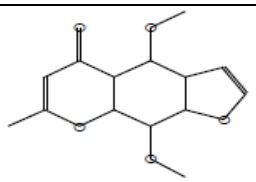
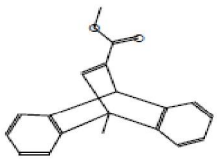
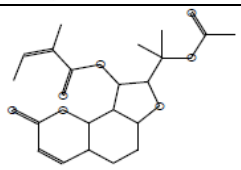
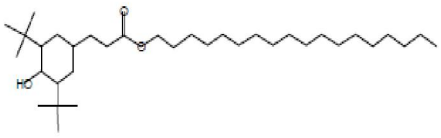
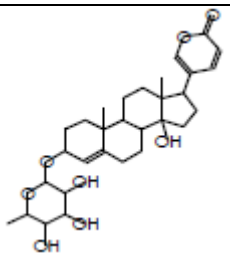
Compound	Formula		M. F.	M.W.
Visnagin RT 26.87	C13H10O4, MW 230, CAS# 82-57-5, Entry# 28748 5H-Furo[3,2-g][1]benzopyran-5-one, 4-methoxy-7-methyl		C13H10O4	230
Benzopyran RT 28.47	5H-Furo[3,2-g][1]benzopyran-5-one, 4-hydroxy-9-methoxy-7-methyl-		C13H10O5	246
P Camphorene RT 29.55	C20H32, MW 272, CAS# 20016-72-2, Entry# 34430 4-(6-Methylhepta-1,5-dien-2-yl)-1-(4-methylpent-3-en-1-yl)cyclohex-1-ene		C22H36O	2 332
Khellin RT (30.46)	C14H12O5, MW 260, CAS# 82-02-0, Entry# 207738 5H-Furo[3,2 g][1]benzopyran-5-one, 4,9-dimethoxy-7-methyl-		C14H12O5	260
DITRIETHYLSILYL ETHER RT (33.58)	C12H30OSi2, MW 246, CAS# NA, Entry# 135305		C16H11N	217
Z-Cnidimine RT (43.55)	C21H22O7, MW 386, CAS# 15591-75-0, Entry# 53377		C21H22O7	386
Benzenepropanoic acid, RT(52.78)	3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester C35H62O3, MW 530, CAS# 2082-79-3, Entry# 33752 2,6-Di-tert-butyl-4-[(2 octadecyloxycarbonyl)ethyl]phenol		C35H62O3	530
Proscillaridie RT (52.78)	C30H42O8, MW 530, CAS# 466-06-8, Entry# 186860 $:03[M+H]+		C35H62O3	530

Table 7: The antibacterial activity of khella Seed extracts (50 mg/ml) based on the growth inhibition of bacterial isolates by disc diffusion method

Khella extracts		*B. cereus*	*E. coli*	*L. monocytogenes*	*Ps. aeruginosa*	*Staph. aureus*	*S.typhi*
AKSE	**IZD**	13.6 ±0.65	15.3 ±0.46	12.32±0.44	9±0.43	11.5±0.78	8.35±0.46
	MGI	71.8±0.51	76.4±0.64	64.93±0.34	56.76±0.28	70.2±0.45	46.6±0.53
MKSE	**IZD**	42.2±0.97	34.5±0.85	49.8±2.3	36.0±0.57	45.8±0.64	45.3±0.75
	MGI	100±00	96.4±0.72	92.2±0.57	94.0 ±0.62	100±00	100±00

IZD = inhibition zones diameter; MGI = mean growth inhibition percentages
AKSE = Aqueous Khella Seeds extract; MKSE=Methanolic khella Seeds extracts. Each value calculated as mean of three replicates ± stander error.

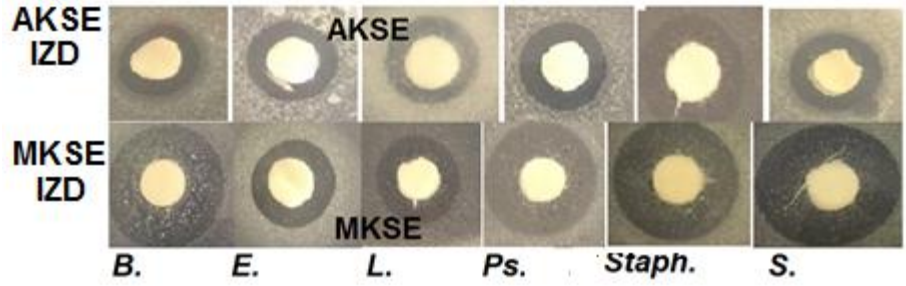


Fig. 1: Agar plate showing the antibacterial activity of Aqueous Khella Seeds extract (AKSE) and Methanolic khella Seeds extract (MKSE) (10 mg/ml/disc) based on the growth inhibition of bacterial isolates by inhibition zones diameter(IZD) mm. B = *B. Cereus, E= E.Coli, L= L. Monocytogenes, Ps= Ps. aeruginosa, Staph= Staph.aureus and S= S.typhi*

Table 8: MICs (mg/ml) of aqueous and methanolic Seed extracts of Khella against bacterial isolates.

Extracts / Bacterial isolates		MIC Conc.(mg/ml)											
		Aqueous Khella Seeds extract						Methanolic khella Seeds extract					
	C	50	25	12.5	6.25	3.15	1.15	50	25	12.5	6.25	3.15	1.15
B. cereus	**1.3**	0.15	0.65	0.95	1.05	1.15	1.25	0.09	0.16	0.43	0.62	1.07	1.15
E. coli	**1.5**	0.25	0.79	0.95	1.15	1.28	1.34	0.15	0.35	0.73	1.15	0.25	1.25
L. monocytogenes	**1.2**	0.17	0.62	0.87	0.91	1.05	1.15	0.09	0.25	0.58	0.74	0.97	1.02
Ps. aeruginosa	**1.4**	0.15	0.72	0.95	1.11	1.25	1.28	0.20	0.45	0.75	0.85	1.07	0.05
Staph. aureus	**1.3**	0.68	0.75	0.85	1.05	1.15	1.25	0.08	0.18	0.42	0.67	1.05	1.15
S. typhi	**1.4**	0.65	0.88	1.03	1.15	1.20	1.25	0.15	0.35	0.59	0.75	0.95	1.24

C = Control

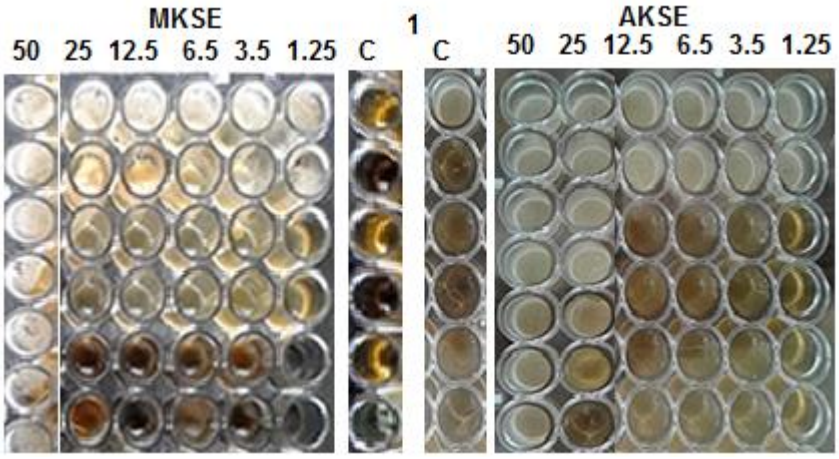


Fig. 2: Microtiter ELISA plate showing Aqueous (AKSE) and Methanolic (MKSE) Khella Seeds extract MIC by microdilution method against (C1: control &MIC, 1 =*B. cereus*.

The (MIC) is defined as the lowest concentration of a drug/compound that will inhibit the visible growth of an organism *in vitro* after overnight incubation. In recent years, (MICs) have been used in Phytotherapy, the use of plants for medical purposes, Thus, determination of the MICs has become the main factor for the scientific studies regarding the feasibility of bioactive components of the plants in industry (Watase-Renee and Reppun-Thomas, 2011). Based on the previous results most tested foodborne bacteria were sensitive in low concentrations to total and basic alkaloid fraction of *T. vulgaris*, *B. cereus, M. luteus* and *S. typhi*, *L. monocytogenes, K. pneumoniae, Ent. cloacae, E. coli* and *Staph. aureus*. The highest (MICs) values recorded for *Ps. aeruginosa* and *P. vulgaris* (Fu *et al.,* 2007)

3.9. Anti virulence activity of MKSE:

3.9.1. Biofilm formation of isolated bacteria:

A biofilm positive phenotype was defined as OD ≥ 0.17 at O.D.570 nm.The results presented in Table (9) showed that, four bacteria isolates exhibit strong biofilm formation with (1.7 ; 1.5 ; 1.3,1.4

OD at 620 nm) for *B.cereus, Ps.aeruginosa, Staph. aureus* and *S.typhi* respectively. Moderate biofilm formation by two bacterial isolates (0.8; 0.7 OD at 620 nm) for *E.coli* and *L. monocytogenes* respectively.

3.9.2. The antibiofilm activity of MKSE at (MIC) were assessed against biofilm formation of *B.cereus*, *E.coli*. *L. monocytogenes*, *Ps. aeruginosa*, *Staph.aureus* and *S.typhi*. The enhanced antibiofilm at different MIC concentrations (6.25; 12.5; 6.25; 12.5; 6.25; 6.25 (mg/ml) with biofilm reduction percentages were 43, 70, 62, 43, 31 and 62 % respectively (table, 8). The positive control at 0 (mg/ml) showed the antibiofilm formation effect at (0.62, 0.73, 0.58, 0.75, 0.67 and 0.75 mg/ml) were 0.74, 0.45, 0.46, 0.80, 0, 90 and 0.53 at 570 nm O.D.), with biofilm reduction percentages were 43, 70, 62, 43, 31 and 62 % respectively.

Table 9: Ant virulence activity (mg/ml) of methanolic Seed extract of Khella against bacterial isolates. Biofilm formation assay using (Tissue culture plate method by ELISA reader)

	B. ceres	***E. coli***	***L. monocytogenes***	***Ps. aeruginosa***	***Staph. aureus***	***S. typhi***
Growth (O.D.620nm)	1.7	0.8	0.7	1.5	1.3	1.4
Control negative (O.D.620nm)	0.08					
Biofilm (O.D.570nm)	0.74	0.45	0.46	0.80	0.90	0.53
Biofilm production	Strong	Moderate	Moderate	Strong	Strong	Strong
%Biofilmreduction	43	70	62	43	31	62

Interpretation of biofilm formation was classified as, strong biofilm 0.7 to 1.0, moderate biofilm 0.3 to 0.4 and weak biofilm ≥ 0.3(O.D.570).

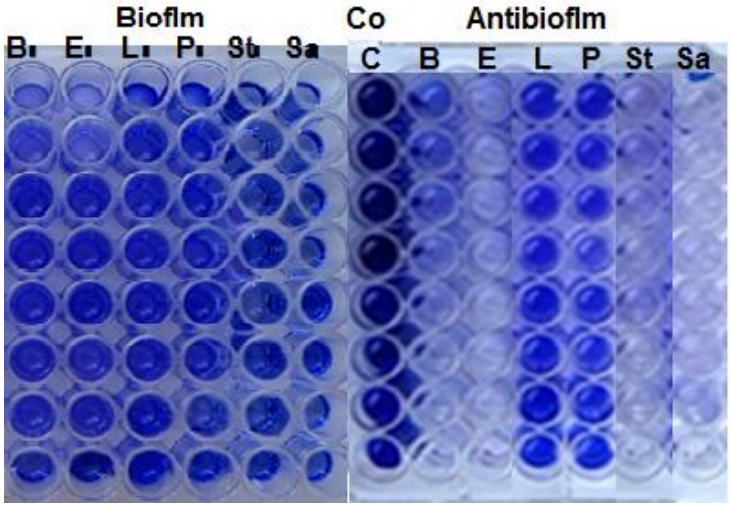


Fig. 3: Microtiter ELISA plate showing biofilm formation and antibioflm of bacterial isolates were grown overnight in polystyrol microtiter wells in TSB supplemented with 2% glucose. The cells that adhered to the plate after washing were then visualized by staining with crystal violet

3.9.3. Time kill of isolated bacteria:

The MKSE was evaluated for its ability as bactericidal agent against of isolated bacteria in broth media at different time intervals 4h to determine the time kill assay (table, 10). The results exhibited the effectiveness of the bactericidal activity of MKSE at 6.25 ; 12.5 ; 6.25 ; 12.5 ; 6.25 ; 6.25 (mg/ml) minimum Bactericidal Concentration (MBCs)for *B.cereus* ; *E.coli ; L. monocytogenes ; Ps.*aeruginosa ;*Staph.aureus and S.typhi*.respectively. The time kill was recorded between (0-12 h) for Gram positive bacteria (*B. cereus, L. monocytogenes* and *staph. aureus*) and (0-16 h) of Gram negative bacteria (*E. coli and Ps. aeruginosa*) while between (0-8 h) for *S. typhi*. These data concluded that, the time kill

assay for Gram positive bacteria was faster than Gram negative bacteria *except S.typhi* was faster than *E. coli and Ps. aeruginosa* (Table 10).

These results were not agreed with (Okorondu *et al.,* 2010 and 2011) who reported that, total phenols which could be responsible for the antioxidant and antimicrobial activities. Most probably because of the structural similarity between khellin and psoralen, *A. visnaga* has photosensitizing ability and it was considered useful as a photosensitizer in patients with psoriasis (Abdelfattah *et al.,* 1983).

Ethanolic and aqueous extract of the *A. visnaga* were tested against eight pathogenic microorganisms. The most active extract against Gram positive bacteria was ethanol extract with minimal inhibitory concentration value (Khalfallah *et al.,* 2011) and (Semyaril *et al.,* 2011). The inhibition zones obtained with Khella Seeds extract against *Ps. aeruginosa*, *Salmonella spp* and *Staph. aureus* were greater than those obtained by other workers. Gram negative bacteria known to be more resistant to antibiotics than Gram positives ones. For instance, the resistance could be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism (Ceylan, and Fung, 2004). This antimicrobial activity attributed to similarity in the mechanism of action of this plant extract and antibiotics. While, the Khella Seeds extract extract showed greater antimicrobial activity than antibiotic especially ampicillin. The antimicrobial activity of the crude extract might be due to the presence of active compounds including alkaloid, quinines, tannins, flavonoides, saponins and iridoids (Nilforoushzadeha *et al.,* 2008). Active compounds of plants extract have been shown to cause disruption of the cellular membrane, inhibition of ATPase activity, and release of intracellular ATP and other constituents of several microorganisms such as *E. coli*, *E. coli* O157:H7, *L. monocytogenes*, *Lactob. sakei*, *Ps. aeruginosa*, *S. enteritidis*, and *Staph. aureus* (Moreno *et al.,* 2006 and Raybaudi-Massilia *et al.,* 2009) reported that, the antimicrobial action of plant extract compounds was related to inactivation of cellular enzymes, which depended on the rate of penetration of the substance into the cell or caused by membrane permeability changes. Increased membrane permeability is a major factor in the mechanism of antimicrobial action, whereas compounds may disrupt membranes and cause a loss of cellular integrity and eventual cell death.

Table 10: Time kill assay of Bacterial isolates treated with methanolic seed khela extract optical densities held at 37°C for 24h intervals 4h

Time (h)	Bacterial isolates											
	B. cereus		***E. coli***		***L. monocytogenes***		***Ps. aeruginosa***		***Staph. aureus***		***S. typhi***	
	Non	**Treated**	**Non**	**Treated**	**Non**	**Treated**	**Non**	**treated**	**Non**	**Treated**	**Non**	**Treated**
0	0.05	0.04	0.04	0.03	0.08	0.06	0.07	0.05	0.06	0.05	0.04	0.03
2	0.12	0.10	0.07	0.05	0.12	0.12	0.10	0. 08	0.14	0.09	0.11	0.05
4	0.23	0.07	0.12	0.07	0.27	0.08	0.27	0.09	0.25	0. 04	0.24	0.03
8	0.38	0.03	0.28	0.05	0.35	0.05	0.31	006	0.43	0.04	0.32	0.00
12	0.58	0.00	0.57	0.02	0.56	0.00	0.48	0.04	0.68	0.00	0.58	0.00
16	0.82	0.00	0.84	0.00	0.84	0.00	0.66	0.00	0.92	0.00	0.92	0.00
18	1.05	0.00	1.07	0.00	1.15	0.00	0.95	0.00	1.13	0.00	1.12	0.00
20	1.39	0.00	1.29	0.00	1.36	0.00	1.12	0.00	1.39	0.00	1.45	0.00
22	1.75	0.00	1.45	0.00	1.81	0.00	1.75	0.00	1.55	0.00	1.85	0.00
24	2.15	0.00	1.85	0.00	2.05	0.00	2.03	0.00	1.75	0.00	2.24	0.00

Bacterial growth non treated ($O.D._{620}$ nm)
Bacterial growth treated with methanolic seed khela extract. ($O.D._{620}$ nm)

3.9.4. Effect methanolic extract:

The antimicrobial effect of methanolic seed khela extract at 12.5 and 6.5 mg/ml MIC on morphological structure *Staph. aureus and Ps. aeruginosa* incubated for 24h were examined by Scanning Electron Microscopy (SEM). Treated each, *Staph. aureus and Ps. aeruginosa* were observed changes in the cells from illustrated in (Fig. 4) directly seemed that, severe damage appeared to be shrunk, empty, and the remains were flaccid. Furthermore, most of them appeared to be stuck together and melted. *Ps. aeruginosa* showed giant cells and appendages on cell surface. Non-treated cells (control) were intact and showed a smooth surface. Generally, SEM images observations confirmed the physical damage and considerable morphological alteration to pathogenic bacteria

treated with methanolic of Seed Khella aqueous extract at MICs . The active plant components bind to the cell surface and then penetrate to the target sites, possibly the phospholipid bilayer of the cytoplasmic membrane and membrane-bound enzymes (Shan *et al.,* 2007 and 2011). Some cells damage deformity in the cell walls and cytoplasmic membrane was the loss of structural integrity and the ability of the membrane to act as a permeability barrier (Packiyasothy&Kyle 2002 and Filipowicz *et al.,* 2003). The distortion of the cell physical structure would cause the expansion and destabilization of the membrane and would increase membrane fluidity, which in turn, would increase passive permeability (Ultee *et al.,* 2002). Most of the Gram negative cells and the Gram positive cells appeared to be shrunk and even some were empty, and the remains were flacci (Lopez *et al.,* 2005 and Shan *et al.,* 2005).

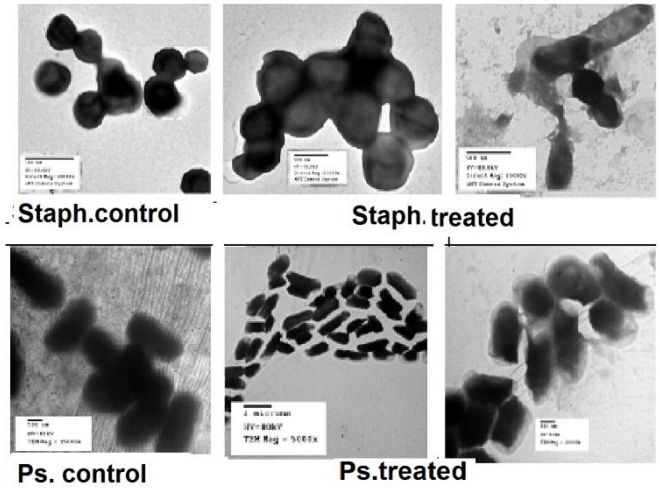


Fig. 4: Scanning Electron Microscope (SEM) showing *Ps. aeruginosa* and *Staph. aureus* treated with methanolic Khella Seed extract.

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