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Invivo and invitro effect of Moringa oil and Moringa microemulsion on MRSA strain isolated from broiler chicken

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ABSTRACT

Methicillin-resistant Staphylococcus aureus (MRSA) is responsible for staph infection that is difficult to be treated because of resistance to some antibiotics. This study designed to evaluate the potential use of Moringa oil and microemulsion (ME) as an alternative choice to antibiotics to control MRSA in broiler chickens and production of enterotoxins.

S. aureus strains were isolated from broiler chicken farms in Dakhlia, Egypt with percentage of 22.2%. All isolates were screened for antibiotic resistance to 14 antibiotics using disk-diffusion method, and the antimicrobial resistance of *S. aureus* isolates was most frequently noted against Cephalexin (80%), Cefotaxim (75%), Erythromycin (75%). The *S. aureus* isolates were examined for the presence of *mec*A gene to detect the MRSA and for the presence of genes responsible for enterotoxins production. MRSA was identified by positive amplification of the *mec*A gene in 42.5% (17 of 40) of *S. aureus* isolates. Out of 40 examined isolates, One isolate exhibited the enterotoxin B (2.5%).

Moringa ME was prepared and characterized that the nanodroplet was determined mainly using high-resolution transmission electron microscopy (TEM) which size had 10.13 ± 0.19 nm with a narrow size distribution, (polydispersity index: 0.259) which the zeta potential had a-5.61±3.7mV, conductivity 0.083 ms/cm and had fatty acid components Linoleoylchloride (80.17%) which had antibacterial activity by using gas chromatography–mass spectrometry (GC-MS/MS) and the effect on cell viability% was assessed by SRB (sulforhodamine B) assay was IC_{50}> 100 ug/ml.

The MIC of Moringa oil and ME on tested *S. aureus* isolates were 0.50 ml/l and 0.25 ml/l. A total of 55 commercial one day old Arbor Acres broiler chickens were haphazardly divided to five groups of 10 birds. The birds were examined for S. aureus free. The five groups were the positive control, negative control, Moringa oil (0.5 mL/L water/3days), Moringa ME (0.25 mL/L water/3days) and antibiotic groups (for 3days). The treated groups with moringa oil and ME exhibited lower lesions, mortality and *S. aureus* count in addition to higher growth performance compared to the positive control group.

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Our data showed that, Moringa oil and Moringa ME could be a substitutive choice for the control of S. aureus infections in poultry farms.

INTRODUCTION

S. aureus is an opportunistic bacteria affect human and other animal species. Mainly thisbacteriais responsible for food poisoning and is the third largest source of food related illness all over the world **(Ali *et al.* 2017)**. *S. aureus* can affect all avian species causing synovitis, Arthritis, osteomyelitis, chondronecrosis, gangrenous dermatitis, bumble foot, omphalitis, septicemia **(Abdul Aziz *et al*. 2013)**. MRSA was first recorded in 1961. MRSA is mediated by PBP2a (penicillin binding protein PBP2a), that is a 78 KDa protein. That protein is often heterogeneously expressed in staphylococci. It shows low affinity to the b-lactum antibiotics. The *mec*A gene is responsible for this protein encoding and existon a large mobile genetic element termed staphylococcal chromosomal cassette mec (SCCmec**) (Ali et al. 2017)**.

Poultry meat consumption is increasing worldwide; that reached 14.2 kg per capita per year. Among poultry meat products, chicken carcasses, processed products, andcuts are the most consumed (~75% of total poultry meat) then turkey (~25%) and, to a lesser extent, followed by duck **(Filières et al. 2017)**. Because of the ability of *S. aureus* to colonize the skin and nares of animals, several foods of animals' origin such as poultry, swine, and cattle have been expected to be reservoirs for *S. aureus* contamination **(Sankomkai et al. 2020**).

S. aureus is documented as one of the most important foodborne pathogens in both fresh and ready-to-eat products and responsible for many infections around world **(Diep *et al.* 2006**). Staphylococcal enterotoxins (SEs) are formed in foods under favorable conditions become a potential biological hazard **(Maktabi *et al*. 2021**).

SEs are not degraded by cooking (heat stable), and also persist through harsh environmental conditions such as freezing and drying **(Loir et al. 2003)**. The SEs amount that essential for establishment of typical food poisoning symptoms is very low, ranging from 20 ng to 1 µg **(Normanno et al. 2007**).

Moringa oleifera (M.oleifera) has been used as ingredient of Indian diet since centuries. It has been used for treating malnutrition, inflammation, fungal infection, bacterial infectionand diarrhea. The presence of some phytochemical substances especially a short polypeptide found in MO seed extracts was reported to directly act on pathogen resulting in growth inhibition via disrupting cell membrane synthesis or synthesis of essential enzymes. (**Ruttarattanamongkol and Petrasch 2015).**

Although, it was so difficult to exactly define the responsible component (s) for the antimicrobial properties. Some authors attributed this antimicrobial effect to the compounds moringin, 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate and 4-(α-L-rhamnosyloxy) phenylacetonitrile **(Abdalla et al. 2016)**. The Moringa seeds have an appreciable amount of oil (up to 40%), Moringa oil is well-known Commercially as "Behen oil," as it contains significant amounts of palmitic along with behenic acid and stearic acids in addition to oleic acid (more than 70%) **(Zhao and Zhang 2013).**

Microemulsion is composed of oil, surfactants, cosurfactants and water. Microemulsions have many advantages such as good thermodynamic stability, long shelf life, low viscosity, transparency, enhance drugs solubility, ease of manufacturing and enhanc-

ing effect on transdermal delivery of drug compared to other conventional formulations. Because of their ability to incorporate large amount of drug, microemulsion components combined synergistically to increase drug flux and enhanced rate of permeation. So, a short time ago more attention has been focused on microemulsions for transdermal delivery of drugs **(Vibhute et al. 2015)**.

This study aimed to determine the antibiotic resistance profile of *S. aureus* and MRSA recovered from broiler chicken and to evaluate M. oleifera oil and its ME as a natural alternative to antibiotics against *S. aureus* isolates. Also detection of enterotogenic *S. aureus* strain and determination the enterotoxins in muscle and ovals of broiler chicken.

MATERIALS AND METHODS

1. Samples collection

A total of 180broiler chicken samples collected from chicken farms distributed in Mansoura, Egypt. Samples were collected from lung (60), livers (60) and joints (60) of diseased and freshly dead chickens under aseptic conditions during the period of Augest to November 2020. Every sample was packed individually into a sterile polyethylene bag, marked, then transferred directly in an ice box to the reference laboratory for Veterinary Quality Control on poultry production, Egypt, for conventional bacteriological analysis. The lung, liver and joint from each bird was pooled as one sample.

2. Bacterial isolation, identification:

S. aureus isolation and identification was done according to **BAM, 2001** and **ISO 6888-3:2003(en).** Twenty-five grams of collected sample were weighed into sterile stomacher bags containing 225 mL of sterile Buffered Peptone water that incubated at 37°C for 18 hours. One loopful of the broth was streaked on Baired-Parker agar supplemented with egg yolk-tellurite emulsion and incubated at 37° C/48 hours. Only 5 suspected colonies was picked up then inoculated into test tubes containing 5 ml of sterile brain heart infusion broth then incubated at 37°C /24 h for further confirmation based on Gram staining, catalase test and coagulase test.

3. Antimicrobial susceptibility testing

The antibiogram of forty *S. aureus* isolates were done by Kirby-Bauer disc-diffusion test. *S. aureus* tested against 14 antibiotics (Oxoid Ltd) and the interpretation according to (**CLSI, 2020**): Ampicillin (AMP) (10 µg/ disc), Cefoxitin (Fox) (30 µg/disc), Ampicillin/Sulbctam (SAM) (20 µg/disc), Amoxicillin + Clavulinic acid (AMC) (30 µg/disc), Cefotaxim (CTX) (30 µg/disc), Ceftriaxone (CRO) (30 µg/disc), Cephalexin (CL) (30 µg/ disc), Erythromycin (E) (15 µg/disc), Gentamicin (CN) (10 µg/disc), Neomycin (N) (30 µg/disc), Ciprofloxacin (CIP) (30 µg/disc), Chloramphenicol (C) (30 µg/disc), Tetracycline. (TE) (30 µg/disc), Trimethoprim-sulfamethoxazole (SXT) (23.75 µg/disc). Cefotaxim (CTX) (30 µg/disc) disc diffusion test was used for detection Oxacillin resistant strains according to **CLSI (2020)**

4. Molecular analysis

4.1 Molecular confirmation of MRSA and determination of interotoxigenic *S. aureus*

4.1.1. DNA extraction

DNA was extracted from *S. aureus* isolates were tested for the presence of *mec*A gene. DNA extraction was performed using QIAamp DNA mini kit (Qiagen- Germany- GmbH). 200 µl of samples suspension incubated with 10 µl of proteinase K and 200µl of lysis buffer at 560C for 10 minutes. Then 200 µl of 100% ethanol was added to the lysate. The samples then washed and centrifuged. Nucleic acid was eluted with 100µl of elution buffer.

4.1.2. PCR amplification

Primers were provided from Metabion (Germany) and listed in table (1). Primers were used in a 25µl reaction containing 12.5µl of Emerald AMP Max PCR master mix (Takara, Japan), 6µl of DNA template,1µl of each primer of 20 pmol concentrations and 4.5µl of water. The reaction was completed in an applied bio system 2720 thermal cycler.

4.1.3. Analysis of PCR products

The product of PCR was separated via gel electrophoresis using 1.5% agarose gel (Applichem, Germany, GmbH) stained with Ethidium bromide.

For gel analysis of interotoxigenic determinants of *S. aureus*, 30 µl of the multiplex PCR products were added in each gel slot and to determine the fragment sizes, Generuler 100 bp ladder (fermentas, Thermo) was used.

The gel was visualized through a gel documentation system (Alpha Innotech, Biometra) and the data was evaluated by computer software.

Table (1) Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Sea	GGTTATCAATGTGCGGGTGG	102	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Mehrotra et al. (2000)
	CGGCAC-TTTTTTCTCTTCGG							
Seb	GTATGGTGGTG-TAACTGAGC	164						
	CCAAATAGTGACGAG-TTAGG							
Sec	AGATGAAGTAGTT-GATGTGTATGG	451						
	CACACTTTTA-GAATCAACCG							
Sed	CCAATAATAGGA-GAAAATAAAAG	278						
	ATTGG-TATTTTTTTTCGTTC							
See	AG-TTTTTTCACAGGTCTCC	209						
	CTTTTTTTTCTTCGGTCAATC							
mecA	GTA GAAATG ACT GAA CGTCCG ATA A	310						McClure et al. (2006)
	CCA ATTCCA CATTGT TTC GGTCTA A							

5.1. Preparation, characterization and cytotoxicity assay of Moringa ME.

Moringa oil was kindly purchased from CAP-PHARM®, Cairo, tween 80 was obtained from the Sigma-Aldrich Co.). Double-distilled and deionized water was filtered before use. Moringa oil ME (oil in water) was prepared in the Nanomaterials Research and Synthesis Unit by using moringa oil (20 ml), Tween 80 (30 ml), and distilled deionized water (50 ml) were mixed for half hour in a homogeneous blender 1500 watt, and then distilled water was slowly added to the mixed oil phase according to (**Rao and McClements, 2011).**

High-resolution transmission electron microscopy (HRTEM) monitoring is carried out using a JEM 1400F HRTEM at a beam energy of 300 keV to characterize the microemulsion and measure electrical conductivity, zeta potential (surface charge), and both size droplet and distribution (polydispersity indexes PDI) of microemulsion using Zetasizer Malvern Instrument(Corp, Malvern, UK) **(Sorour et al. 2021).**

Moringa oil and moringa micro-emulsion components using GC-MS at Nawah Scientific Inc.

The Vero: Green Monkey cell line was donated by Nawah Scientific Inc. for cell culture (Mokatam, Cairo, Egypt). Cells were grown in DMEM (Dulbecco's Modified Eagle Medium) media supplemented with 100 unit/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum in a humidified and 5% (v/v) CO2 atmosphere at 37 °C. Also, viability of cell was determined using SRB (sulforhodamine B) analyze at various concentrations (0.01, 0.1, 1, 10, and 100 ug/ml), as described by **Skehan et al. (1990**).

5.2. Determination of minimum inhibitory concentration (MIC):

MICs of tested materials were assessed using the broth microdilution method in Mueller-Hinton broth (MHB) in accordance with **(CLSI. 2020)**.

S. aureus isolates were adjusted to be $5x10^5$ colony forming units (CFU/mL). Two-fold serial dilution in the same type of broth media of tested of the two tested materials (Moringa oil and ME) was diluted to yield concentrations, Sterile distilled water was added in wells of negative control. Bacteria were added to a 96-well microtiter plate and incubated at 37°C for 24 hours. MIC was defined as the lowest concentration which inhibited the visible bacterial growth.

6. Determination of antibacterial activity of moringa oil and moringa ME in commercial broiler chicken model as a treatment trial:

6.1 Ethical approval

The experimental study was approved by the Ethical Committee of the Animal Health Research Institute, Ministry of Agriculture, Giza, Egypt.

6.2 Experimental animals:

The experimental study was conducted in December 2020 for nearly 4 weeks (days 4 – 25) on 50 commercial one day-old Arbor Acres broiler chicks, that were randomly assigned to five groups (10 birds each).

Additionally, five chicks were examined for *S. aureus* free. This study was conducted in battery cages in Laboratory Animal Unit (biosafety level two), Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Animal Health Research Institute Giza, Egypt under controlled ventilation and light condition in RLQP where each group was placed in individual isolators. Chicks were provided with adequate diets amount and clean drinking water along the experimental period. A starter diet was administrated during 0 to 13 days, Moreover, grower diet was fed till the day 26. The feed was according to the nutrient requirements of chicks **NRC (1994).**

The five groups were the positive control (*S. aureus* strain was recovered from a naturally occurring case of MRSA), negative control (uninoculated), Moringa oil, Moringa ME and antibiotic groups. All groups of chicks except the negative control in isolators were exposed to 100 ml of 2.9 x 10^7 CFU/ ml *S. aureus* suspension on day 4 by aerosol, in the morning and afternoon with a 4 h interval between each exposure. Isolator air extraction fans were switched off for a total of 10 min during and after exposure according to **McNamee (1999).**

Then give the studied materials (Antibiotic, Moringa oil and ME**)** on days 8, 9 and 10 in water. Samples (lung, upper respiratory tract and muscles) were taken and pooled at days 11, 18 and 25 for enumeration of coagulase-positive staphylococci using the methods described by **ISO 6888-3:2003(en)** as shown in Figure (1).

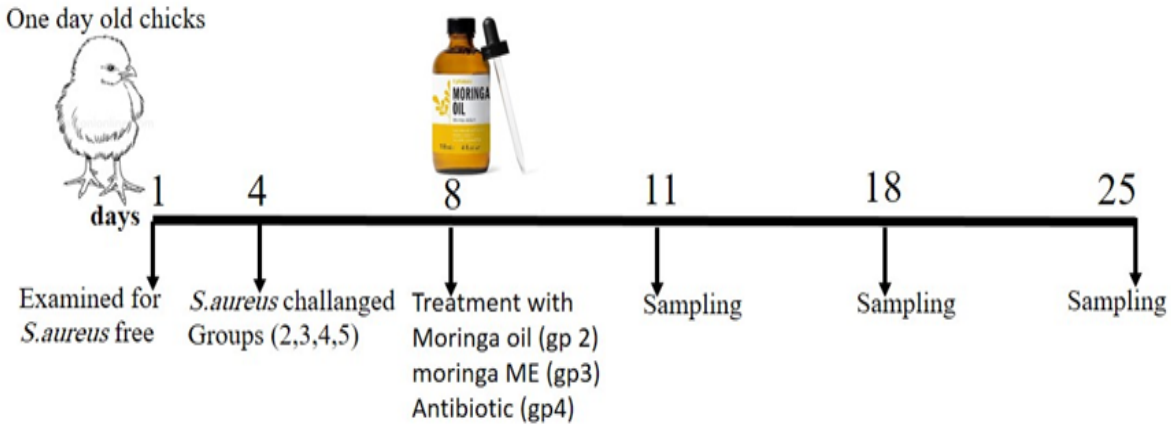


Figure 1. Schematic outline of the experimental design

7. Detection of SEB by RPLA (Zouharova and Rysanek 2008):

The SEB (Staphylococcal enterotoxins B) presence in chicken was determined by the reversed passive latex agglutination (RPLA) method with SET-RPLA (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) by following the manufacturer's instructions

RESULTS

1. Total incidence of *S. aureus* from chicken:

S. aureus was detected in 40 sample from 180 different broiler organs (Joints, livers and lungs) with percentage of 22.2% by direct plate culture methods and enrichment culture method. Moreover, the isolates were confirmed as *S. aureus* by different biochemical tests.

2. Antimicrobial resistance pattern of *S. aureus* isolates

The most common resistance pattern was CTX (75%), E (75%), TE (50%), SXT (50%), AMP (47.5%), In contrast, most of samples showed high sensitivity to CIP (72.5%), AMC (72.5%), SAM (67.5%) and CN (70%). Meanwhile, FOX which applied for detection of mecA-mediated methicillin (oxacillin) resistance and the percentage was (25%) (Table 3). See table (3).

3.1. Molecular confirmation of MRSA:

In this approach, PCR was used to detect methicillin resistant strains (MRSA) strains among 40 tested *S. aureus* isolates. Out of the 40 tested *S. aureus* strains, 17 isolates only confirmed to be MRSA as *mec*A gene was found. MRSA was isolated from 20% (8/40) total examined samples.

3.2. Molecular determination of determination of enterotoxigenic *S. aureus*: Only one isolate showed the *Seb* gen which responsible for production of enterotoxin B in chicken in muscle and organs and negative for *Sea , Sec, Sed, See* genes.

4. Characterization of moringa oil ME:

Micro-emulsion characterization of the nanodroplet was mainly determined by TEM which size had 10.13 ± 0.19 nm with a narrow size distribution, (polydispersity index: 0.259) which indicated that greater homogeneity can be realized **(Fig 2a).** The zeta potential is an indicator stable suspensions are generally taken by using dynamic light scattering (DLS) had a -5.61± 3.7mV, conductivity 0.083 ms/cm.

On the confluent surface of Vero cells, results for moringa oil ME had different concentration (0.01, 0.1, 1, 10, 100 ug /ml) after 3 days of inoculation, the effect on cell viability% was assessed by SRB assay was 90.59%,90.19%, 84.74%,83.85%,79.28%, respectively in 100 ug/ml and IC_{50}> 100 ug/ml. **(Fig.2 b).**

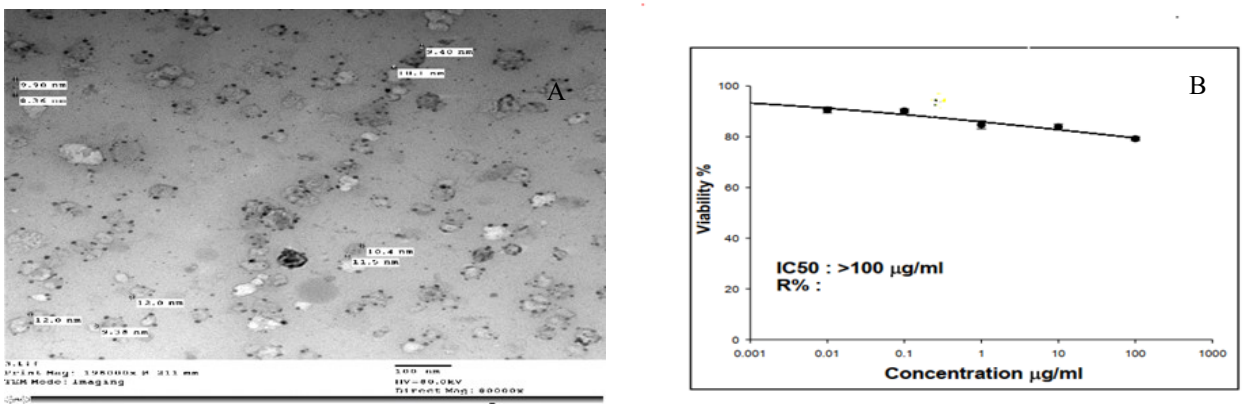
When GC-Mass analyzed the moringa oil had 37 total components which were cis-Vaccenic acid (53.35%), n-Hexadecanoic acid (10.15%), Hexadecanoic Acid, 2,3-Dihydroxy

Table 3. Results of antimicrobial susceptibility testing of *S. aureus* isolates to different antibiotics

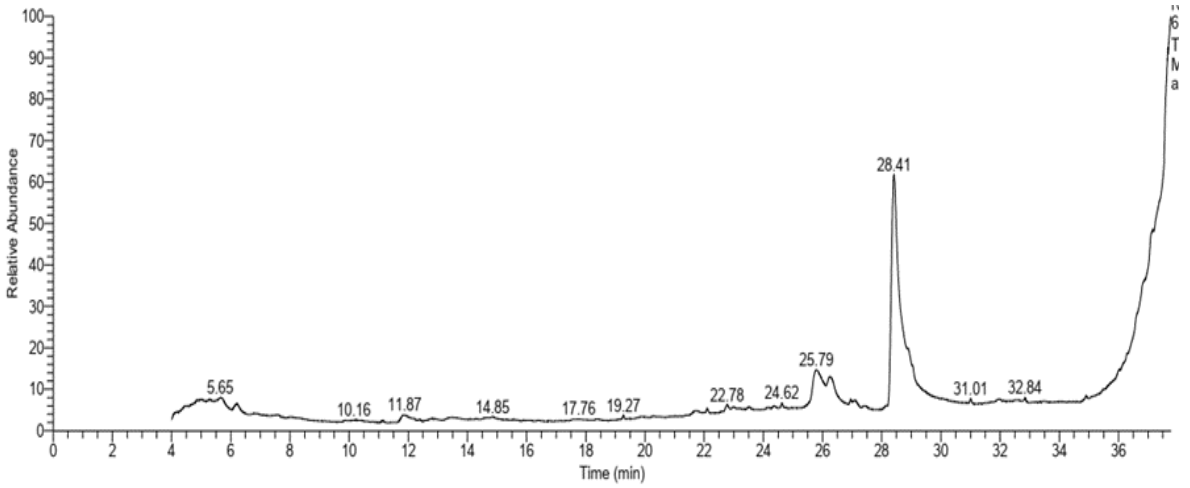
Antimicrobial class		Antimicrobials	*S. aureus* isolates (no = 40)		
			R	I	S
β-lactams	Penicillins	Ampicillin (AMP)	19 (47.5%)	6 (15.0%)	15 (37.5%)
	β -lactams inhibitors	Ampicillin/Sulbctam (SAM)	13 (27.5%)	0	27 (67.5%)
		Amoxicillin-Clavulinic (AMC)	11 (27.5%)	0	29 (72.5%)
	Cephalosporins	Cefotaxim (CTX)	30 (75%)	5 (12.5%)	5 (12.5%)
		Ceftriaxone (CRO)	6 (15%)	21 (52.5%)	13 (32.5%)
		Cephalexin (CL)	32 (80.0%)	1(2.5%)	7 (17.5%)
		Cefoxitin (FOX)	10(25%)	0	30 (75%)
Macrolides		Erythromycin (E)	30 (75%)	5 (12.5%)	5 (12.5%)
Aminoglycosides		Gentamicin (CN)	10(25%)	2 (5%)	28 (70%)
		Neomycin (N)	15 (37.5%)	3 (7.5%)	22 (55.0%)
Quinolones		Ciprofloxacin (CIP)	7 (17.5%)	4(10%)	29(72.5%)
Phenicols		Chloramphenicol (C)	17 (42.5%)	5 (12.5%)	18 (45%)
Tetracyclines		Tetracycline (TE)	20 (50%)	4(10%)	16 (40%)
Sulphonamides, DHFR inhibitors and combinations		Trimethoprim–Sulfamethoxazole (SXT)	20 (50%)	7 (17.5%)	13 (32.55%)

propyl Ester (5.04%), E-2-Decenal (2.04%), 2-Bromotetradecanoic Acid (1.17%), 4-Isopropenyl-1-Methyl-1-Cyclohexene (2.26%) , 3',4',7-Trimethylquercetin (1.13%), 1-Chlorooctadecane (1.11%), 2,4-Dodecadienal (1.29%), Oleic Acid (4.99%), Isooctyl Chloride (2.17%) and other compound. as shown in **(Fig.3 a)**.

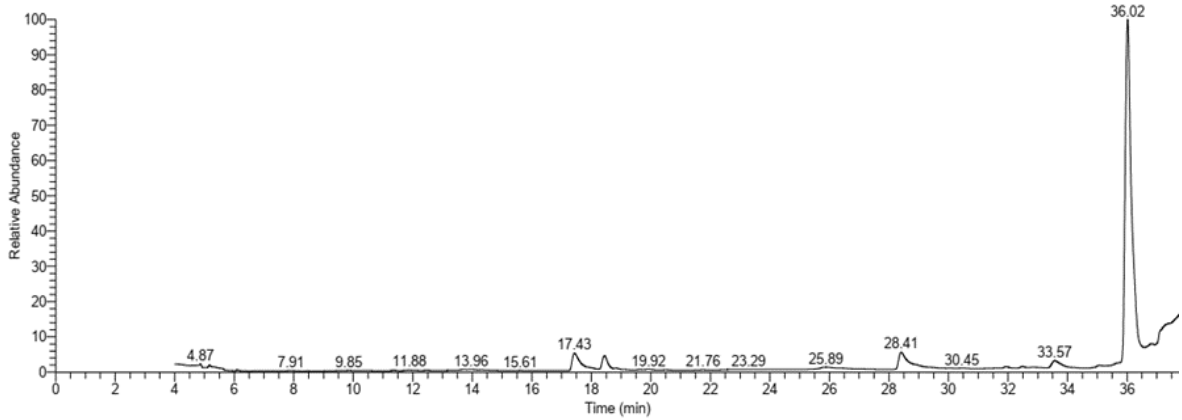
While moringa microemulsion has 7 fatty acid components which are Linoleoyl chloride (80.17%), 9-Oximino-2,7-diethoxyfluorene (0.84%),, 3-Benzodioxole, 4-methoxy-6-(2-propenyl)- (4.24%), (-)-Spathulenol (3.52%), cis-Vaccenic acid (3.97%), Glycerol 1-palmitate (2.17%), and 1,3 diolein (5.08%) as shown in **(Fig.3 b)**.



(**Figure 2**):A: TEM of moringa micro-emulsion size had 10.13 ± 0.19 nm with a narrow size distribution
B: Cell viability % of moringa micro-emulsion effect on Vero cells



(Fig. 3a): Mass Spectra Profile of GC-MS analysis of moringa oil



(Fig. 3b): Mass Spectra Profile of GC-MS analysis of moringa micro-emulsion

5. MIC of moringa oil & moringa ME

Based on MICs values Moringa oil & ME exhibited a wide spectrum of actions against both two MRSA & MSSA strain and the concentration values ranged from 6.25 to 50 ml/l against *S. aureus*. A minimum concentration of the 50 ml/ml moringa oil inhibited the growth of all cultivated MRSA strains probably. Also, it was noted that the moringa oil microemulsion demonstrated bactericidal with an MIC of 3.12 to 12.5 ml/l. However, the *mecA* gene was detected in all phenotypically cephalexin, Ampicillin, and oxacillin resistant *S.aureus* isolates as shown in table (4).

Table 4. Comparative resistance of *S. aureus* isolates to different antimicrobial agents, MRSA presence, MIC (μg/ml) of moringa oil and its microemulsion against *S. aureus* isolates

Sample no	antimicrobial susceptibility	*mec*A	MIC of moringa oil (ml/ml)	MIC of microemulsion (ml/l)
1	Cl, AMC, TE, C, SAM, AMP, E, SXT, N, Fox	+ve	50	12.5
2	CL, AMC, CRO, TE, CTX, E, N	-ve	12.5	6.25
3	CL, AMC, TE, C, SAM, AMP, E, SXT, N, Fox	+ve	50	12.5
4	Cl, AMC, TE, C, SAM, AMP, E, SXT, N	-ve	50	12.5
5	Cl, TE, C, CTX, SAM, AMP, E, SXT	-ve	50	12.5
6	Cl, CRO, E, SXT	-ve	12.5	6.25
7	Cl, TE, C, CTX, SAM, E, CTX, SXT	-ve	50	12.5
8	Cl, AMC, TE, CTX, SAM, AMP, E, CTX, SXT, N, Fox	+ve	50	12.5
9	Cl, CN, TE, CTX, E	-ve	12.5	6.25
10	TE, CTX	-ve	12.5	3.12
11	Cl, CTX	-ve	6.25	1.56
12	CTX, E	-ve	12.5	3.12
13	Cl, CTX	-ve	12.5	6.25
14	C, CTX, AMP, E, CIP	-ve	12.5	6.25
15	CTX, E	-ve	6.25	1.56
16	Cl, AMC, CRO, TE, CTX, SAM, AMP, E	-ve	50	12.5
17	CRO, C, CTX, E	-ve	12.5	3.12
18	Cl, CRO, TE, CTX, E, SXT , N	-ve	12.5	6.25
19	CRO, CTX, SXT	-ve	12.5	3.12
20	Cl, CRO, CTX, AMP, E, SXT	-ve	12.5	6.25
21	Cl, CRO, TE, C, CTX, AMP, E, CIP,	-ve	50	12.5
22	Cl, AMC,CRO, TE, C, CTX, E, SXT, N, Fox	+ve	50	12.5
23	CN, C, E	-ve	6.25	1.56
24	Cl, CN, AMC, CRO, TE, C, CTX, SAM, AMP, E, CIP, N	+ve	50	12.5
25	Cl, AMC, TE, C, CTX, SAM, AMP, E, CIP, N, Fox	+ve	50	12.5
26	Cl, CRO, CTX	-ve	12.5	3.12
27	Cl, CRO, AMP, E	-ve	50	12.5
28	Cl, CN, CRO, SAM, CIP	-ve	12.5	6.25
29	Cl, CN, TE, CTX, SAM, AMP, E, Fox	+ve	50	12.5
30	Cl, CRO, CTX	-ve	12.5	6.25
31	Cl, CN, CRO, TE, C, CTX, AMP, E, CIP, Fox	+ve	50	12.5
32	Cl, C, CTX, AMP, E, CIP, N	-ve	50	12.5
33	Cl, CN, CRO, C, CTX, SAM, AMP, E, CIP	-ve	50	12.5
34	Cl, CTX, CIP	-ve	12.5	6.25
35	Cl, CN, TE, CTX, C, AMP, E, CIP	-ve	50	12.5
36	Cl, AMC, CRO, CTX	-ve	12.5	6.25
37	CRO, CTX, N	-ve	12.5	6.25
38	Cl, CN, CRO, TE, CTX, SAM, AMP, E, CIP, N, Fox	+ve	50	12.5
39	Cl, CN, AMC, CRO, TE, C, CTX, AMP, E, N	-ve	50	12.5
40	Cl, CRO, N	-ve	12.5	6.25

AMP (Ampicillin), FOX (cefoxitin), SAM (Ampicillin/Sulbctam), AMC (Amoxicillin-Clavulinic), CTX (Cefotaxim), CRO (Ceftriaxone), CL (Cephalexin), E (Erythromycin), CN (Gentamicin), N (Neomycin), CIP (Ciprofloxacin), C (Chloramphenicol), TE (Tetracycline), SXT (Trimethoprim–Sulfamethoxazole).

6. Determination of antibacterial activity of moringa oil and moringa oil ME in commercial broiler chicken model as a treatment trial:

The five groups of positive control, negative control, Moringa oil (0.5 ml/mL water/3days), Moringa ME (0.25 mL/mL water/3days) and antibiotic groups (were treated orally by 1 ml Ciplrotril 100 mg per 2 litres of drinking water for 3 days (VAPCO) were conducted to study the antimicrobial activity of moringa oil and its ME on chicken model.

Most deaths occurred in positive group. Out of 10 examined birds 2 chicks died between 2 and 7 days post infection with total mortality rate 20%. In all chickens that died, S. aureus was counted from bone marrow, upper respiratory tract liver and lung.

No signs were detected in negative control, moringa oil, moringa oil ME and antibiotic groups (one, two, three, and five) during the experimental period. Signs were only observed in the positive control group (five) occurred only after inoculation with MRSA strain. The onset varied from 48 to 72 hours post infection. The initial signs observed were fever, depression, anorexia, reluctance to move and recumbence.

The same, gross lesions were noted in the positive control group (five), leg and breast muscles were occasionally congested. On gross examination of the visceral organs, the livers were congested and enlarged. Moreover, small white foci were observed on liver surfaces. The lungs were commonly consolidated and the air sacs were cloudy. Osteomyelitis did not develop.

Treatments of groups 2 and 3 with moringa oil and moringa ME in groups 2 and 3 were significantly increased the BW gain using one-way ANOVA test ($p<0.05$) compared to the antibiotic group (group 5) and positive control (group 5) (table 4) and reduced total S. aureus count to zero till the end of study. While in antibiotic group (Ciprofloxacin), *S. aureus* count was $8.3*10^2$ at day 18 and $1.6 *10^2$ at day 25. On the other hand, S. aureus recovery rate in the positive control was $7.2*10^9$, $1.2 *10^9$ and $3.5 *10^6$ at days 11, 18 and 25 respectively.

Table (5) Body weight of broiler chickens during the experimental treatments.

Group	Body weight gain			
	0 day	11days	18 days	25 days
Group 1 (negative)	42.9 ± 0.45	335.5± 0.85	719.7± 0.52	1266± 0.81
Group 2 (moringa oil)	43.1±0.31	319.8± 0.75	688± 1.9	1237.5± 1.19
Group 3 (moringa ME)	42.8±0.38	327.9± 0.31	704.8± 1.35	1250.25± 0.85
Group 4 (Antibiotic)	42.9±0.43	316.9± 0.62	676.8± 1.42	1228.75± 0.62
Group 5 (positive)	42.9±0.43	282.0± 0.89	659.4± 1.19	1231.12±0.74

7. Determination of enterotoxin in chicken in muscle and organs: the examined samples (6 samples/groups) not showed a positive reaction in the SET-RPLA.

DISCUSSION

The *S. aureus* infection has come to be a serious problem in poultry industry and create major animal and human problems **(Lowder *et al.* 2009).** The *S. aureus* isolation rate in the current study 22.2 % comes in accordance with previous finding of **Badr *et al.* 2012** who reported 18.8% of *S. aureus* from chicken in Egypt and **Mamaza *et al.* 2019** who recorded *S. aureus* from chicken with the percentages of 31.5% in north-eastern Nigeria. However, a higher prevalence of 48.4% was recorded by **Benrabia *et al.* (2020)** in Algeria. The isolation percentage may differ according to the isolation methodology, regional variations, and application of control programs.

Regarding antibiogram of *S. aureus,* the majority of *S. aureus* species were resistant to 3 or more than 3 kind of antibiotics, so it catego-

rized as multidrug resistance bacteria (**Bianchi *et al.* 2014**). This result comes in agreement with those of **Mamaza *et al.* 2019** in Nigeria, **Ali *et al*. 2017** in Bangladesh and **Khusnan *et al.* 2016** in Yogyakarta, who reported multiple resistances across antibiotics families to *S. aureus* isolates. The development of antibiotics resistance by *S. aureus* is related to acquisition of determinants by horizontal gene transfer of mobile genetic elements and can also develop by mutations resulting in alteration of the drug-binding sites on molecular targets and by the expression of endogenous efflux pumps **(Foster, 2017).**

MRSA is a famous pathogen with a serious effect on both human and veterinary public health **(Benrabia *et al*. 2020)**. we used cefoxitin diffusion disc as a surrogate for phenotypically detection of mecA-mediated methicillin (oxacillin) resistance **(CLSI, 2020)** and positive strains are reported as Oxacillin resistant not cefoxitin resistant (25%). Then molecular typing of *S. aureus* isolates to be confirmed as MRSA and the prevalence of MRSA was 20.0% (8/40) in this study. This result was nearly similar to previous studies: 18% in Malaysia (**Abdulkadir *et al*. 2007**), 27.5% in Algeria (**Benrabia *et al.* 2020**). A lower percentages of MRSA Detection with percentage of 10.6% has also been reported by **Persoons *et al*. (2009)** in Belgium, and 8 % **Geenen *et al*. 2013**) in The Netherlands. Also, **Abolghait et al. (2020)** recorded that prevalence of MRSA was 5.5 % in Egypt .While, a higher percentage of 92% MRSA were identified by **Ahmed *et al*.)2021)** from broiler farms in Egypt. This variation between countries could be due to antibiotic selection, application biosecurity measures and infection control practices

The association of *S. aureus* with antimicrobial resistance profiles can provide useful information for the clinical treatment of infection caused by this microorganism **(Kot *et al*. 2019)**. In current study, all MRSA isolates were resistant to cephalexin, Ampicillin, Ampicillin/Sulbctam, oxacillin and Vancomycin. This nearly similar to **Benrabia *et al*. 2020** who recorded that all MRSA isolates were resistant to cefoxitin, amoxicillin/clavulanic acid, and oxacillin from *S. aureus* isolated from broiler chicken in Algeria and **(CDC, 2013)** who reported that 100% of isolates carrying mec-A gene were phenotypically resistant to oxacillin. While a high percentage of MRSA isolates showed resistance to gentamicin and tetracycline in Italy **(Cheng *et al*. 2013)**. However, the molecular typing of MRSA isolates obtained from poultry is necessary to be confirmed as MRSA than antibiotics.

Unlike the majority of other secreted toxins produced by *S. aureus*, the SEs require only minute quantities to be toxic in humans. Additionally, the SEs have a remarkable tolerance to extreme denaturing conditions, such as low pH **(Bergdoll, 1983**), heating **(Asao *et al.,* 2003**) and proteolytic digestion **(Regenthal *et al.* 2017)**. These combined qualities make the SEs, especially SEB, potential bioterrorism agents **(Madsen, 2001)**. In our paper, no one of the examined *S. aureus* isolates exhibited the gene which responsible for production of enterotoxin A, C, D and E according to **Atanassova *et al*. (2001)** using SET-RPLA and **EOS (2005)**. While, only one of the tested *S. aureus* isolates exhibited the gene which responsible for production of enterotoxin B. This was similar to that reported by Enterotoxin (SEs A, B, C, and D) have frequently been identified in red and poultry meat products **(Balaban and Rasooly, 2000; Normanno *et al*. 2007; Nagwa and Hafez, 2018)** but unlike **Hassan *et al*. (2016)** who failed to detect enterotoxins B in meat products samples.

The characterization of moringa oil and ME is a basic stage on the way to determine the efficacy of the moringa oil and ME in the control of *S. aureus* infection. Our moringa oil ME formulation (25% oil/water) have PDI, zeta potential, and droplet size of 0.234, -24 mV ±4.19 and 29.96±1.56 nm, respectively that similar to **Lv *et al*. (2017)** who reported similar notes concerning the lower droplet size and PDI of moringa oil ME and this may be due to the type of surfactant used; tween 80 (70 nm) has a significant influence on polydispersity index and droplet size.

Quercetin and its derivatives' is a polyphenolic flavonoid that may be chemo-protective agents found in plant oil extract which inhibited *P. aeruginosa* and *S. aureus* at concentrations of 20 mcg/mL, while it inhibited *E. coli* and *P. vulgaris* at concentrations of 300 and 400 mcg/mL, respectively **(Jaisinghani, 2017**).

Long chain fatty acids, such as linoleoyl chloride, n-Hexadecanoic acid, glycerol 1 palmitic acid, and cis 13-Docosenamide acid, have been shown to inhibit gram-positive bacteria growth at low concentrations **(Dahiru *et al*. 2021)**.

The opportunities of treating of MDR MRSA infections using available antibiotics are very limited **(Kot *et al.* 2019)**. M. oleifera has antimicrobial properties against different bacterial pathogens **(Kou *et al.* 2018).** The moringa oil and ME efficacy against MRSA strains was evaluated by means of MIC. The results revealed that the MICs of moringa oil and ME were 0.5 mL/L and 0.25 mL/L in MRSA strains, respectively. These results are in harmony with those obtained in previous studies in Egypt **(Mohamed *et al.* 2020)** and in Saudi Arabia **(Amina *et al*. 2019)**.While in Greece**, Lalas *et al.* 2012** recorded the MIC of Moringa Peregrinaon seed oil against *S. aureus* isolates was 3.5 mg/mL. On the other hand, in Nigeria **Abdulrasheed *et al.* 2015** stated that Moringa oleifera seed oil did not inhibit the tested bacteria including *S. aureus*, even at 100% concentration. The variation in antimicrobial activity of Moringa oil described by different studies could be attributed to the oil extraction method they used and the differences in Moringa species.

Application of natural herbs with a view to enhancing production performance and health status has created an important demand in poultry production **(Mahfuz *et al.* 2019).** The severity of gross lesions, signs and mortalities that were detected in the challenged chickens with *S. aureus* (2.9×10^7) in the positive control group (group5) came in accordance with a previous report of **Kibenge *et al.* 1983 and Perpetua *et al.* 1999**. Our study displayed the reduction of the severity of signs lesions and mortalities in treated groups with Antibiotic, moringa oil and ME. However, the results show that birds treated with moringa oil and ME had the highest values of live body weight gain, lowest morbidity percentage with marked reduction of *S. aures* count compared to the positive control group. This results were supported by **(Mahfuz *et al.* 2019**) and **(Osama *et al*. 2020).** This results may be attributed to that Moringa oleifera seeds have a variety of nutrients (phosphorus, calcium, carbohydrates, protein, and vitamins A, C, and E) and bioactive compounds (alkaloids, phenols, flavonoids, tannins, and saponins) with both antimicrobial and antioxidant properties **(Mune *et al.* 2016).** The seed bioactive substances are reported to elicit probiotic effects, growth-stimulating which result in improved broiler performance **(Elbushra et al. 2019).**

CONCLOUSION:

Our findings showed that the prevalence of MRSA in poultry are worrying and growing day by day with the increasing trends of usage of antibiotics. Microemulsion of Moringa oleifera oil was successfully developed using generally accepted safe trial against *S. aureus* infection. This microemulsion system has shown acceptable parameters as compared to pure oil and antibiotics. The performance of chicks which supplemented with moringa ME showed a higher weekly WG in chicks. Moreover, a marked reduction of *S. aureus* count in both treated groups with moringa oil and ME compared to the positive control and group of antibiotic was recorded. Microemulsion shown promising anti- bacterial activity over study period.

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