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Comparison between antimicrobial activity of thymus and cumin extracts and their nanoparticle on Salmonella enteritidis

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ABSTRACT

In this research, extracts of cumin and thyme were evaluated and synthesized and their nanoparticles were used as an antibacterial alternative to antibiotics. the research aimed to compare the effect of aqueous and oil extracts and their nanoparticles on *Salmonella enteritidis* and some genesby determining the (minimum inhibitory concentration) (MIC) by using the resazurin micro-dilution method: MIC values showed the effect of thyme oil at 100%, thymus oil micro emulsion 20%, oil Cumin 100%, oil mixture (thyme and cumin) 20%, thyme oil 20% and cumin oil 20% at the lowest concentrations (0.09, 0.156, 100, 10, 0.625, 20) mg/ml respectively while the aqueous cumin extract 20%, cumin oil micro-emulsion 20%, aqueous cumin extract 100%, and aqueous extract of cumin nanoparticles 20% had no effect. The effect of the substances on the qacA gene was very good, while it did not affect on the invA gene. The cell membrane was also broken and the bacterial cells were destroyed by examining the bacteria under the microscope.

1. INTRODUCTION

Because of the widespread use incorrect of antibiotics. Multi drug-resistant Pathogenic microbes have emerged. resistance among intestine microbiota .bacterium resistant to antibiotics can be transmitted from infected animals to people like Salmonella spp. (Abeer et al., 2021) Essential oils (EOs) derived from therapeutic aromatic herbs might be a viable alternative to antibiotics. The antibacterial and anti-biofilm ability of (15) EOs was evaluated against Salmonella enterica serovar Enteritidis ATCC 13076 planktonic and biofilm-associated cells (S. enteritidis) (Yuliany Guillín et al., 2021) Novel antibacterial chemicals generated from natural sources are in high demand.EOs from Thymus cerebellum and Thymus vulgarisLwere shown to be effective against multidrug-resistant Salmonella bacteria collected from a range of animal samples (Valeria et al., 2020)

Li and Jiang (2004) observed that cumin seeds yielded 3.8% essential oil. While (Behera et al. 2004) showed that cumin seeds contained 5.6 ± 0.05%essential oil (Stahl-Biskup, 2004) stated that Thyme herbs' dried plant material has 1-2.5% of essential toil in it. Meanwhile. the essential oil consists of thyme ranged from (1.5%) to (5%) and it is mostly composed of phenols. (Raghavan, 2007).On the other hand, thyme (Thymus vulgarisL.) yielded approximately 0.6%of essential oil as mentioned by (Burdock, 2010). Gas chromatography-mass spectrometry (GC-MS) was used to examine phenols , flavonoids, and tannins concentrations in The essential oils and individual phenolic compounds, and the antioxidant activity of cumin. in roots, stems, leaves, and flowers

(Iness. 2010) Some harmful bacterial strains, like Salmonella enteritidis, Salmonella choleraesuis and Salmonella typhimurium, can be inhibited by thyme essential oil (Penalver et al., 2005) tested the effectiveness of eight types' of plant essential oils as natural antimicrobials against gram-negative bacteria *Salmonella enteritidis* using the agar disc diffusion method. The oils tested include oregano, mastic thyme, clove, rosemary, tea tree, coriander, laurel, and sage (Idoya. 2012)The essential oils are efficient against a variety of diseases due to the presence of various aldehydes, phenolics, terpenes, and other antibacterial components. The type, makeup, and direction of an essential oil's functional groups determine its reactivity (Mallappa. 2016).

Using plant extracts from Morus alba and Artemisia herba-alba, green gold nanoparticles (AuNPs) were created and examined for antibacterial efficacy against selected

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pathogens using polymerase chain reaction (PCR) to identify antibiotic resistance genes to Salmonella spp. (Abeer et al., 2021)

This research aims to detect the antibacterial activity of thymus and cumin extract in addition to detecting the antibacterial effect of nanoparticles made from those extracts.

2. MATERIAL AND METHODS

1-Tested microorganisms

Clinical isolates which were previously identified biochemically and serologically as *Salmonella enteritidis* were obtained from the bacteriology unit of the poultry diseases department, Animal Health Research Institute, Egypt. Salmonella enteritidiswas used as gram-negative bacteria. Picking a colony of relevant bacteria using a sterile wire loop and suspending it in a 5 cc Brain heart infusion liquid medium resulted in a standardized bacterial suspension. The dilutions were used to make bacterial stock solutions that could be used in various antibiotic experiments. For standardization of each inoculum, Using fresh broth, each active culture's optical density was adjusted to 0.1 at 625 nm, yielding a standard inoculum of (106) colony forming units (CFU) per (m1) (Nakahara and Alzoreky, 2003)

2-Preparation of aqueous extracts of thyme and cumin:

With a few adjustments, the decoction procedure described by Abdelfadel et al., (2016) was used to make the aqueous extracts

In a 250 ml flask, water extract was made by combining 20 g of each dried spice with 100 ml of sterile distilled water. The blend was vigorously stirred and left to filter for 24 hours in a temperature of 25 4 oC. To prepare hot water extracts, 20g of each dried spice was combined with (100 ml) of sterilized distilled water in a(250 ml) flask, and the mixture was boiled for 15 minutes to extract the flavour and mimic typical cooking conditions. The supernatant was centrifuged after being passed through muslin cloth (30000g, 15 min).

3- Chemicals:

Cumin (Cuminum cyminum) and thyme (Thymus vulgaris) oil extracts were obtained at National Research center (NRC), Tween (80) was obtained from the (Sigma-Aldrich Co). Double-distilled and deionized water was filtered before use .

4-Preparation, characterization and cytotoxicity assay of nanoemulsion.

The nanoemulsion was prepared in the Nanomaterials Research and Synthesis Unit by using oils (20 ml) from Cumin or thyme oils or mix (10 ml from each oil), 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) .

Transmission Electron microscopy (TEM) monitoring is carried out using [JEM (1400F) HRTEM] at ray power of (300) keV to characterize the nanoemulsion and measure electrical conductivity zeta potential (surface charge), and using(Zetasizer Malvern Instrument)(Corp, Malvern, UK) to Determination size droplet and distribution (polydispersity indexes PDI) of microemulsion (Sorour et al. 2021) .

Oils and nano-emulsion components using gas chromatography–mass spectrometry (GC-MS) at Nawah Scientific Inc. It was ascertained analysis of the mass spectrum Utilizing the database of 1GC/MS-MS in National Institute of Standard and technology (NIST) has extra than (62,000)style . in NIST library which The spectrum of the unkown components stockpiled . The components of the test materials' names, molecular weights, and structures were determined. (Bagavathi and Ramasamy, 2012)

The Vero Green Monkey cell stripe was donated by [Nawah Scientific Inc] for cell culture [Egypt , Cairo, Mokatam], Cells were grown by DMEM Dulbecco's Modified Eagle Medium media with (100 mg/ml) streptomycin (100 unit/ml) penicillin (10%). heat-inactivated fetal cow serum in wet and(5% v/v) CO2 atmosphere at (37 °C). Also, viability of cell was determined using (SRB) sulforhodamine B analyze at unlike concentrations (0.01, 0.1, 1, 10, 100) ug/ml as described on the report of (Allam et al., 2018).

5-Molecular analysis

DNA was extracted from *Salmonella enteritidis* isolates and was checked for the organization of inv A and qac A / B genes.

DNA was extracted using a QIAamp DNA mini kit (Qiagen-Germany- GmbH).and PCR amplification by Metabion (Germany) furnished the primers . by used DreamTaq Green PCR Master Mix (2X) thermoscientific Company(USA ,cat., No.K1080)and DNA samples were amplified in 50 µl. reaction volumes in a 0.2 ml. eppendorf tube, containing 25 µl PCR Master Mix which was composed of:(10X buffer, 10mM d NTPs mixture, Taq polymerase), 1µl of each primers, 2 µl target DNA, adjustment of the final volume to 50 µl with sterile deionizer water, then following thermal cycling profile as shown in Table.1

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

Primer name	Primers sequence 5`-3`	mplicon Size	Primary denaturation	Amplification (40 cycles) Secondary denaturation	Annealing	Extension	Final extension	References
Salmonella invA	*F TGTTACGGCTATTTTGACCA*	521 bp	94˚C 5 min.	93˚C 60 sec.	42˚C 60 sec.	72˚C 120 sec.	72˚C 7 min	Swamy et al., 1996
	R CTGACTGCTACCTTGCTGATG		One cycle	30 cycles	30 cycles	30 cycles	One cycles	
Quaternary ammonium compound *qac*A *qac*B	*F GCAGAAAGTGCAGAGTTCG*	361 bp	94˚C 45sec.	94˚C 30 sec.	53˚C 40 sec.	72˚C 40 sec.	72˚C 7 min	Noguchi et al. (2005)
	R CCAGTCCAATCATGCCTG		One cycle	35 cycles	35 cycles	35 cycles	One cycles	

Determination of antibacterial activity by MIC using Resazurin microtiter assay (REMA)

The REMA technique was used in accordance with Martin and Palomino's instructions. (Martin et al., 2003). Resazurin was made at 0.015 percent by dissolving 0.015 g, vortexed, and filter sterilized (0.22 um) filter before being preserved at 4°C for a maximum of 2 weeks. Each well of the 96-well plate received 100 l MHB broth. 100 µl of stock solution of each aqueous extracts and their couple combinations in ratio 1:1 was added, and subsequent two-fold dilutions were performed in columns on the plate, yielding concentrations of 100 to 0.781 mg/ml for each extract. A 100 µl of each bacterial inoculum of concentration 1-3×105 CFU/ml was added to every well at (37) degrees Celsius the plates were incubated for (24) hours . After incubation, each well received 30µl of resazurin solution, and the plates were re-incubated for 1-2 hours. When the color shifts from blue to pink, resazurin and bacterial growth are inhibited. The MIC was determined to be the lowest dose of antibiotic that change in color from colorless to blue color , which indicates bacterial growth .

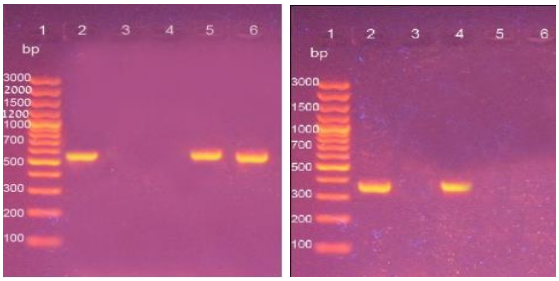
7-Transmission Electron Microscopy (TEM): done using a JEM 1400F HRTEM with a 300 k V beam energy Determine the impact of the chemical on the morphological change of bacterial cell due to reaction between nanoemulsion and salmonella entertides .

3. RESULTS

Molecular confirmation of salmonella entertidis

In this approach, PCR were used to found as invA gene and qacA/B genes detection were found in salmonella entertidis isolates .

3/2isolates samples are produced a single band on invA PCR, with molecular sizes ranging from 521 bp, while qacA/Bgene detect on 361 bp in 1/3 samples as in figure.(1)



Fig(1) A B

Fig A : Molecular diagnosis of Salmonella by PCR procedure based on invA gene sequences showing positive bands at 521 bp.
Lane 1: DNA Ladder , Lane 2: control positive ,Lane 3: control Negative ,Lane 4-6: samples

Fig B. : Results of PCR assays for identification of qac A/B gene of Salmonella.
Lane 1: DNA Ladder, Lane 2: control positive , Lane 3: control Negative ,Lane 4-6: samples

Characterization of micro-emulsions:

The results of HRTEM (Fig.1a,b,c) revealed that droplets size of Thymus oil, C**um**in oil and mix oil micro-emulsion which measured the 31.72 ±1.56 nm, 30+68 ±1.56 nm,21.8 ±1.56 nm, respectively. There no aggregation, size homogeneity and spherical nature.

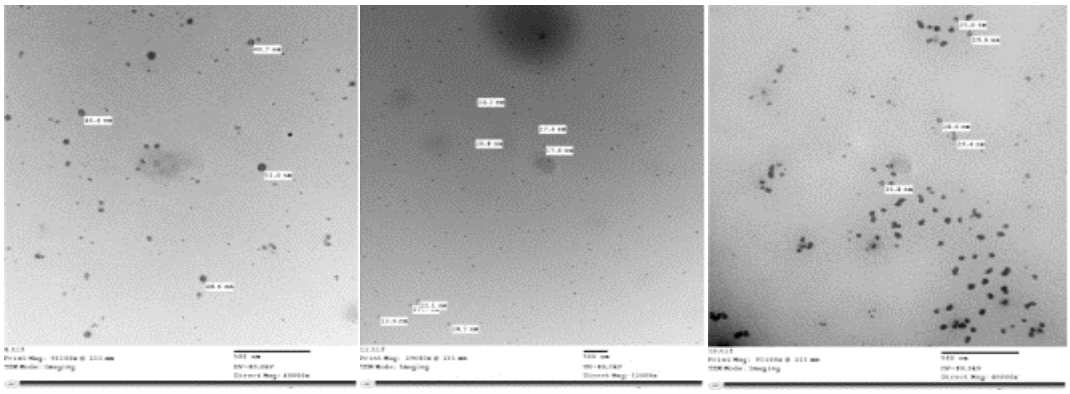


Fig1: a-Thyme b-mix c-cumun

Thymus oil, Cumin oil micro-emulsion (20% oil, in water) and mixed (10%Thymus oil + 10% Cumin oil) (Oil/water) which measured the conductivity, viscosity, polydispersity index (PDI) and zeta potentials were 0.158 ms/cm, 0.8872, 0.258 and -4.68 mV± 3.99, respectively.Cumin oil micro-emulsion was 0.171 ms/cm, 0.8872, 0.222 and -3.75 mV± 5.79, respectively. Mixed oil micro-emulsion was 0.163 ms/cm, 0.8872, 0.199 and -1.19 mV± 10.55, respectively.

The SRB test was used to measure cell survival.within 96-well plates, aliquots of a (100) µL cell comment (5x103 cells) were brood in full media for 24 hours. Another aliquot of (100) µL of medium containing different doses of medicines was used to treat the cells.After(72) hours , After replacing the media with (150µL) of (10%) TCA and incubating the cells for(one)h in (4 °C), the cells were fixed. After the TCA solution was withdrawn, distilled water was used to wash the cells five times. Aliquots of a (70) µL SRB sol (0.4% w/v) were added, and they were then brood for (10) min at dark environment at room temperature.Plates were cleaned 3 times with acetic acid at 1% before being let to air dry overnight.

Then, Protein restrictive SRB dye was dissolved 150µL in TRIS (10 mM), and the absorbance was assessed in (540) nm by using a BMG LABTECH®- FLUOstar Omega microplate reader (Ortenberg, Germany).After 72 h incubation, the results of viability % with different concentrations ranging (0.01, 0.1, 1,10, 100 ug/ml) of thymus micro-emulsions were 98.34±0.382,98.21±0.22, 96.42± 0.45, 95.80 ±0.16, 97.56±0.58.Also, cumin micro-emulsions were 98.59±0.36,98.21±0.22, 96.42 ± 0.45, 90.40± 0.903, But the mix micro-emulsions were 97.3±1.3,98.21±0.22, 96.42 ± 0.45, 81,29±2.09; Therefore IC50: >100 ug/ml.as shown in Fig.(3).

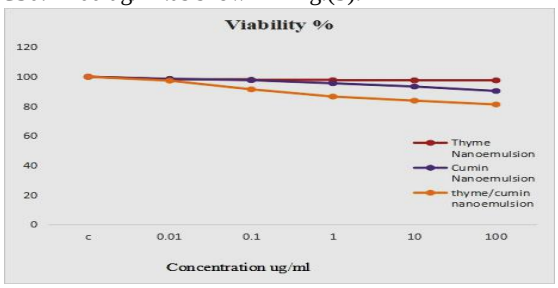


Figure (3): Cell viability% of thyme nanoemulsion , cumin nanoemulsion and (thyme+cumin) mix nanoemulsion was assessed by SRB-assay .

Chemical composition of thyme cumin and (thyme+cumin) mix nanoemulsion:

Thyme oil has 9 chemical components include are O-cymene (4.98%), Decenal (1.55%), Dodecadienal(1.33%), Phenol (24.68%),Palmitica acid(24.24%), Octade cadienoic acid (11.54%),, Trimethylsiy l (26.07%), , Glycidyl Oleate (1.62%),and Enoyloxy (1.26%). While, the 20% thyme nanoemulsion had 7 chemical components which are Octadecenoic acid (1.13%), oleic acid(1.30%), Ethanaminium (2.95%),2-Hydroxy-3-[(9E)-9-OCtadec (23.36%), Glycidyl olcate(5.63%), Enoyloxy(27.44%) and 1,2,3propanetriylester (32.94%) as shown in (Fig.4 a,b).

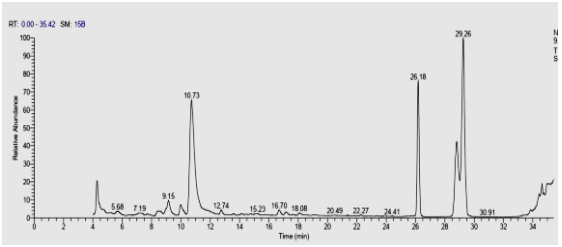
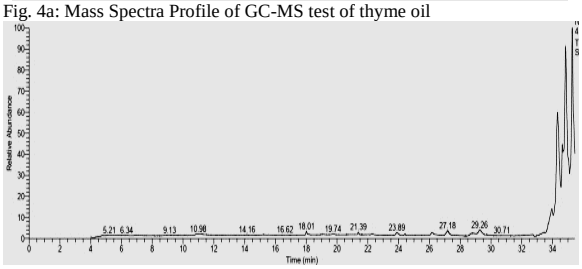
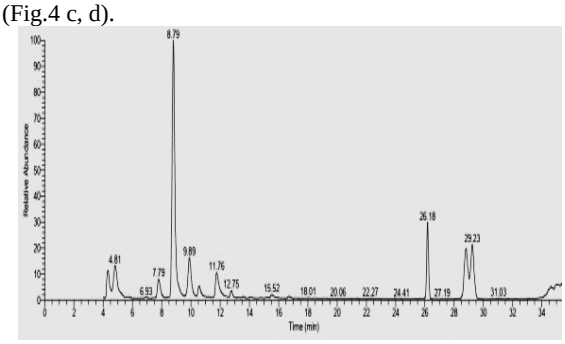


Fig. 4a: Mass Spectra Profile of GC-MS test of thyme oil

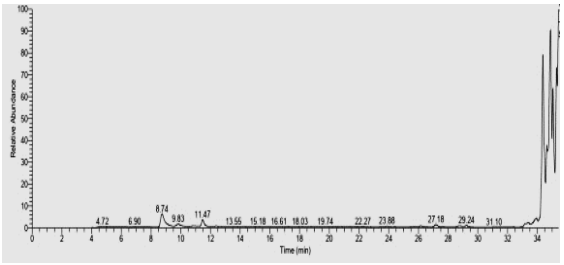


(Fig. 4b): Mass Spectra Profile of GC-MS test of thyme nano-emulsion

Cumin oil had 9 chemical components which are Propanal, 2-methyl-3-phenyl(41.87%), cymene (5.74%), C-terpene (5.7%), Estragola (3.34%), 2-Caren-10-al (6.91%), Eugenol (4.29%), Palmitic Acid (14.06%), Linolsaeure (8.31%) and Linoelaidic acid, trimethylsilyl ester (6.79%). While, the 20% cumin nanoemulsion 8 chemical components which are Anthole (anise camphor) (3.48%), pyrrolidin (1.55%), Dodecadienal(1.33%), oleyl oleate (33.19%),9-Hexadecenoic Acid (30.53%), Oleyl palmitoleate |(12.44%), Cetyl linoleate (9.74%) and 1,3-Diolein (2.3%) as shown in (Fig.4 c, d).

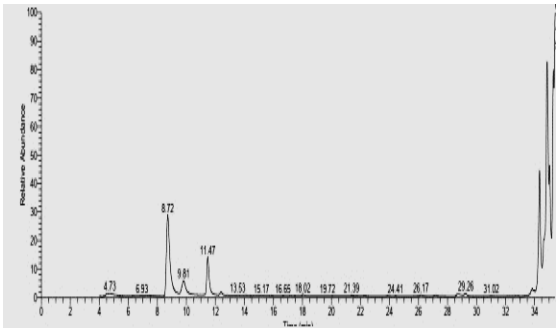


(Fig. 4c): Mass Spectra Profile of GC-MS test of cumin oil



(Fig. 4d): Mass Spectra Profile of GC-MS test of cumin nano-emulsion

While, the mix (10 % thyme +10% cumin) nanoemulsion 8 chemical components which are Anthole (anise camphor) (17.06%), Thymol (2.97%), 2,5-Dimethoxy-p-cymene (7.32%),9-Hexadecenoic Acid (19.91%), Oleyl palmitoleate |(11.87%), Behenyl palmitoleate (31.54%) and 1,3-Diolein (5.7%) as shown in (Fig.4 e).



(Fig. 4e): Mass Spectra Profile of GC-MS analysis of mix (10 % thyme +10% cumin) nano-Emulsio

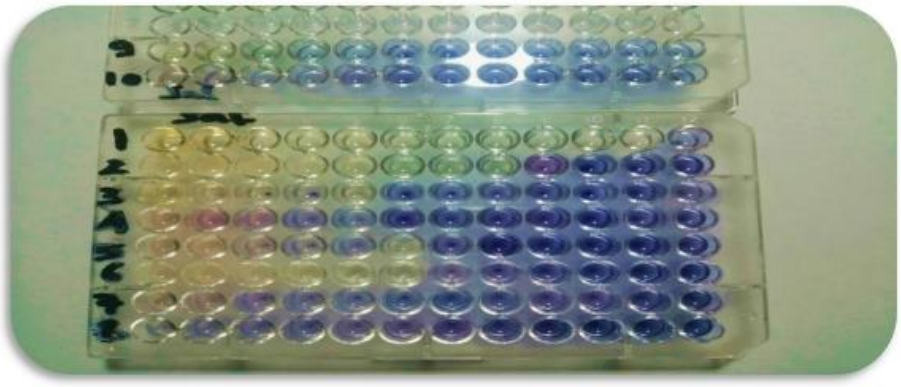
Table (2) inhibition zone of *Salmonella enteriditis* against plants extracts and micro emulsions ,MIC

NO	material	concentration	Sall .1	Sall.2	Sall.3	Concentration MIC
1	Thymus oil	100%	17 ml	18	20	0.09 µg/ml
2	Thymus oil micro emulsion	20%	13 ml	12	16	0.156 µg/ml
3	Cumin oil	100%	12	10	12	100 µg/ml
4	Cumin oil micro emulsion	20%	7	-	_	-
5	Mix thymus ,cumin oil micro emulsion	20%	10	8	-	10 µg/ml
6	Thymus oil	20%	12	12	13	0.625 µg/ml
7	Cumin oil	20%	10	11	9	20 µg/ml
8	Cumin aqueous extract	20%	7	-	7	-
9	Cumin aqueous extract	100%	8	-	-	-
10	Nano cumin aqueous extract	20%	-	-	-	-

Disc diffusion test:-

By measure the diameter of the inhibitory region of bacterial upgrowth surrounding the disc, plant extracts and nanoemulsion were tested for their antibacterial activity against the *Salmonella entertidis* by using the disc diffusion technique. and Minimum inhibitory concentration (MIC) as shown in table (2). Figure (5 ,6)

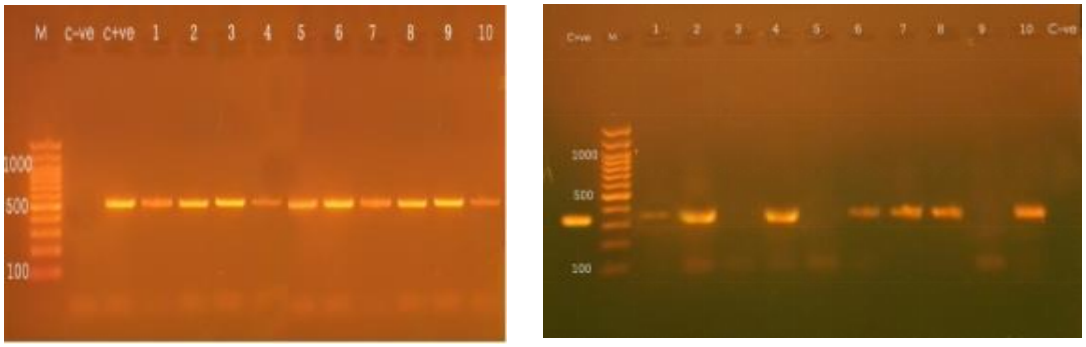
Figure(5):disc diffusion method of plants extract and nanoparticles



Fig(6):-MIC of extracts plant and nanoparticles against *Salmonella*

Results of PCR assays for identification of invA and qacA/B genes of Salmonella entertidis after different treatments:

It was shown that the substances had a greater impact on the *qac*A while they did not affect of the *inv*A gene after being administered to the bacteria (figure 7 A and B)

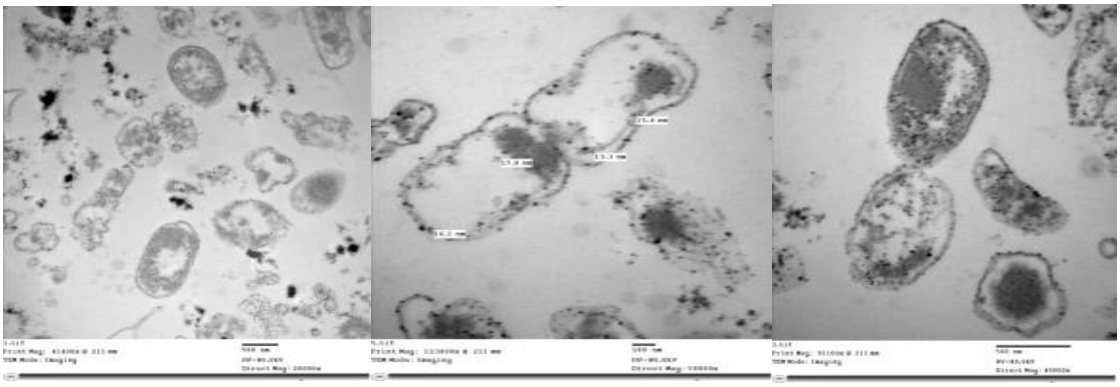


(A) **(B)**

Fig (7) A: Molecular diagnosis of *Salmonella* by PCR procedure based on *inv*A gene sequences showing positive bands at 521 bp.
Lane M: DNA Ladder ,Lane +ve: control positive , Lane -ve: control Negative , Lane 1: treatment A conc. 8MIC(thyme oil 100%) , Lane 2: treatment A conc. 9 , Lane 3: treatment B conc. 8MIC (thyme oil nano emulsion 20%) ,Lane 4: treatment B conc. 9 , Lane 5: treatment C conc. 2MIC (cumin oil 100%) , Lane 6: treatment C conc. 3 , Lane 7: treatment F conc. 6MIC(thyme oil 20%) , Lane 8: treatment F conc. 7 , Lane 9: treatment G plate 2 conc. 2MIC (cumin oil 20%) , Lane 10: treatment G plate 2 conc. 3
Fig.B: Results of PCR assays for identification of *qac*A/*B* gene of *Salmonella*.
Lane M: DNA Ladder , Lane +ve: control positive , Lane -ve: control Negative , Lane 1: treatment A conc. 8MIC, Lane 2: treatment A conc. 9 ,Lane 3: treatment B conc. 8MIC ,Lane 4: treatment B conc. 9 ,Lane 5: treatment C conc. 2MIC , Lane 6: treatment C conc. 3 , Lane 7: treatment F conc. 6MIC ,Lane 8: treatment F conc. 7 , Lane 9: treatment G plate 2 conc. 2MIC ,Lane 10: treatment G plate 2 conc. 3

Transmission electron microscope (TEM)
Morphological changes of *Salmonella enteritidis* after treatment of Thymus oil micro emulsion and Mix thymus ,cumin oil micro emulsion Shaw on fig (8 a,b,c)

Fig(8 a): control salmonella under TEM



Fig(8 b): *salmonella* after treatment of Thymus oil micro emulsion

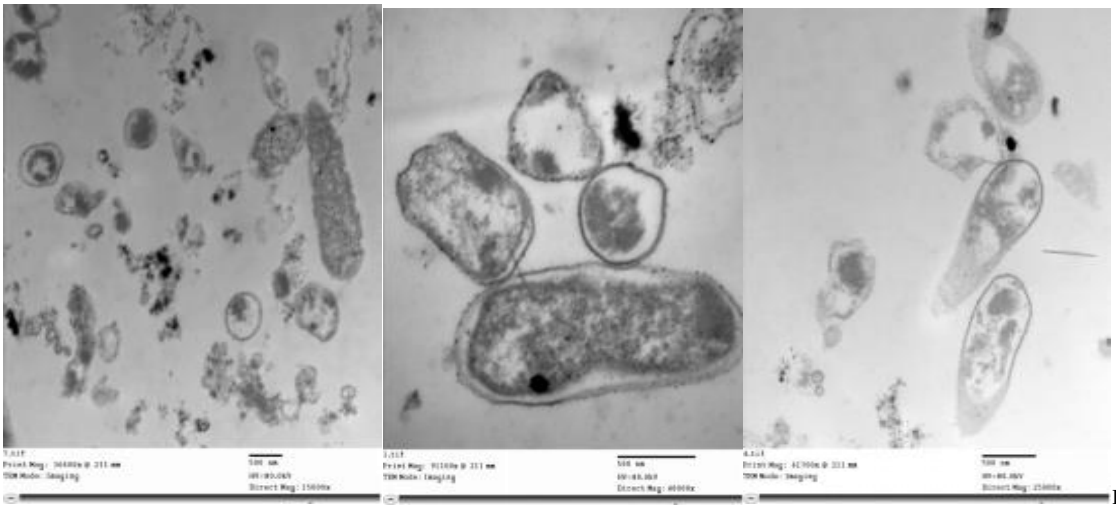


Fig (8 c):*salmonella* after treatment ofMix thymus ,cumin oil micro emulsion

4. DISCUSSION

The purpose of this search is to compare the antibacterial action of the thymus, cumin extracts, and their nanoemulsions in terms of strength and concentration to the impact of the material in the nano state and the normal state of the aqueous plant extract and oil on *Salmonella enteritidis* bacteria. The lack of effective therapies for food-borne infections, particularly for multidrug-resistant (MDR) Salmonella spp., causes treatment failure globally. they thus sought supplementary and alternative treatments for these resistant foodborne bacteria. Salmonella was present in 6 percent of the 100 samples of meat products, and it was exclusively serotyped as S. Enteritidis according to the antibiotic susceptibility assays (Mohamed et al., 2021)

The results of this study indicated that the essential oil has more antimicrobial activity than the aqueous extract and this agrees with the result of (Mohammed et, al 2021)that showed that the whole examined oils had antimicrobial effects with varying degrees against *S. aureus* and *S. Typhimurium.* Thyme oil, at 1%, demonstrated the greatest percentage of reduction on *S. aureus*, followed up by clove oil and cumin oil, however, clove oil demonstrated the greatest percentage reduction with *S.Typhimurium*. The impact of 15 Eos like Thymus vulgaris (TV) as antibacterial and anti-biofilm were assessed on (*Salmonella enterica*), *Salmonella enteritidis* ATCC 13076 and the result showed that the interactions between the compounds work in concert to provide antimicrobial activity (Yuliany et al., 2021).

It was found that the effect of the substance in the nano state is stronger than the ordinary aqueous extract and essential oil even in a lower concentration. this result agrees with (Chang et al., 2012) who found Physically stable thyme oil nanoemulsions were tested for their antimicrobial action against an acid-resistant yeast, Zygosaccharomyces bailii (ZB), and appeared that thyme oil nanoemulsions had an appreciable impact as the antimicrobial agent. and with (Sonam et al ., 2017) who shows essential oil-functionalized nanoparticles (NPs) have important antimicrobial action against multidrug-resistant pathogens because of an improvement in chemical stability and solubility, a reduction fast evaporation, and a minimization of degeneration of action of the essential oil components.

In our research, the results of the effect of substances on the *inv*A virulence gene were negative and this result is consistent with (Morshdy et al., 2022) The *inv* A gene was investigated in all 5 isolates of *S. Typhimurium* after treatment with thymus oil

This study demonstrates the influence of the nanoemulsion state on cell membrane damage and destruction. this agrees with the result of (Aljabeili, et al 2018 and Alaa Eldin et, al 2022) Biofilm control by thyme essential oil (EO) usage has increased to prevent biofilm production by set bioactive components, including thymol and carvacrol, which play essential roles like antibacterial and antioxidative agents, there were found that the result of thyme essential oil matched with Gonelimali et.al (2018). Also (Rajapandiy et, al .2018) have demonstrated the antimicrobial action of thyme, the changes in inner pH (int), and membrane possibility were measured in Staphylococcus aureus cells after exposition to the thyme extract, and their results set that the plant extract influenced the cell membrane of Gram-positive bacteria, as demonstrated by a decrease in pHint with cell membrane hyperpolarization. ,

Our result indicated that ,the damage of bacterial cell membranes was noticed after the treatment of bacteria with thyme oil nanoemulsion, , this also noted with (Yesim et al., 2020).

plant extracts are of big value as natural antimicrobials and can be used safely as food preservatives. Fourier-transform infrared (FTIR) spectroscopy revealed that nanoemulsion (NE) handling the functional groups of lipids, proteins, and nucleic acids in bacterial cells. Scanning electron microscopy (SEM) showed hurt to the cell membranes and walls of NE-treated bacteria this agreed with the result of (Yuliany et al,2021) which found The biofilm treated with the essential oil EO under Scanning Electron Microscopy showed sparse and morphologically patchy cells with medium size of 1450 ± 199 nm, to *salmonella enteritidis*

Conclusions. The plant extracts and nanoemulsions used in this study were very successful in reducing bacterial contamination and can be used as an alternative to chemical preservatives for the prevention and control of foodborne infections as well as food preservation without the health risks associated with chemicals.

5. REFRENCES

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