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MACRO AND NANO *ECBALLIUM ELATERIUM IN VITRO* ACTIVITY AGAINST MULTIDRUG-RESISTANT *SALMONELLA TYPHI* WITH GENETIC DETECTION AND SEQUENCING

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ABSTRACT

Multidrug-resistant (MDR) *Salmonella typhi* is involved in a number of illnesses such as typhoid fever. This research sought to recognize and quantify *Salmonella typhi* in clinical stool samples by serotyping, with detection and sequencing of *blaCMY-2* gene in charge of cephalosporin resistance, *DHFR* gene responsible for sulfonamide resistance and *acrB* and *rplD* genes responsible for macrolide resistance and use of *Ecballium elaterium* nanoparticles as an all-natural remedy for MDR *S. typhi* strains. *S. typhi* was discovered in 76/113 (67.25%) of the collected specimens. 41/76 samples were multidrug-resistant. However, men were likely more prone to infection than women. The findings showed that 95.12% of *S. typhi* isolates harbored the *blaCMY-2* gene, 92.68% harbored the *DHFR* gene, 48.78% harbored the *acrB* gene, and 85.36% harbored the *rpID* gene while 31.70% of them harbored the four genes. Agreement of the four genes sequences showed mutations ranging between deletion, insertion, transversion, and inversion mutations. GC-MS illustrated that the Biochanin B compound was the most popular component, with a percentage of 19.31% in *E. elaterium* essential oil. 10mg/ml of *E. elaterium* nanoparticles were found to be the minimal inhibitory concentration (MIC). *E. elaterium* nanoparticles damaged the cell wall, nuclear material, and cytoplasmic structures of MDR *S. typhi*. Moreover, they had a more significant cell viability effect on human gastric epithelial cell line (GESI) than chloramphenicol drug. The IC50 of *E. elaterium* nanoparticles was 1230.05±72.9ug/ml, and the chloramphenicol drug was 73.6924±4.05ug/ml, showing that chloramphenicol was more cytotoxic on GES1 normal cells than *E. elaterium* nanoparticles. *E. elaterium* nanoparticles have high effect against MDR *S. typhi* and more safe than using chloramphenicol drug.

Keywords: *S. typhi, E. elaterium,* Multi-drug resistant, Cytotoxicity

INTRODUCTION

Salmonella serovar Typhi (S. typhi), a part of the Family *enterobacteriaceae* among the most major Gram-negative motile bacterium that causes typhoid, is the main cause of viral gastroenteritis **(Elumalai *et al*., 2014).**There are no cutting-edge ways to avoid typhoid or enteric fever other than the use of antibiotics, making it one of the most frequent health issues globally. In places with poor sanitation and hygiene, just one enteric illness for which fatality levels has not been reduced is typhoid dropping **(Hardjo Lugito and Cucunawangsih, 2017).**
S. typhi has recently developed greater renitence to many antimicrobials, including chloramphenicol, ampicillin, sulfonamides, and tetracycline. As a result, the bacteria are now considered multidrug-renitent **(Das *et al*., 2017).** Multi-drug resistant *S. typhi* is classified as *S. typhi* insulate, renitent to three separate types of antibiotics **(Aljanaby, 2013).**
Ceftriaxone is one of the most recent antibiotics introduced for medical use; Caution should be expressed about the 2 gene of *blaCMY*, which confers *Salmonella* renitence to cephalosporins **(Mthembu *et al*., 2019).** The resistance to trimethoprim is due to interfering with folate synthesis in Gram-negative bacteria. Being competitive and dihydrofolate reductase binding (*DHFR*), which catalyzes the production of dihydrofolate to tetrahydrofolate, it exhibits bacteriostatic behavior. Although trimethoprim can also attach to *DHFRs* from eukaryotic cells, the medication has a higher affinity for bacterial enzymes **(Chiu *et al*., 2004).**
Ecballium elaterium fruits' diluted aqueous extract has long been used as an analgesic and anti-inflammatory for chronic sinusitis. It has various applications, such as treating rheumatic diseases, dropsy, hypertension, cancer, fever, and liver disorders **(Chan *et al*., 2010).** The plant has been prepared for the production of nanoparticles using the literature review as a research method **(El Moussaoui *et al*., 2019).**
In order to stop and manage MDR *S. typhi* strains, this research seeks to isolate, identify multi-drug resistant *S. typhi* isolates in clinical stool separates from Egypt, detect special genes involved in various medicines defenses additionally their sequences, and assess the impact of various *E. elaterium* nanoparticle concentrations. Moreover, the study has conducted the cytotoxicity effect of *E. elaterium* nanoparticles compared with the drug of choice on the human gastric epithelial cell line (GES1).

MATERIAL AND METHODS

Bacterial isolation & identification from collected samples

Distinct clinical stool specimens (113) were collected during June 2019 to January 2021. From various hospitals in Egypt: 84 samples from Abbassia Fever Hospital and 29 samples from Abo El-Reesh Al Mounira Hospital with research ethics approval (no. 30-2020/10) by the Egyptian National Center for Research and Health Development.
Patients' ages ranged from 18 to 45 years old. This study included male and female patients to evaluate the connection between gender, age, and the frequency of *S. typhi* infection Stool tests were obtained and handled utilizing sterilized, established procedures in a moisture container **(Inusa *et al*., 2018)**. Salmonella Shigella agar and MacConkey media (Oxoid, England) were used to culture the samples, incubated at 37°C for a day **(Amsalu *et al*., 2021)** and purified in the same medium.
First, the system of VITEK 2, diversion 9.02, from BioMerieux in the United States, verified the bacterial identity **(Henning *et al*., 2015)**. sérotyping technique (SSI Diagnostica, Denmark) was used to identify *S. typhi* **(Diep *et al*., 2019)**.

Detection of multidrug-resistant *S. typhi*

The *S. typhi* isolates sensitivity to eleven broad spectrum antibiotics from eight different classes was assessed, including penicillins (ampicillin 10µg), macrolide (azithromycin 15µg), cephalosporins (cefaclor 30µg, cefoperazone 75µg and cefepime 30µg), quinolones (ciprofloxacin 5µg and nalidixic acid 30µg), aminoglycosides (gentamycin 10µg), sulfonamides (trimethoprim/sulphamathoxazole 1.25/23.7µg), phenicols (chloramphenicol 30µg) and cyclines (doxycycline 5µg) (Oxoid, England). On Mueller- using Hinton agar plates 0.5 McFarland inoculum that had been quickly grown, antibiotic discs were first positioned. The plates were then incubated for a further 24 hours at 37°C. The width of the inhibitory zone was then weighted and contrasted with the Clinical & Institute for Laboratory Standards CLSI recommendations **(Humphries *et al*., 2021).**

Detection of *blaCMY-2, DHFR, acrB,* and *rplD* genes obtaining *S. typhi* DNA

To extract the bacterial DNA fragment, a QIAamp DNA Mini Kit (Cat. No. 51304 and 51306, QIAGEN, USA). On a 5 ml brain-heart broth medium, *S. typhi* was freshly cultured for 24 hours at 37°C **(Oates *et al*., 2012).**
The use of PCR analysis to find the *blaCMY-2* gene is in charge of cephalosporin resistance, *DHFR* gene is in charge of sulfonamide resistance and *acrB* & *rplD* genes answerable for macrolide resistance. Primer pairs were designed from patterns in publicly accessible sources like Genbank (https://www.ncbi.nlm.nih.gov) and shipped in lyophilized form Willowfort Co., Birmingham Research and Development Park, UK (Table 1).

Table 1 The sequences of the specific primers used to detect *blaCMY-2, DHFR, acrB* and *rplD* genes

Primer	Sequence (5'-3')		Tm˚C	Product size (bp)	GenBank accession no.
blaCMY-2	Forward	AAACAGTGGCAGGGTATCCG	57.4˚C	648	JN714983
	Reverse	ATGCACCCATGAGGCTTTCA	57.3˚C		
DHFR	Forward	GATGGCTGCGAAAGCGAAAA	56.7˚C	413	LT904889
	Reverse	AGTGTTGCTCAAAAACAACTTCG	54.6˚C		
acrB	Forward	CAAAGGCGATCATGGCGAAG	56.9˚C	930	NC004631
	Reverse	TATTCCCAGCGGGAAGAGGA	57.8˚C		
rplD	Forward	TCCGAAACTACCTTCGGTCG	56.6˚C	509	MF150849
	Reverse	TCGAAGGCGATCAGGCTAAC	57.0˚C		

Replication of the PCR was done in a 25µl Dream Taq Green PCR master mix (2Xconcentration), forward primer (10pmol/l) 2µl, reverse primer (10pmol/l) 2µl and 5µl of DNA extracted in a total volume of 50µl with sterile H_2O Diethyl pyrocarbonate (DEPC) treatment. The cycling circumstances for gene identification were created using the Veriti 96-well Thermal Cycler from applying Biosystems in the US **(Su *et al*., 2021).**
After that, a 1.5 per agarose gel (made by Vivantis, USA) was used to electrophorese the PCR results (Bio-Rad Laboratories, Hercules, CA, USA) at 100V for approximately 30 minutes in 1 X TBE buffer. The gels were then stained with 2µl of 10 mg/ml ethidium bromide (Sigma, USA). UVP-gel documentary system (MultiDoc-It™ system) was used to do data analysis (ww.totallab.com, Ver.1.0.1). Purified PCR results were measured spectrophotometrically at 312 nm and with micro spin filtering (SYNGENE Model 680XHR, U.K) **(Hall and Beiko, 2018).**

Sequencing of DNA gene fragments

ABI 3730xl DNA sequencer" and analysis software v3.1 were used for the in comparison to arrangement. The four genes' sequences and homology were compared to the GenBank database applying the Basic Local Alignment Search Tool (BLAST) and BLAST nucleotide (BLASTN 2.2.13) programs (GATC Company, Germany) **(Ibrahim *et al*., 2016).**

Preparation of *Ecballium elaterium* extract and nanoparticles

Mature *E. elaterium* fruits were purchased from El-sheikh Zowaid, North Sinai, then it was identified in the National Research Center, Egypt. Fresh plant samples were dried by air in a dim area at room temperature (28°C) for 7 days **(Mahmoud M. Saker, 2012)**. The dried herbs were pulverized into a powder by grinding device **(Ahmadi *et al*., 2017).** 30-40 g of dried components was added 200 ml of sterile distilled water at room temperature also the mixing was left for 24 h. The extract was evaporated till it became dry at 37°C **(Adwan *et al*., 2011).**
E. elaterium was collected, rinsed with deionized water multiple times, and room temperature dried afterward. They were processed into a powdery form by a ball milling instrument (TENCAN, Changsha Tianchuang Powder Technology Company, China). In the grinding method, 5 g of *E. elaterium* was placed in nanotubes and ground into extremely fine powders for four hours. The ball milling process's collision of the tiny stiff balls in a covert container process would generate localized high pressure **(Rabiee *et al*., 2020)**.

Characterization of synthesized nanoparticles

TEM (Transmission Electron Microscope) at 80.0 Kv of accelerating voltage was used (JEOL JEM-1400 series TEM, Japan) to study the morphological size of *E. elaterium* nanoparticles. First, 1mg of *E. elaterium* nanoparticles was suspended in 10ml of distilled water, and then 2 µl drops of nanoparticles were placed onto a parafilm and directly put on electron microscope (E.M.) grids. Finally, the filter paper was used to wick away specimen drop and placed in a Petri dish.

Antibacterial screening of *E. elaterium* extract and nanoparticles on MDR *S. typhi*

To test the antibacterial effects of various solvent extracts and nanoparticles, agar well diffusion method has been used. Filter paper discs (about 6 mm in diameter), each consisting 50 µl of the extract solution, were placed on the agar surface. Agar plates are then kept the following day at 37°C under the proper conditions, depending on the test microorganism. Measurements were made on the diameter of growth inhibitory zones **(Daoud *et al*., 2019).**
Serial dilutions of *E. elaterium* nanoparticles were prepared to the MDR *S. typhi* that was subcultured overnight on Mueller-Hinton agar to examine their impact. The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were specified by using dilutions of *E. elaterium* nanoparticles made in D.W. (1, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100µg/ml) **(Macé *et al*., 2017).**
Tubes contained 95 µl of Nutrient broth, 100 µl serial *E. elaterium* nanoparticles dilutions were inoculated with five µl of microbial inoculum. Negative control tubes contain bacterial suspension without *E. elaterium* nanoparticles, while positive control tubes only contain *E. elaterium* nanoparticle suspension. For 24 hours, every tube was incubated at 37°C. MIC and MBC values were calculated spectrophotometrically using optical density as a gauge at 600 nm on a spectrophotometer (T80 UV/VIS Spectrometer, PG instrument, United Kingdom). Experiments were conducted in triplicate **(Mutlu-Ingok *et al*., 2021).**

Chemical characterization of *E. elaterium* nanoparticles in conjunction with gas chromatography-mass spectrometry (GC–MS)

Following the steps outlined in the European Pharmacopoeia, the dried *E. elaterium* plant sample (500g) was hydro-distilled for 4 hours using a Clevenger-style apparatus. The tests of essential oils were kept at 4ºC in the darkness **(Mahdavi *et al*., 2017).** An Agilent 7000 series Triple Quad Gas Chromatograph linked up to a Mass Spectrometer (GC-MS fitted with an Elite-5MS (5% diphenyl / 95% dimethyl polysiloxane)) was applied to perform gas chromatography-mass spectrometry (GC-MS) to chemically analyze the specific oil. The National Bureau of Standards and Technology (NIST) database, which contains over than 62,000 patterns, was used for mass spectrum interpretation. The molecular weight and structure of the test material's constituent parts were determined **(Neelamegam and Ezhilan, 2012).**

Detection of the effect of *E. elaterium* nanoparticles on MDR *S. typhi* by using transmission electron microscopy (TEM) and protein profile SDS-PAGE

The effect of *E. elaterium* nanoparticles on MDR *S. typhi* were specified by TEM images (JEOL JEM-1400 series TEM, Japan) to study the morphology of bacteria as a control sample compared to *S. typhi* treatment with (*E. elaterium* nanoparticles). First, the bacterial was inoculated onto 2 ml brain-heart broth, then 1mg *E. elaterium* nanoparticles were added and incubated for 24 hrs at 37ºC. Centrifuging the mixture at 7500 rpm for 10 min., the palette was preserved in glutaraldehyde and osmium tetroxide after the excess was eliminated, and dehydration in alcohol. Next, the test had an epoxy resin coating. The thickness of the microtome sections was set between 500 and 1000 m (Leica Ultracut UCT Ultramicrotome). Toluidine blue (1X) staining was applied to small fragments and examined by Camera Leica ICC50 HD.
The effect of *E. elaterium* nanoparticles on MDR *S. typhi* was selected by protein profile (lane 2) compared to *S. typhi* as a control (lane 1) using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The bacteria were inoculated into 2 ml brain-heart broth, then 1mg *E. elaterium* nanoparticles added, then incubated for a day at 37°C. After centrifuging the mixture for 10 minutes at 7500 rpm, the supernatant was removed. SDS-PAGE was done by Biometra 010-130 minigel-twn, Germany**.**

Examination assay of cytotoxicity of *E. elaterium* nanoparticles compared with chloramphenicol drug

The American Type Culture Collection provided the human gastric epithelial cell line (GESI), and the cells were increased in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) (Grand Island, NY, USA), 10 ug/ml of insulin (Sigma), and 1% penicillin-streptomycin (Sigma). Cell plates (well plates at a density of $1x10^3$cells/well) were placed in a 96-well plate with 100 ul of the tested substance per well and 100 l of full growth media for a day **(Chen *et al*., 2018).**

Cytotoxicity was calculated using the original enzymatic reduction modification of viability assay 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to generate blue crystals named formazan **(Elshal *et al*., 2022).**
First, the culture medium was deleted from a centrifuge tube, and the cell layer was rinsed with 0.25% (w/v) Trypsin 0.53 mM EDTA solution to remove all traces of serum that contained trypsin inhibitor. Next, 2.0ml of trypsin EDTA solution was added to the flask, and cells were observed under an inverted microscope until the cell layer was dispersed. Subsequently, 6.0ml of complete growth medium was added, and cells were aspirated by pipetting gently, then centrifuged at 125 xg for 5 min. The supernatant was discarded, and the cell pellet was in a new growing media suspended; finally, For 24 hours, cultures were kept at 37°C. Following an elevated microscope inspection of the plates, the MTT assay was conducted.
Multiwell plates worked well for the MTT *in vitro* cytotoxicity measurement technique. Each vial of MTT [M-5655] to be used was reconstitute with 3 ml of medium and reconstituted MTT were added in an amount equal to 10% of the culture medium volume. Cultures were returned to the incubator for 2hrs based on the maximal cell density and the type of cell, and then The formed formazan crystals were eliminated by adding MTT solubilization solution [M-8910] in an amount equal to the volume of the original culture medium. Different concentrations of cells were incubated with *E. elaterium* nanoparticles (4.0, 16.0, 63.0, 250.0, 1000.0 ug/l) and Chloramphenicol drug (0.4, 1.6, 6.3, 25.0, 100.0 ug/l), dissolved in 10% FBS for 24 hrs. Spectrophotometrically (BioTek Instruments, Inc.Winooski, VT, USA) measured absorbance at wavelength 450 nm, and the plates were measured.

Statistical analysis

The experimental data were expressed using the mean ± standard error of the mean (SEM). Additionally, one-way ANOVA was performed to analyze the data gathered, and a significant difference was defined as one with a p-value of 0.05 or lower **(Elshal *et al*., 2022).**

RESULTS AND DISCUSSION

Salmonella typhi causes an intestinal disorder called typhoid fever. Fever, malaise, stomach pain, and constipation are common symptoms in patients **(Umair and Siddiqui, 2020).** Typhoid fever is estimated to cause between 11 and 21 million cases worldwide and 128,000 to 161,000 fatalities per year **(WHO, 2019).**

Collection data with co-relation to patient gender and age

The current study showed that seventy-six isolates out of 113 (67.25%) were identified as *S. typhi*. Males were more influenced by *S. typhi* than females with a rate of 62 and 38% respectively and the age rate was categorized as 18-25 (male 90% & female 10%), >25-35 (male 65% & female 35%), and >35-45 (male 54% & female 46%). It is obvious that males were infected by *S. typhi* more than females in each category (Fig. 1). **Anook and Farooq** conducted a similar investigation **(2017).** They found that *S. typhi* grew 57.9% in 119 clinical stool samples from Al-Samawah general hospital in Iraq from March 2017 to February 2018, and illustrated that the frequent pathogens isolates from the patients admitted to hospitals in Iraq were higher in males (55.0%) than in females (45.0%). This result was in contradiction with **Inusa *et al.* (2018),** who found that females (55.5%) had a statistically significant higher frequency of *S. typhi* than males (44.5%).

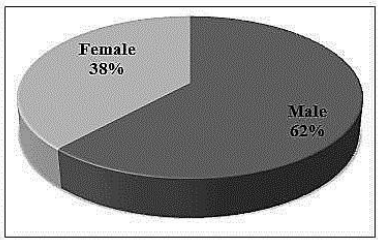

Figure 1 Percentage of *S. typhi* in clinical samples with co-relation to patient gender

Detection of MDR *S. typhi*

Despite the development of improved antibacterial medications, enteric fever remained a serious health issue **(Zaki and Karande, 2011).** In addition to gaining resistance to effective medications like ciprofloxacin, *S. typhi* also became resistant in comparison to antibiotics like co-trimoxazole, ceftriaxone, and ampicillin **(Inusa *et al*., 2018).**. In the current study, *S. typhi* isolates were evaluated for antibiotic susceptibility (Fig. 2). Fifty-four percent (41 out of 76) of them were found to be multidrug-resistant strains. *S. typhi* strains were resistant to macrolides, sulfonamides, penicillins, and cephalosporins. **Ghurnee *et al.* (2021),** found that *S. typhi* most renitent to the antibiotics ciprofloxacin (99.52%) and nalidixic acid (81.60%). *S. typhi* were noted in the research by **Klemm *et al.* (2018)** to be highly resistant to cephalosporin and fluoroquinolone.

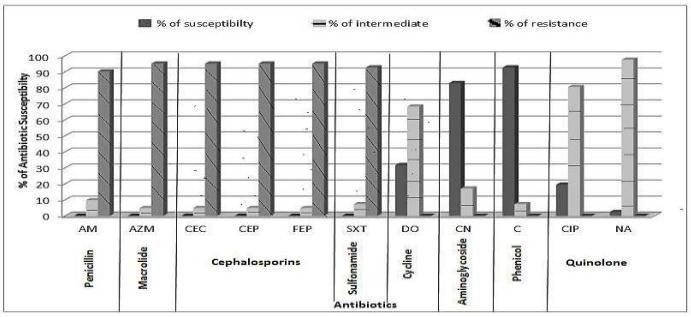

Figure 2 Percentage of susceptible, intermediate and resistant *S. typhi* isolates to different antibiotic groups (AM (ampicillin), AZM (azithromycin), CEC (cefaclor), CEP (cefoperazone), FEP (cefepime), CIP (ciprofloxacin), NA (nalidixic acid), CN (gentamycin), SXT (trimethoprim/sulphamathoxazole), C (chloramphenicol) and DO (doxycycline))

Results of genes in charge of multidrug-resistance and mutations in DNA gene fragments

In the current study, the existence of the *blaCMY-2*, *DHFR*, *acrB*, and *rplD* genes responsible for multidrug-resistance in *S. typhi* strains was determined by PCR in this work. The PCR product revealed that 39 strains out of 41 (95.12%) harbored the *blaCMY-2* gene, 38 isolates (92.68%) harbored the *DHFR* gene, 20 isolates (48.78%) harbored the *acrB* gene, and 35 isolates (85.36%) harbored the *rplD* gene, whereas thirteen isolates (31.70%) harbored the four resistance genes (Fig. 3, 4, 5 and 6).
S. typhi strains resistant to cephalosporins are common worldwide, and this is probably related to the synthesis of the CMY enzyme (s) **(Li *et al*., 2007).** *S. typhi* strains harboring *blaCMY-2* were associated with community-acquired infections **(Liebana *et al*., 2012).** Previous research showed that CMY-2-carrying plasmids may around self-transfer several strains alone but infrequently co-transfer numerous genes **(Li *et al*., 2008)**. The discovery of the plasmid-encoded *DHFR* gene in *S. typhi* provided proof that this specific *DHFR* is widely distributed. According to the study, there are many *S. typhi* strains in Solan, Himachal Pradesh, that are resistant to trimethoprim, co-trimoxazole, and sulfanilamide. India. Therefore consideration must be given to controlling and preventing the MDR strains that emerged in this region and became a severe hazard to public health **(Neha *et al*., 2016).** The overexpression of the *acrB* gene resulted in the AcrAB-TolC efflux pump turning on. The establishment of a multi-resistant phenotype in *Salmonella* was caused by the transporter protein *acrB*, which may extrude a variety of unrelated chemicals from bacterial cells **(Giraud *et al*., 2006).**
Series alignment was performed for each detected gene and compared to the World Wide Web's gene bank database, revealing the presence of several types of mutation (Fig. 3, 4, 5 and 6). Table 2 illustrated different types and numbers of mutations found in the 4 detected genes. In the case of the *blaCMY-2* gene, the deletion mutations were recorded at nine query positions, while the inversion mutations were detected at thirty-one query positions. At the same time, transversion mutations were recorded at sixty query positions. The insertion mutations were recorded at eleven query positions. Nevertheless, in the case of the *DHFR* gene, one type of mutation was substitution (inversion and transversion mutations).

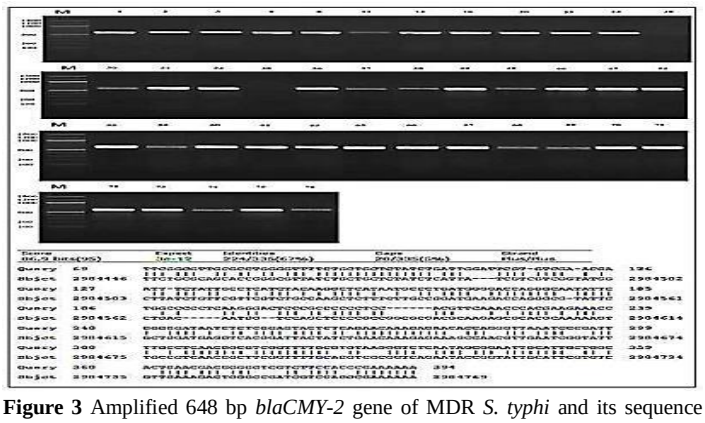

Figure 3 Amplified 648 bp *blaCMY-2* gene of MDR *S. typhi* and its sequence alignment compared to GenBank. M as Marker & 1, 2, 3, ….to 41 as no. of sample. Deletion mutations were recorded at nine query positions, while the inversion mutations were detected at thirty-one query positions. At the same time, transversion mutations were recorded at sixty query positions. The insertion mutations were recorded at eleven query positions

There were three types of mutations in the *acrB* gene were deleted at three query positions, substitution (inversion recorded at twelve query positions and transversion were recorded at thirteen query positions) and insertion mutations were recorded at one query position. One type of deletion mutation was recorded at three query positions in the *rplD* gene. It's possible that Egypt's excessive or incorrect usage of antibiotics is to blame for the abnormalities in both genes.

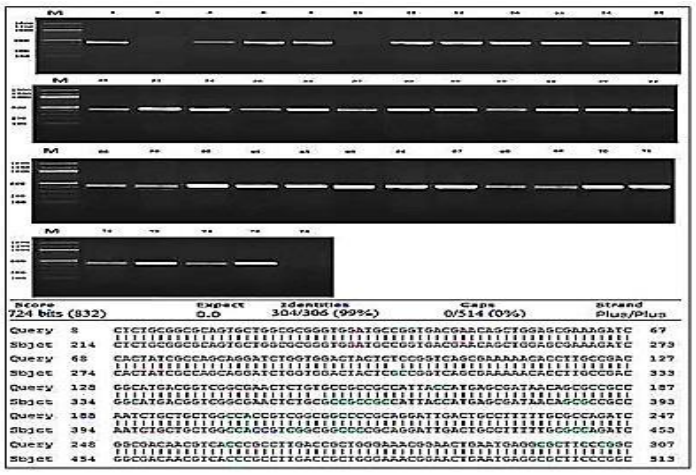

Figure 4 Amplified 413 bp *DHFR* gene of MDR *S. typhi* and its sequence alignment compared to GenBank. M as Marker & 1, 2, 3, ….to 41 as no. of sample. One type of mutation was substitution (inversion and transversion mutations)

Figure 5 Amplified 930 bp *acrB* gene of MDR *S. typhi* and its sequence alignment compared to GenBank. M as Marker & 1, 2, 3, ….to 41 as no. of sample. Deletion mutations were recorded at three query positions, substitution (inversion recorded at twelve query positions and transversion recorded at thirteen query positions) and insertion mutations recorded at one query position

Table 2 Mutation types detected in complex region of *blaCMY-2, DHFR, acrB and rplD* genes of MDR *S. typhi* compared to GenBank data base

Multi-drug resistance genes	Types of mutations*			
	Substitution (Inversion)	**Substitution (Transversion)**	**Deletion**	**Insertion**
blaCMY-2 gene	Q76: T-C; Q80: G-A; Q110: G-A Q122: A-G; Q124: C-T; Q126: A-G Q133: A-G; Q138: C-T; Q141: A-G Q145: A-G; Q149: G-A; Q168: G-A Q169: G-A; Q179: A-G; Q186: T-C Q209: C-T; Q220: T-C; Q223: A-G Q225: A-G; Q234: G-A; Q239: C-T Q247: A-G; Q280: A-G; Q284: G-A Q289: A-G; Q324: G-A; Q329: A-G Q360: A-G; Q361: C-T- Q375: T-C Q377: G-A	Q72: G-T; Q77: T-A; Q83: T-G Q86: G-C; Q90: T-A; Q106: G-C Q127: A-C; Q136: G-C; Q137: C-G Q147: A-C; Q154: A-T; Q156: A-T Q157: A-C; Q159: G-T; Q160: C-G Q163: T-G; Q187: G-T; Q189: C-A Q198: G-T; Q206: C-A; Q214: T-G Q221: T-A; Q222: C-A; Q224: C-G Q232: A-C; Q238: C-G; Q241: G-C Q242: G-T; Q249: T-G; Q250: C-G Q253: T-A; Q258: G-T; Q263: C-A Q266: A-T; Q279: C-A; Q285: G-C Q294: C-G; Q295: C-G; Q296: G-T Q301: T-G; Q302: G-C; Q304: C-G Q313: G-T; Q325: T-G; Q328: A-C Q330: G-C; Q335: T-A; Q336: C-G Q341: G-C; Q345: A-T; Q354: G-C Q355: C-G; Q358: G-T; Q366: C-A Q370: G-T; Q374: G-C; Q376: C-G Q380: T-G; Q385: C-G;Q386: C-G	Q117-118: -C Q122-123: -T Q129-130: -A Q216-217: -4G Q216-217: -2C	Q111: +G Q112: +A Q113: +T Q180: +A Q191: +C Q192: +C Q193: +C Q194: +T Q195: +C Q201: +A Q202: +C
DHFR gene	Q151: T-C	Q100: T-G	--	--
acrB gene	Q764: A-G; Q784: T-C;; Q785: A-G Q789: C-T; Q807: A-G; Q808: T-C Q828: T-A; Q833: A-T; Q836: C-T Q837: G-A; Q840: C-T; Q847: C-T	Q679: C-G; Q746: T-A; Q755: C-A Q758: T-G; Q763: A-C; Q781: A-T Q793: G-C; Q796: C-A; Q805: A-C Q806: A-C; Q815: G-C; Q843: T-G Q850: G-C	Q9-10: -G Q14-15: -T Q16-17: -A	Q39: +A
rplD gene	--	--	Q10-11: -C Q463-464: -T Q463-464: -G	--

*Q: Query position, A: Adenine, G: Guanine, T: Thymine and C: Cytosine.

Antibacterial screening of *E. elaterium* extract and nanoparticles on MDR *S. typhi*

Traditional medicine utilized *Ecballium elaterium* extensively to treat various illnesses and health conditions, such as constipation, rheumatism, Hepatoprotective and proapoptotic effects, according to **Bohlooli *et al*, (2012)** and **El Naggar *et al*., (2015).** According to **Greige-Gerges *et al.* (2007),** this plant's fruit juice is particularly high in lipids, proteins, carbohydrates, and minerals. It also contains a number of bioactive compounds, such as triterpenoids (cucurbitacins), polysaccharides, tannins, gum, and peptides **(Attard and Attard, 2008).**

Ecballium elaterium extract and nanoparticles were tested for their antibacterial activity on MDR *S. typhi* strains contained four resistance genes (*blaCMY-2, DHFR, rplD* and *acrB*) by filter paper disc method. Figure 7 illustrated that the diameter of the growth inhibition zone of *E. elaterium* nanoparticles (22mm) on MDR *S. typhi* was larger than that of *E. elaterium* extract (16mm). Characterization of *E. elaterium* nanoparticles carried out using a transmission electron microscope (TEM) showed that their size was 16.3 nm. **Adwan *et al*. (2011),** cleared that the educator of *E. elaterium* exhibited antibiotic efficacy against multidrug-resistant bacteria (15mm).

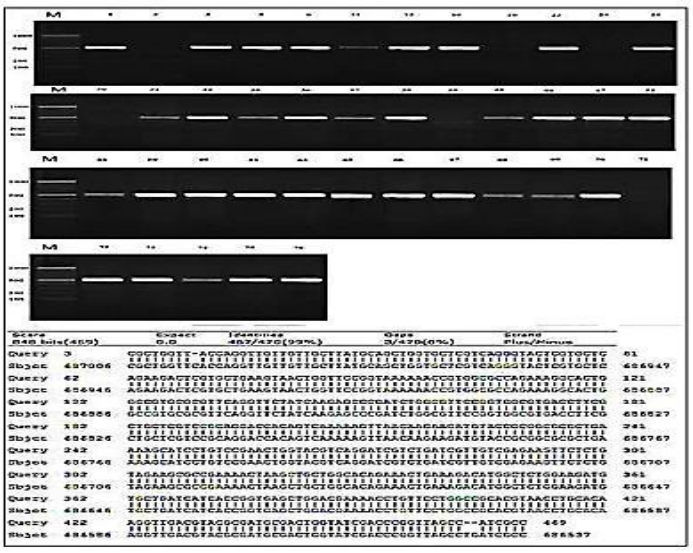

Figure 6 Amplified 509 bp *rplD* gene of MDR *S. typhi* and its sequence alignment compared to GenBank. M as Marker & 1, 2, 3, ….to 41 as no. of sample. One type of deletion mutation was recorded at three query positions

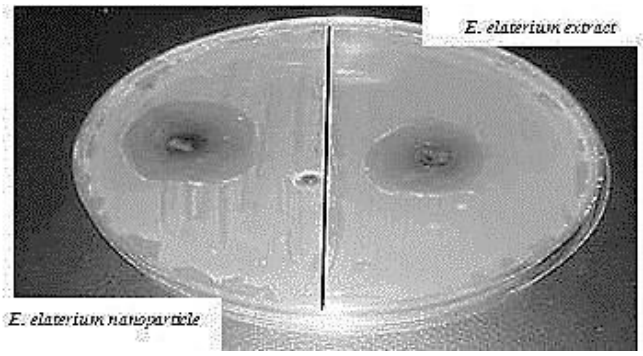

Figure 7 Bioassay of *E. elaterium* extract and nanoparticles on MDR *S. typhi* by agar well diffusion method

In the current study, for the bacterial strains carrying the *blaCMY-2, DHFR, rplD* and *acrB* genes, the minimal bactericidal concentration (MBC) was 20 µg/ml Nano *E. elaterium*. The strain with four genes has a minimum inhibitory concentration (MIC) was 10 µg/ml shown in Table 3.

Table 3 Effect of different concentrations of *E. elaterium* nanoparticles on MDR *S. typhi*

Concentration of *E. elaterium* nanoparticles (µg/ml)*	**Antibacterial Activity of *E. elaterium* nanoparticles on MDR *S. typhi* harboring four genes (optical density at 600 nm)**
1	0.899
10	0.898 (MIC)
20	0.967 (MBC)
30	1.072
40	1.076
50	1.168
60	1.218
70	1.229
80	1.354
90	1.366
100	1.378

***Positive control (treated *S. typhi*) 0.754nm and negative control for (*S. typhi* without treatment) 1.488nm

Chemical characterization of essential oil of *E. elaterium* nanoparticles

The essential oil of *E. elaterium* nanoparticles was chemically characterized by gas chromatography-mass spectrometry (GC–MS), the chemical structure and concentrations of each component of the essential oil of *E. elaterium* were determined. Figure 8 illustrated the concentrations of chemical compounds detected in *E. elaterium* essential oil. Cyclohexylmethylsilane compound with a percentage of 6.53%, 2-Cyclohexene-1-ol (7.50%), Bicyclo(3.1..1)heptan-6-one, 2-hydroxy-(1.alpha.,2.beta.,5.alpha.) (4.31%), Methyl 2-((2- benzyloxy carbonyl amino)-4-methylpentanoyl amino)-4-methylpentanoate (8.54%), Methyl-d3 2-propenyl ether (6.32%), Benzenepropanoyl bromide (2.66%), 2-ethyl-5-methylfuran (3.0%), 5- methyl furfural (3.29%), Benzaldehyde (6.76%), Hexanal dimethyl acetal (4.55%), Biochanin B (19.31%), Phenylacetaldehyde (12.16%), Cyclohexasiloxane, dodecamethyl (1.18%), 2- trimethylsiloxy-6-hexadecenoic acid, methyl ester (1.25%), 1,8-bis((trimethylsilanyl)ethynyl)- 9,10-anthraquinone (4.36%), 2,2,5,8,8-pentamethyl-5-((trimethyl-sily)oxy)-3,7-dioxa-2,8-disilanonane (0.65%), 1,2-benzenedicarboxylic acid, bis(2-methoxyethyl) ester (5.27%) and eicosapentaenoic acid, Cis-5,8,11,14,17 (0.93%).

According to GC-MS in the current investigation, Biochanin B compound (19.31%) was the most prevalent compound. This outcome was in contradiction with **Zhao *et al*., 2018**, by increasing ubiquitinated adapter proteins LRSAM1, NDP52, and p62 are expressed, making it easier to identify intracellular bacteria, biochanin B, which could induce autophagy, limited the proliferation of invading *Salmonella*. Biochanin B increased the death of intracellular *Salmonella* through autophagy in HeLa and THP-1 cells, according to a gentamicin protection experiment. The host's immune system was strengthened by Biochanin B, which also increased the quantity of *Salmonella* killed by autophagy during invasion**.**

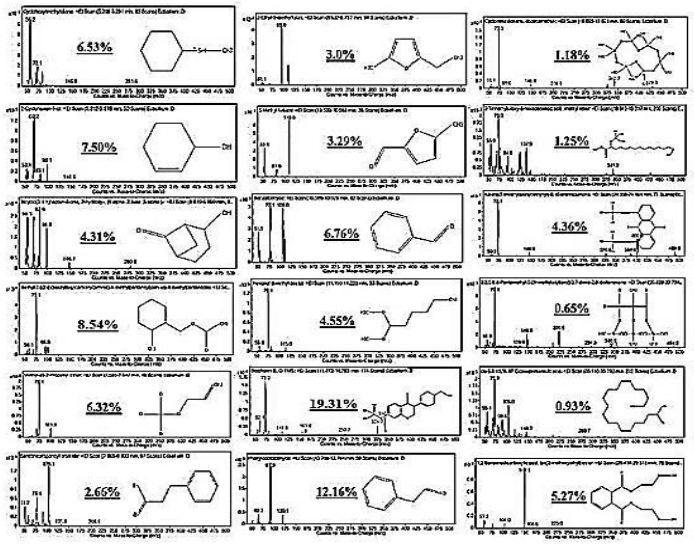

Figure 8 Diagrams showing GC-MS for chemical composition, structure, and concentrations of *E. elaterium* essential oil

The effect of *E. elaterium* nanoparticles on MDR *S. typhi* by using transmission electron microscopy (TEM) and protein profile SDS-PAGE

The effect of *E. elaterium* nanoparticles on MDR *S. typhi* strains harboring four genes was determined by TEM and Protein profile SDS-PAGE. After being treated with *E. elaterium* nanoparticles, the cell wall was damaged (the nuclear materials and cytoplasmic structures were not seen) (Fig. 9). The molecular weight of each band in the protein profile of MDR *S. typhi* (lane 1) compared to the treated bacteria (lane 2) was determined and molecular weights are indicated (Table 4).

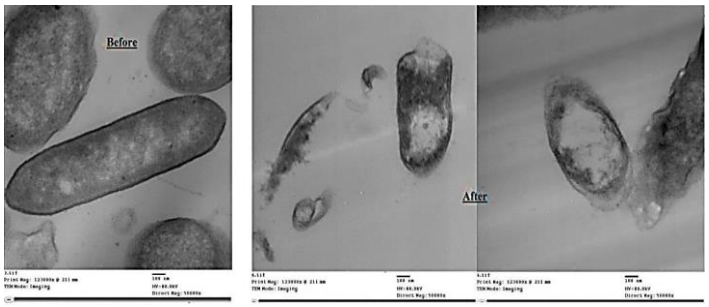

Figure 9 Images of TEM shows MDR *S. typhi* strain compared with after adding *E. elaterium* nanoparticles

Table 4 Molecular weight of each band in SDS-PAGE

Control			MDR *S. typhi* have 4 resistance genes			MDR *S. typhi* + *E. elaterium* nanoparticles		
Lane	**Band No.**	**M.W. (KDa)**	**Lane**	**Band No.**	**M.W. (KDa)**	**Lane**	**Band No.**	**M.W. (KDa)**
M	1	100	1	1	100.00	3	1	71.61
M	2	75	1	2	96.11	3	2	67.53
M	3	63	1	3	84.35	3	3	57.78
M	4	48	1	4	69.92	3	4	44.50
M	5	35	1	5	65.65	-	-	-
M	6	20	1	6	57.24	-	-	-
M	7	17	1	7	52.99	-	-	-
M	8	11	1	8	48.12	-	-	-
-	-	-	1	9	43.48	-	-	-
-	-	-	1	10	39.55	-	-	-
-	-	-	1	11	31.22	-	-	-
-	-	-	1	12	20.46	-	-	-
-	-	-	1	13	19.37	-	-	-
-	-	-	1	14	16.74	-	-	-
-	-	-	1	15	12.16	-	-	-
-	-	-	1	16	11.00	-	-	-

Results of cytotoxicity of *E. elaterium* nanoparticles compared with chloramphenicol drug

The viability of cells was detected by a reduction assay of MTT. These results showed that chloramphenicol 30µg (the drug of choice that treat MDR *S. typhi*) had a more potent inhibitory activity towards GES1 normal cells than *E. elaterium* nanoparticles.

The inhibitory concentration that is half as large (IC50)was used to measure the cytotoxicity of *E. elaterium* nanoparticles compared to chloramphenicol. The results showed that *E. elaterium* nanoparticles were 1230.05±72.9 ug/ml, and the chloramphenicol drug was 73.6924±4.05 ug/ml. Consequently, chloramphenicol was more cytotoxic on GES1 normal cells than *E. elaterium* nanoparticles (Table 5).

Table 5 Cytotoxicity to GES1 normal cells with *E. elaterium* nanoparticles and chloramphenicol drug

Sample	**Nano *E. elaterium* / GES1**					**chloramphenicol / GES1**				
Concentrations of dilutions (ug/ml)	1000	250	63	16	4	100	25	6.3	1.6	0.4
Mean value	0.2443	0.297	0.335	0.3837	0.4267	0.266	0.352	0.4407	0.466	0.5237
% Percentage	50.832	61.79	69.695	79.82	88.766	44.681	59.13	74.02	78.275	87.962
Cytotoxicity IC50	1230.05 ug/ml					73.6924 ug/ml				
S.D. (±)	72.9					4.05				

CONCLUSION

Using natural compounds is safer for humans than using chemical compounds, *E. elaterium* nanoparticles have a higher infectivity against MDR *S. typhi* that contain (*blaCMY-2*, *DHFR*, *rplD*, and *acrB*) resistance genes and have lower cytotoxicity on GES1 normal cells than chloramphenicol (the most effective drug on MDR *S. typhi*), so we are recommended to use natural products in the treatment of multidrug-resistant bacteria.

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Conflict of Interest: The authors have no financial conflicts of interest to declare.

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