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# Antibacterial Efficacy of Macro and Nano *Achillea millefolium* L. against Multidrug-resistant *Salmonella typhi* with Genotyping Analysis

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TYPHOID fever is one of many illnesses caused by *Salmonella typhi* (*S. typhi*), especially multidrug-resistant (MDR) *S. typhi*. This research aimed to isolate and quantify MDR *S. typhi* by detecting specific genes responsible for MDR *S. typhi* and the use of *Achillea millefolium* leaves (*A. millefolium* L.) essential oil, extract, and nanoparticles separately as all-natural remedies for MDR *S. typhi* strains. *S. typhi* was discovered in 52/84 (61.90%) of the collected specimens from Abbassia Fever Hospital, Egypt. 24/52 samples were multidrug-resistant. However, men were likely more susceptible to infection than women. The findings showed that 91.66% of *S. typhi* isolates harboured the *blaCMY-2* gene, 87.50% harboured the *DHFR* gene and 50.0% harboured the *acrB* gene, while 33.33% of them carried the three genes. Gas chromatography-mass spectrometry (GC–MS) exhibited that the cyclohexane,1-methylene-4-(1-methylethyl) compound was the most popular component, with a percentage of 44.5% in *A. millefolium* L. essential oil. 30µg/mL of *A. millefolium* L. nanoparticles was found to be the minimal bactericidal concentration (MBC) and the minimal inhibitory concentration (MIC) was 20µg/ml. Consequently, as detected by transmission electron microscopy (TEM), *A. millefolium L.* nanoparticles damaged the cell wall and cytoplasmic structures of MDR *S. typhi*. The IC50 of *A. millefolium* L. nanoparticles against human gastric epithelial cell line (GES1) was 552.023±32.7ug/ml, and the meropenem drug was 151.386±8.32ug/mL, showing that meropenem was more cytotoxic on GES1 normal cells than *A. millefolium* L. nanoparticles. *A. millefolium* L. nanoparticles have high effect against MDR *S. typhi* and more safe than using meropenem drug.

**Keywords:** *A. millefolium,* Cytotoxicity, Nanoparticles, *S. typhi.*

## Introduction

*Salmonella* species are primarily motile, rod-shaped, flagellated (peritrichous flagella-all around the cell body), potentially harmful to domestic or wild humans, with cell lengths ranging from 2.0 to 5.0m and diameters between 0.7 and 1.5m. *S. typhi* is primarily spread by contaminated food and water. When someone gets typhoid fever, these bacteria reside in their blood and intestinal system, making typhoid disease more common in densely populated areas. Through tainted food and drink, they enter the body, multiply, and spread throughout the bloodstream (Mahdi & Motaweq, 2023).

Multidrug-resistant (MDR) *S. typhi* is classified as *S. typhi* insulate, resistant to three separate types of antibiotics (Aljanaby, 2012). Antibiotics are a well-known tool in the fight against typhoid fever because they have significantly decreased a number of infectious illnesses death rates and morbidity (Mohd et al., 2021). However, excessive antibiotic prescription and abuse in human medicine have

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accelerated the establishment and dissemination of multidrug-resistant strains of *S. typhi* (Ramatla et al., 2021).

One of the most recent antibiotics to be used in medicine is ceftriaxone; caution should be expressed regarding *the blaCMY 2* gene, which makes *Salmonella* resistant to cephalosporins (Mthembu et al., 2019). Trimethoprim resistance results from the drug's interference with the synthesis of folate in Gram-negative bacteria. It has bacteriostatic properties due to its competitiveness and binding to the enzyme dihydrofolate reductase (DHFR), which catalyses the conversion of dihydrofolate to tetrahydrofolate. Trimethoprim has a higher affinity for bacterial enzymes, even though it can also bind to DHFRs in eukaryotic cells (Chiu et al., 2004).

Additionally, quinolone-resistant *S. typhi* is caused by point mutations in the quinolone resistance-determining region (QRDR), which is home to the DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes (Wan Makhtar et al., 2021).

Traditional medicine has a long history of using the species *Achillea millefolium* leaves as a natural treatment for injuries, bleeding, headaches, inflammation, pneumonia, rheumatoid arthritis, spasmodic diseases, flatulence, and dyspepsia. The beneficial effects of *A. millefolium* L. extracts on health have been supported by several scientific types of studies, and a number of recently published reviews have summarized these findings (Ali et al., 2017; Mohammad et al., 2017).

This study's objective was to detect and quantify *S. typhi* in clinical stool samples using the serotyping method, with the detection of *blaCMY-2*, *DHFR* and *acrB* genes responsible for MDR in *S. typhi* and the use of *A. millefolium* L. essential oil, extract and nanoparticles as an all-natural treatment for MDR *S. typhi* strains. Additionally, the study has been conducted to detect the cytotoxicity effect of *A. millefolium* L. nanoparticles compared with the drug of choice on the human gastric epithelial cell line (GES1).

## Materials and Methods

*Sample collection*

Different clinical stool isolates (84) were obtained from Abbassia Fever Hospital during June 2019 to January 2021 with study ethics approval (no. 30-2020/10) from the Egyptian National Centre for Study and Health Development.

*Bacterial isolation and identification*

The samples were cultivated using *Salmonella Shigella* agar and MacConkey media (Oxoid, England), incubated at 37°C for one day, and then purified using the same medium (Amsalu et al., 2021).

First, BioMerieux's VITEK 2, diversion 9.02, technology in the US confirmed the bacterial identity (Henning et al., 2015). *S. typhi* was identified using the sérotyping method (SSI Diagnostica, Denmark) (Diep et al., 2019); after adding 20 µl of antisera to the glass slide, culture was transferred from a number of colonies to the drop of antiserum, and everything was thoroughly mixed. Tilting the slide took ten seconds. The response can be observed by holding the slide up to a light source while it is set against a dark background. An obvious agglutination indicates a good reaction. Persistent homogenous milky turbidity is a sign of a negative reaction.

*Detection of multi-drug resistant S. typhi*

The susceptibility of the *S. typhi* isolates to eleven broad-spectrum antibiotics from eight different antibiotic groups was evaluated, penicillins (ampicillin 10µg), carbapenems (meropenem 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) and cyclines (doxycycline 5µg) (Oxoid, England). On Mueller-Hinton agar plates that had been inoculated with 0.5 McFarland *S. typhi*, antibiotic discs were first positioned. After that, the dishes were incubated for 24h at 37°C. Next, the average inhibitory zone diameter was determined and compared to the CLSI's (Clinical & Institute for Laboratory Standards) recommendations (Humphries et al., 2021).

*Detection of resistant genes using a polymerase chain reaction*

*S. typhi* was freshly cultured on a 5mL brain-heart broth medium at 37°C for one day, and the bacteria's DNA fragment was extracted using a QIAamp DNAMini Kit (Cat. No. 51304 and 51306, QIAGEN, USA) (Mahmoud et al., 2023).

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).

Replication of the PCR was done in a 25µl Dream Taq Green PCR master mix (2Xconcentration), forward primer (10 pmol/l) 2µl, reverse primer (10 pmol/l) 2µL and 5µL of DNA were extracted for a total of 50µl using sterile $H_2O$ and diethyl pyrocarbonate (DEPC). The cycling conditions for gene identification were created using the Veriti 96-well Thermal Cycler from applying Biosystems in the US (Mahdi & Motaweq, 2023), then electrophoresed on a 1.5 per agarose gel (manufactured by Vivantis, USA) at 100V for roughly 30min in 1 X TBE buffer. Next, 2µL of Ethidium bromide (10 mg/ml) was used to colour the gels (Sigma, USA). Data analysis was carried out using the MultiDoc It™ system and UVP-gel documentation system (ww.totallab.com, Ver.1.0.1). Spectrophotometric measurements of the purified PCR data were made at 312 nm and with micro spin filtering (SYNGENE Model 680XHR, U.K.) (Hall & Beiko, 2018).

*Preparation of Achillea millefolium L. essential oil, extract, and nanoparticles*

*A. millefolium* leaves were acquired from Cairo University's Faculty of Pharmacy Experimental Farm. Fresh plant samples were dried by air for 7 days at 28°C in a dark environment. The dried herbs were pulverized into a powder by grinding device (Ahmadi et al., 2017). The dried *A. millefolium* L. plant sample (400g) was hydro-distilled for 4h using Clevenger-style equipment. The essential oil testing was conducted in the dark at a temperature of 4°C (Mahdavi et al., 2017).

*A. millefolium* leaves were collected, thoroughly cleaned with deionized water, and then allowed to air dry. They were subjected to a ball grinding device to turn them into a powdery state (Rabiee et al., 2020) (TENCAN, Changsha Tianchuang Powder Technology Company, China). The grinding procedure involved placing 10g of *A. millefolium* L. in nanotubes and pulverizing it into an incredibly fine powder for four hours. Small, stiff spheres colliding in a hidden container during the ball milling process would produce localised high pressure that would eventually turn into nanoparticles.

The filter paper disk diffusion technique has been used to evaluate the antibacterial properties of different solvent extracts, essential oils, and nanoparticles separately. Filter paper discs (about 6mm in diameter) were placed on the agar surface, each containing 50µl of the extract solution, essential oil, and nanoparticles individually. Agar plates are then incubated at 37°C for 24h. The inhibitory zone diameter was assessed (Daoud et al., 2019).

*Characterization of synthesised nanoparticles*

Particle size distributions of *A. millefolium* were determined using Dynamic Light Scattering (DLS) in the range between 0 and 1000 nm using DLS, Nicomp Nano Z3000 System (Entegris Ltd, USA). 10 mg of *A. millefolium* nanoparticles were added to 5 ml distilled water, incubated for 5 min. and measured at 23˚C by DLS.

**TABLE 1. The precise primer sequences utilized to find the *blaCMY-2*, *DHFR* and *acrB* genes**

| Primer | | Sequence (5'-3') | Tm $^0$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 | | |

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 *A. millefolium* nanoparticles. First, 0.5mg of *A. millefolium* nanoparticles were suspended in 5ml of distilled water and then 2µL 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 the specimen drop and placed in a Petri dish.

*Antibacterial screening of A. millefolium nanoparticles on MDR S. typhi*

Serial dilutions of *A. millefolium* nanoparticles were prepared for the MDR *S. typhi*, which was sub-cultured over an entire night on Mueller-Hinton agar to assess their effects. The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were specified using dilutions of *A. millefolium* 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 brain heart broth, and 100 µl serial of *A. millefolium* nanoparticles dilutions were inoculated with five µl of microbial inoculum and incubated at 37°C for 24h. MIC and MBC measures were calculated spectrophotometrically using optical density as a gauge at 600 nm on a spectrophotometer (T80 UV/VIS Spectrometer, PG instrument, UK). Experiments were conducted in triplicate (Mutlu-Ingok et al., 2021).

*Chemical characterization of A. millefolium L. nanoparticles in conjunction with gas chromatography-mass spectrometry (GC–MS)*

The specified oil was subjected to GC-MS analysis using an Agilent 7000 series Triple Quad Gas Chromatograph connected to a mass spectrometer (GC-MS fitted with an Elite-5MS (5% diphenyl / 95% dimethyl polysiloxane)). The National Bureau of Standards and Technology (NIST) database, which contains more than 62,000 patterns, was used for mass spectrum interpretation. The molecular weight and structure of the test material's constituent parts were determined (Okhale et al., 2018).

*Detection of the effect of A. millefolium L. nanoparticles on MDR S. typhi using transmission electron microscopy (TEM)*

The morphology of bacteria was studied as a control sample compared to *S. typhi* treatment with (*A. millefolium* L. nanoparticles). First, the bacteria were inoculated onto 4mL brain-heart broth, then 2mg *A. millefolium* nanoparticles were added and incubated for 24h at 37°C. The mixture was centrifuged at 7500 rpm for 10min., the palette was preserved in glutaraldehyde and osmium tetroxide after the excess was eliminated, followed by dehydrated in alcohol. Next, the test had an epoxy resin coating. Toluidine blue (1X) staining was applied to small fragments and examined by a Leica ICC50 HD Camera.

*Examination assay of cytotoxicity of A. millefolium nanoparticles compared with meropenem drug*

The human gastric epithelial cell line (GESI) was obtained from the American Type Culture Collection, and the cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) (Grand Island, NY, USA), 10ug/ml insulin (Sigma), and 1% penicillin-streptomycin (Sigma). Cell plates ($1x10^3$ cells/well) were placed on a 96-well plate with 100ul of the tested chemical in each well and 100ul of full growth media for one 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).

The MTT *in vitro* cytotoxicity measurement technique performed well on multiwell plates. 10% of the growth medium was added to each vial of MTT [M-5655] after it had been reconstituted with 3 ml of media. Cultures were placed back in the incubator for 2h. Following this, formazan crystals were removed 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 *A. millefolium* nanoparticles (4.0, 16.0, 63.0, 250.0, 1000.0ug/L) and meropenem drug (0.4, 1.6, 6.3, 25.0, 100.0ug/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

In many low- and middle-income countries, typhoid fever, an acute febrile illness caused by *S. typhi*, is endemic. Worldwide, it is predicted that typhoid fever caused between 11 and 21 million illnesses and 148,000 to 161,000 fatalities (WHO, 2019).

The current study showed that fifty-two isolates out of 84 (61.90%) were identified as *S. typhi*. Males were more influenced by *S. typhi* than females with a rate of 62 and 38%, respectively.

Similar research was done by Sattar et al. (2017). They discovered that *S. typhi* increased by 59% in 118 clinical samples in Bangladesh. They also showed that male patients (62.7%) were more likely than female patients (37.3%) to have frequent pathogen isolates from their hospital admissions. The research of Inusa et al. (2018) in Nigeria indicated that females (55.5%) had a statistically significant greater frequency of *S. typhi* than males (44.5%), was in direct conflict with this outcome.

According to this study, *S. typhi* isolates were evaluated for antibiotic susceptibility. Forty six percent (24 out of 52) of them were found to be multidrug-resistant strains (Table 2). *S. typhi* strains were resistant to macrolides, sulfonamides, penicillins, and cephalosporins. Ghurnee et al. (2021) found that *S. typhi* was resistant to ciprofloxacin (99.52%) and nalidixic acid (81.60%). Farooq et al. (2019) discovered *S. typhi* to be highly resistant to fluoroquinolones (ciprofloxacin and ofloxacin) at 84 and 87%, respectively, which agree with the findings of other studies that have reported high resistance from different regions of Pakistan. *S. typhi* also became resistant in comparison to antibiotics like co-trimoxazole, ceftriaxone, and ampicillin (Inusa et al., 2018).

*S. typhi* strains resistant to cephalosporins are common worldwide, and this is probably related to the synthesis of the CMY enzyme (s). *S. typhi* strains harbouring *blaCMY-2* were associated with community-acquired infections (Liebana et al., 2012). Previous research showed that CMY-2-carrying plasmids may self-transfer several strains alone but infrequently co-transfer numerous genes (Kumarasamy & Krishnan, 2012). The discovery of the plasmid-encoded *DHFR* gene in *S. typhi* provided proof that this specific *DHFR* is widely distributed. According to another study, done in India, Himachal Pradesh, *S. typhi* was resistant to trimethoprim, co-trimoxazole, and sulfanilamide. 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 *blaCMY-2*, *DHFR*, and *acrB* genes, which are in charge of MDR *S. typhi* strains, were identified by PCR in this work. The PCR result showed that 22 out of 24 strains (91.66%) possessed the *blaCMY-2* gene, 21 isolates (87.50%) possessed the *DHFR* gene, 12 isolates (50.0%) possessed the *acrB* gene, and eight isolates (33.33%) possessed all three resistance genes (Figs. 1-3).

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 (Pérez et al., 2012).

According to Applequist & Moerman (2011), the species *A. millefolium* L. has a long-standing application in traditional Chinese medicine as an antihaemorrhagic, wound-healing agent as well as an efficient aesthetic sedative for sores, skin conditions (wounds), snakebites, and varicose veins. Additionally, *A. millefolium* L. was used as an anti-inflammatory and antibacterial natural cure for skin conditions in Italian, Sicilian, and Sardinian folk medicine (Venditti et al., 2015).

In the current study, *A. millefolium* L. essential oil, extract, and nanoparticles were tested for their antibacterial activity on MDR *S. typhi* strains containing three resistance genes (*blaCMY-2, DHFR* and *acrB*) by the filter paper disc method. Figure 4 illustrates that the mean diameter of the growth inhibition zone of *A. millefolium* L. nanoparticles (16mm) on MDR *S. typhi* was larger than that of *A. millefolium* L. extract (9mm) and *A. millefolium* L. essential oil (12mm).

**TABLE 2. percentages of MDR *S. typhi* that are susceptible (S), intermediate (I), and resistant (R)**

| Antibiotic susceptibility | Penicillin | Carbapenem | Macrolide | Cephalosporins | | | Aminoglycoside | Tetracycline | Sulfonamide | Quinolone | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | AM* | MEM* | AZM* | CEC* | CEP* | FEP* | CN* | DO* | SXT* | CIP* | NA* |
| No. of susceptible isolates | 0.0 | 23 | 0.0 | 0.0 | 0.0 | 0.0 | 19 | 9 | 0.0 | 3 | 1 |
| % of susceptibility | 0.0 | 95.83 | 0.0 | 0.0 | 0.0 | 0.0 | 79.16 | 37.50 | 0.0 | 12.50 | 4.17 |
| No. of intermediate isolates | 2 | 1 | 1 | 1 | 1 | 1 | 5 | 15 | 2 | 21 | 23 |
| % of intermediate | 8.33 | 4.17 | 4.17 | 4.17 | 4.17 | 4.17 | 20.84 | 62.50 | 8.33 | 87.50 | 95.83 |
| No. of resistant isolates | 22 | 0.0 | 23 | 23 | 23 | 23 | 0.0 | 0.0 | 22 | 0.0 | 0.0 |
| % of resistance | 91.67 | 0.0 | 95.83 | 95.83 | 95.83 | 95.83 | 0.0 | 0.0 | 91.67 | 0.0 | 0.0 |

*AM (ampicillin), *MEM (meropenem), *AZM (azithromycin), *CEC (cefaclor), *CEP (cefoperazone), *FEP (cefepime), *CN (gentamycin), *DO (doxycycline), *SXT (trimethoprim/sulphamathoxazole), *CIP (ciprofloxacin), *NA (nalidixic acid).

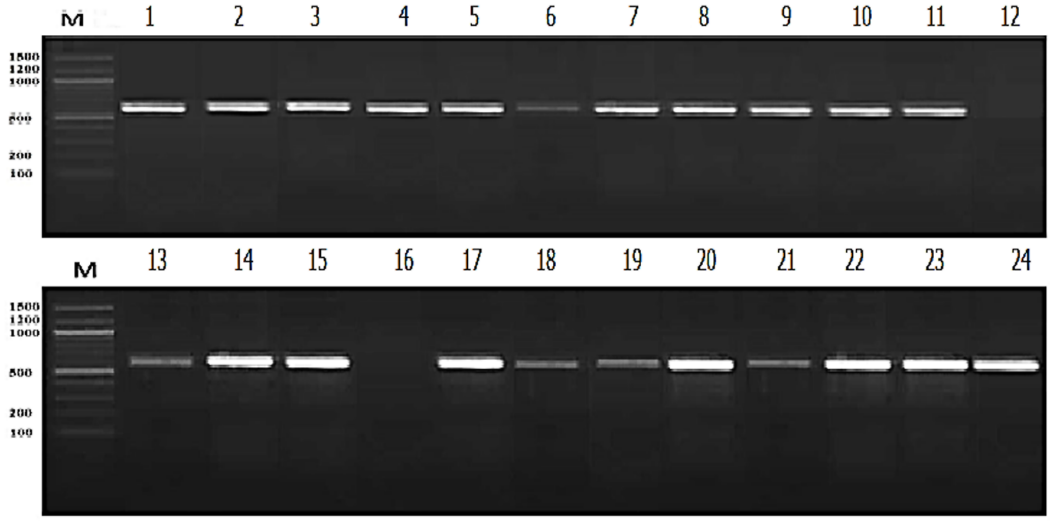


**Fig. 1. Amplified 648 bp blaCMY-2 gene of MDR *S. typhi.* M as Marker & 1, 2, 3, ….to 24 as no. of sample**

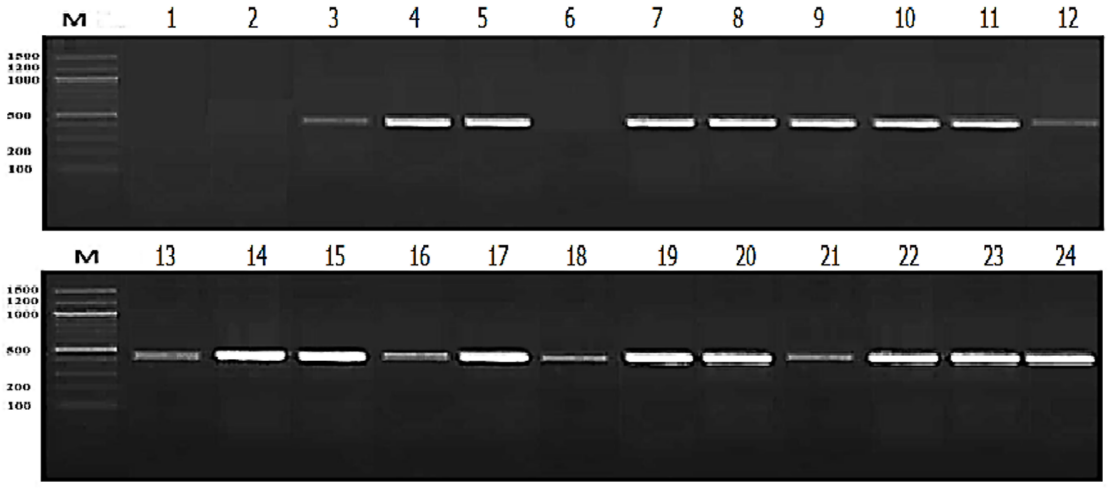


**Fig. 2. Amplified 413 bp DHFR gene of MDR *S. typhi.* M as Marker & 1, 2, 3, ….to 24 as no. of sample**

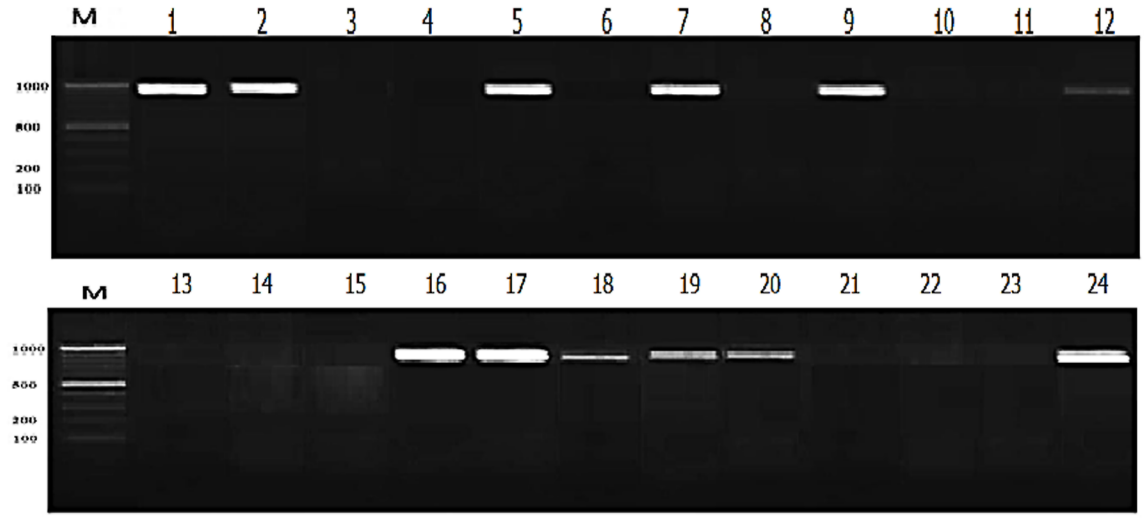


**Fig. 3. Amplified 930 bp acrB gene of MDR *S. typhi.* M as Marker & 1, 2, 3, ….to 24 as no. of sample**

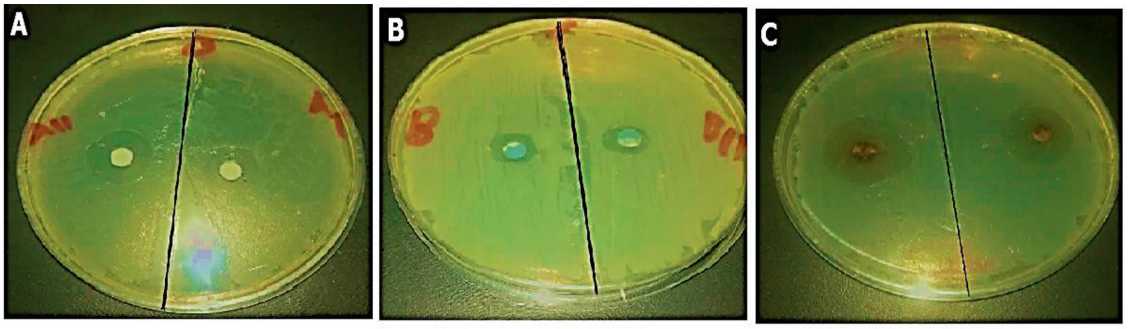


**Fig. 4. Bioassay of *A. millefolium* L. essential oil (A), extract (B), and nanoparticles (C) on MDR *S. typhi* by agar disc diffusion method**

*A. millefolium* L. extract had a weak inhibition zone of antibacterial activity against *Y. enterocolitica* and *S. salivarius* (10-11mm) and a strong activity inhibition zone against *S. aureus* (21mm), according to Grigore et al. (2020) investigation. *A. millefolium* L. was found to have antioxidant and antimicrobial properties *in vitro* against *K. pneumoniae, E. coli, S. enteritidisand* and *P. aeruginosa* confirmed by Frański & Beszterda-Buszczak (2023).

In a closely related study by Kain & Kumar (2020), they discovered that both Gram-positive *B. subtilis* and Gram-negative *P. aeruginosa* were exceptionally resistant to the antibacterial effects of *A. millefolium* L. when compared to *A. millefolium* extract alone, *A. millefolium* L. nanoparticles displayed three times the antimicrobial activity against *B. subtilis* and *P. aeruginosa*, increasing from 10 to 30mm.

Different methods, such as DLS and TEM, are employed in the literature for the analysis of nanoparticles (Arya et al., 2019; OH et al., 2019). In our study, characterization of *A. millefolium* L. nanoparticles carried out using a DLS that showed their size was 12.28nm and using a TEM showed that their size was 2.9nm, which is nearly similar to an investigation was done by Kain and Kumar, who discovered that DLS showed the *A. millefolium* L. nanoparticle size range, the highest intensity was observed at size 10nm. The morphology of the nanoparticles was studied using TEM, which showed that they had a smooth, spherical structure. The sizes of *A. millefolium* L. nanoparticles were also disclosed by TEM, with the smallest size being 4.15nm (Kain & Kumar, 2020).

In the current study, the MBC of nano *A. millefolium* L. was 30µg/mL and the MIC was 20µg/mL for MDR *S. typhi* that harboured the *blaCMY-2*, *DHFR* and *acrB* genes (Table 3).

**TABLE 3. Effect of different concentrations of *A. millefolium L.* nanoparticles on MDR *S. typhi***

| Concentration of *A. millefolium* nanoparticles (µg/mL)* | Antibacterial activity of *A. millefolium* nanoparticles on MDR *S. typhi* harboring three genes (optical density at 600nm) |
|---|---|
| 1 | 0.789 |
| 10 | 0.750 |
| 20 | (MIC) 0.720 |
| 30 | (MBC) 0.890 |
| 40 | 1.051 |
| 50 | 1.094 |
| 60 | 1.098 |
| 70 | 1.131 |
| 80 | 1.155 |
| 90 | 1.177 |
| 100 | 1.198 |

*Positive control (treated *S. typhi*) 0.544nm and negative control for (*S. typhi* without treatment) 1.488nm

GC-MS was used to chemically characterize the essential oil of *A. millefolium* L. nanoparticles, and the chemical structure and quantities of each component were discovered (Fig. 5).

In this study, Cyclohexane,1-methylene-4-(1-methylethyl) compound (44.5%), chamazulene (10.64%) and eucalyptol (8.2%), were the most prevalent compound. Limonene is a derivative of cyclohexane, 1-methylene-4-(1-methylethyl), which has been shown to be an efficient antibacterial agent (AlSaffar et al., 2022).

Eucalyptol is a well-known chemical compound with strong antibacterial potentials, which may be one of the reasons for the evaluated essential oil's low antimicrobial activity, according to Maczka et al. (2021). One of the most significant chemicals for antibacterial activity is eucalyptol (Winska & Mączka, 2020). The sesquiterpene chamazulene is responsible for giving essential oils their bluish hue. This substance comes from the terpenic lactone matricin, which is transformed into chamazulene during the hydrodistillation procedure. According to Singh et al. (2011), it contains antibacterial, antifungal, anti-inflammatory, and antioxidant effects. Ali et al. (2017) and Mohammad et al. (2017) found that most species of *Achillea* have moderate to strong antibacterial activity due to the chemical makeup of their essential oils and extracts as well as their biological activities. However, the three investigations listed above found that the yarrow essential oil had a mild to moderate antibacterial activity profile.

In this research, the effect of *A. millefolium* nanoparticles on MDR *S. typhi* strains harboring three genes was determined by TEM. Cell wall was damaged and cytoplasmic structures were not seen (Fig. 6).

The viability of cells was detected by a reduction assay of MTT. These results showed that meropenem 10µg (the drug of choice to treat MDR *S. typhi*) had a more potent inhibitory activity towards GES1 normal cells than *A. millefolium* L. nanoparticles.

The inhibitory concentration that is half as large ($IC_{50}$) was used to measure the cytotoxicity of *A. millefolium* L. nanoparticles compared to meropenem showing (552.023±32.7ug/mL, 151.386±8.32ug/mL) respectively. This indicates that meropenem was more cytotoxic on GES1 normal cells than *A. millefolium* L. nanoparticles (Table 4).

## Conclusion

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

*Competing interests:* There are no stated conflicts of interest by th authors.

*Authors' contributions:* The authors confirm contribution to the paper as follow: Hamdy A. Elkhateeb: conceptualization, methodology, validation, formal analysis, investigation, writing. Mona I. Mabrouk: methodology, review & editing, supervision, interpretation of results. Amal S. Othman: conceptualization, investigation, review & editing, draft manuscript preparation, supervision, interpretation of results. M.K. Khaled: conceptualization, methodology, investigation, draft manuscript preparation, supervision, project administration. All authors reviewed the results, helped in editing the manuscript and approved the final version of the manuscript.

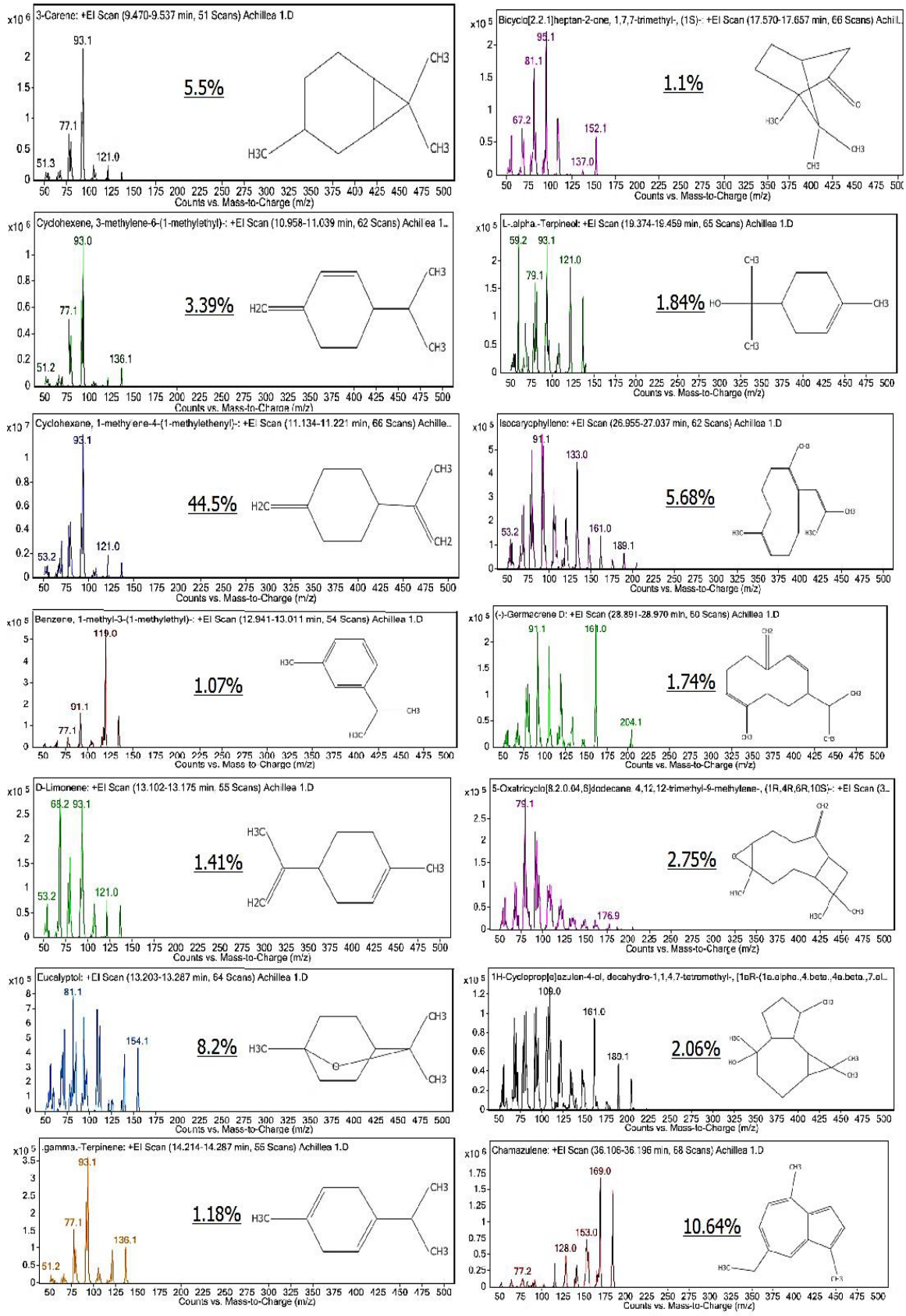


**Fig. 5. Diagrams showing GC-MS for structure, and concentrations of A. millefolium L. nanoparticles essential oil**

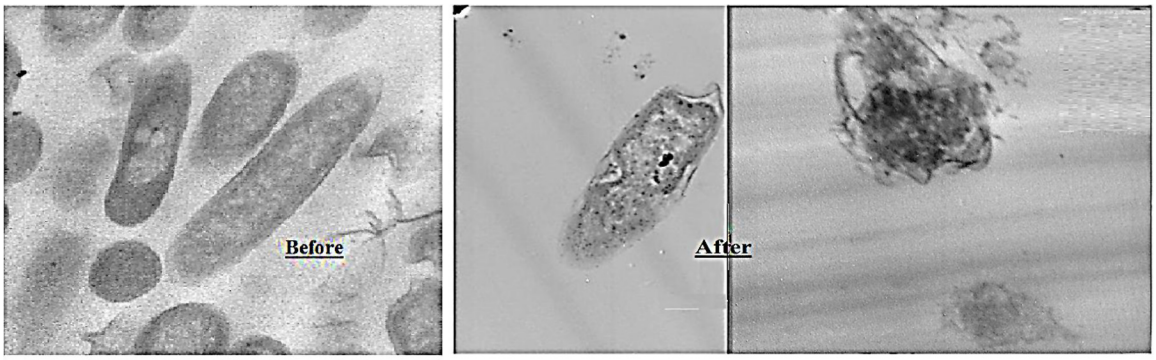


**Fig. 6. Images of TEM showing MDR *S. typhi* strain before and after treatment with *A. millefolium* L. nanoparticles**

**TABLE 4. Cytotoxicity test of *A. millefolium L.* nanoparticles and meropenem drug on GES1 normal cells**

| Sample | Nano *A. millefolium* L. / GES1 | | | | | Meropenem / GES1 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentrations of dilutions (ug/mL) | 1000 | 250 | 63 | 16 | 4 | 100 | 25 | 6.3 | 1.6 | 0.4 |
| Mean value | 0.21833 | 0.27267 | 0.31267 | 0.35867 | 0.4133 | 0.29467 | 0.35967 | 0.41533 | 0.44767 | 0.466 |
| % Percentage | 45.423 | 56.7268 | 65.0485 | 74.6186 | 85.992 | 49.4961 | 60.4143 | 69.7648 | 75.196 | 78.275 |
| Cytotoxicity IC50 | 552.023ug/mL | | | | | 151.386ug/mL | | | | |
| S.D. (±) | 32.7 | | | | | 8.32 | | | | |

*Ethics approval:* The scientific research ethics committee has approved the current research study with a code 30-2020/10 from the Egyptian National Centre for Study and Health Development.

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## دراسة تاثير نبات ايخيليا ميليفوليوم كمضاد لبكتريا السالمونيلا تايفى المتعددة المقاومة للمضادات الحيوية مع التحليل الجزيئى للجينات

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تعد حمى التيفوئيد هي واحدة من العديد من الأمراض التي تسببها السالمونيلا التيفية، وخاصة السالمونيلا التيفية المتعددة المقاومة للمضادات الحيوية. هذه الدراسة تهدف إلى اكتشاف وعزل السالمونيلا التيفية المتعددة المقاومة للمضادات الحيوية واكتشاف الجينات المسئولة عنها واستخدام الزيت العطري والمستخلص وجزيئات النانو لنبات ايخيليا ميلفوليوم كعلاج طبيعى لسالمونيلا التيفية المتعددة المقاومة للمضادات الحيوية. تم اكتشاف السالمونيلا التيفية 52/84 (61.90٪) من العينات التي تم جمعها من مستشفى حميات العباسية، مصر. 24/52 عينة اظهرت مقاومة العديد من للمضادات الحيوية. ومع ذلك، الرجال أكثر عرضة للإصابة من النساء. النتائج تشير إلى 91.66٪ من السالمونيلا التيفية تحتوي على جين blaCMY-2 و 87.50٪ تحتوي على جين DHFR و 50.0٪ تحتوي على جين acrB، و33.33٪ منها تحتوي على الجينات الثلاثة. أظهر GC-MS أن مركب (cyclohexane,1-methylene-4-1-methylethyl) هو الأكثر شيوعًا، بنسبة 44.5٪ في زيت ايخيليا ميلفوليوم. 30 ميكروجرام/ مل من جزيئات النانو ايخيليا ميلفوليوم هو اقل تركيز مبيد للبكتريا بينما 20 ميكروجرام/ مل هو اقل تركيز مثبط للبكتريا. كما اكتشف بواسطه TEM بان تاثير جزيئات النانو ايخيليا ميلفوليوم ياتى بواسطة تدمير جدار الخلية والهياكل السيتوبلازمية لسالمونيلا التيفية المتعددة المقاومة للمضادات الحيوية. ال IC50 لجزيئات النانو ايخيليا ميلفوليوم 552.023 ± 32.7 ميكروجرام/ مل، و لدواء ميروبينيم هو 151.386 ± 8.32 ميكروجرام/ مل، وبذلك فان ميروبينيم اكثر سمية على الخلايا المعوية للانسان GES1 عن جزيئات النانو ايخيليا ميلفوليوم. جزيئات النانو ايخيليا ميلفوليوم لها تاثير عالى ضد سالمونيلا التيفية المتعددة المقاومة للمضادات الحيوية واكثر امانا من استخدام دواء الاميروبينيم.