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Research article

Increasing antimicrobial activity of some plant extracts against antibiotic resistant *Staphylococcus aureus* by using silver nanoparticles

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ABSTRACT

This study was carried out to determine bacteriostatic property of green synthesized silver nanoparticles using *Thymus vulgaris* L., *Zingiber officinale* Roscoe and *Cinnamomum zeylanicum* phenolic plant extracts as reducing agent against multiple drug resistant (MDR) of six isolates of *Staphylococcus aureus* isolated from blood and wound. Several experiments were conducted to study the antibacterial effect of phenolic extracted from plants and combination of phenolic with silver nanoparticles by determining the minimum inhibitory concentrations (MICs). Also, the study included identification of active compounds present in the phenolic extracts by fast liquid chromatography (FLC). The study focused on the characterization and application of silver nanoparticles [Ag-NPs] which indicated a size range of 101.77 nm. The Complete Randomized Design [CRD]was used as experimental design. Data were analyzed by using statistical analysis system- SAS to study the effect of different phenolic plant extracts and the nanoparticles on some bacterial isolates. All plant extracts were showed high activity against all *S. aureus* isolates. Besides, the silver nanoparticles have potent antibacterial activities against all *S. aureus* isolates. The FLC results showed that *T. vulgaris* phenolic extract was the most effective against *S. aureus* isolates than the other tested extracts because of total concentration of the identified phenols was the highest in *T. vulgaris* extract (968.95 µg/ml). The data indicated that the lowest effective concentration for *T. vulgaris* and nanoparticles were 6.25 and 3.125%. Analyses of the interaction data between *T. vulgaris* extract with silver nanoparticles showed the lowest effective concentration were 12.5, 3.125%, respectively. But in case of *C. zeylanicum* and *Z. officinale* were 100, 6.25% and 50, 6.25%, respectively. Finally, using plant extracts with silver nanoparticles as new types of bacteriostatic agent led to decrease concentration for both of them, which reduce their side effects on other living organisms.

Keywords: Multiple drug resistant, phenolic plant extracts, Silver nanoparticles, Staphylococcus aureus,.

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INTRODUCTION

Herbal medicine is a traditional or folk medicine practice based on the use of plants'seeds, berries, roots, leaves, bark, flowers and plant extracts for medicinal Purposes [1]. Prior to the development of modern medicine, traditional medicine systems that have evolved over the centuries among various communities, were still maintained as a great traditional knowledge basis in herbal medicines [2]. According to the World Health Organization reports, as many as 80% of the world's people depend on traditional medicine for their primary health care needs [3]. Some of these plants that have been used medicinally for a long time ago *Zingiber officinale* Roscoe*, Thymus vulgaris* L. and *Cinnamomum zeylanicum. Z. officinale* [Zingiberacea]was used worldwide for different purposes such as a cooking spice, condiment and herbal remedy. The Chinese have used ginger for at least 2500 years as a digestive aid, antinausea remedy, treat bleeding disorders and rheumatism; it was also used to treat baldness, toothache, snakebite, and respiratory conditions [4]. *T. vulgaris* [Lamiaceae]was also one of the plants that have many uses. It adds a distinctive aromatic flavoring to sauces, stews, stuffing, meats, and poultry; it possesses antispasmodic, antiseptic, expectorant, carminative and anti-oxidative properties [5, 6]. *C. zeylanicum* [Lauraceae] being also native to South-East India, was a source of cinnamon bark and leaf and their essential oils. Its sensorial qualities are flavor, slightly sweet, pleasant, warm and bitter, besides being strongly aromatic [7]. Nanoscience can be defined as the study of phenomena and manipulation of materials at atomic, molecular and macromolecular scales where properties differ significantly from those at a larger particulate scale. Nanotechnology is then the design,
characterization, production and application of structures, devices and systems by controlling the shape and size at the nanometer scale [8]. It was anticipated that nanotechnology can have an enormous positive impact on human health. The potential medical applications are predominantly in diagnostics [disease diagnosis and imaging], monitoring, the availability of more durable and better prosthetics, and new drug delivery systems for potentially harmful drugs [9]. The researchers found that silver nanoparticles had a toxic effect on cells, suppressing cellular growth and multiplication and causing cell death depending on concentrations and duration of exposure. The ability of nanoparticles to enter cells and affect their biochemical function makes them important tools at the molecular level. The toxic properties of nanoparticles can in some instances be harnessed to improve human health through targeting cancer cells or harmful bacteria and viruses. Antibiotic therapy in recent years has faced difficulties due to the rapid emergence of multidrug resistance among bacteria causing several life threatening infections, and this in turn, making the future management of infectious diseases uncertain. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for potential antimicrobial activity, and the plant extracts were found to have potential against microorganisms [10,11]. From these microbes resistant to antibiotics, Methicillin-resistant *Staphylococcus aureus* (MRSA) was a major causative agent of nosocomial infections [12]. *S. aureus* is a very common bacterium that lives on our skin and generally causes no trouble. But, if it gets into the blood stream, through a cut or surgery, *S. aureus* can very quickly damage the heart, lungs, brain or poison the entire system [13] and it is an important cause of food poisoning [14]. With this in mind, several experiments were conducted to evaluate and compare the antibacterial activity of phenolic plant extracts against *S. aureus* isolate from blood and wound and studying the effect of silver nanoparticles on the antibacterial activity of *S. aureus.* The antibacterial effect of plants phenolic extracts alone and in combination with Ag-NPs nanoparticles in vitro was evaluated. Finally, identification of some active compounds present in the extracted plants by Fast Liquid Chromatography [FLC].

MATERIALS AND METHODS

Collection of plant samples

Plant samples included *Zingiber officinale* dry rhizomes dry leaves of *Thymus vulgaris* and dry bark of *Cinnamomum zeylanicum* were obtained from local herbarium market in Baghdad city. After the plants were air derided and powdered, it kept at 4°C until further investigations.

Preparation of Phenolic Plant Extracts

Phenols were extracted according to previous studies [15, 16]. 200 gm of plant powder were divided into 2 equal portions, one was mixed with 300 ml of distilled water and another one was mixed with 300 ml of 1% hydrochloric acid. Then, samples were homogenized in electric shaker for 5 min. The two mixtures were transferred to boiled water bath for 30-40 min, then cooled and filtered through muslin cloth and centrifuged with speed of 3000 rpm for 10 min. The two supernatants were mixed. Equal quantity of N-propanol was added to the mixture prior to sodium chloride was added until the solution was separated into two layers. The lower layer extracted in separating funnel with ethyl acetate, and concentrated by using rotary evaporator at 40°C for 1 to 2 h. The upper layer was dried by rotary evaporator at 40°C for 1 to 2 h. The dried material of both layers were mixed and dissolved in 5 ml of 96% ethanol, then left in oven until it turned into powder and kept in refrigerator until use.

Preparation of different concentrations of plant extracts

Stock solutions were prepared by mixing 2 gm from the dried extract with 20 ml of ethylene glycol, and then it

was sterilized with millipore membrane filter (0.22 µm). Then different concentrations of 10, 5 and 1 mg/ml were prepared by mixing known volume from the stock solution with ethylene glycol.

Analysis of chemical composition of the plant extracts by FLC

The analysis of the chemical composition was made by FLC. FLC consists from a mobile phase which is polar and consists of a mixture of solvents such as water and acetonitrile, while the stationary phase comprises of a column which is usually stainless steel and packed with silica particles, a sample of 50 µl was injected into the mobile phase using procedure outlined by Hartley and Buchan and it passes along the stationary phase, the time taken for a sample to pass through the system is recorded as its retention time and is one of the characteristic used to identify the compound, all the compounds were separated and identified using FLC with separation conditions C-18, 3 cm particle size, 50 × 4.6 mm internal diameter of the column, detection U.V. set at 282 nm, flow rate 1.2 ml/min. and 30°C temperature, but the differences were in mobile phase which was 0.1 % acetic acid and THF (tetrahydrofuran) using linear gradient from 0-100% B in 12 min. However, it was deionized water: acetic acid (80:20 V/V) in phenol case of ginger extract. In phenolic compound of cinnamon and thymus the separation conditions C-18, 3 cm particle size, 50 × 2.0 mm internal diameter of the column, detection U.V. set at 280 nm, flow rate 1.2 ml/min. and 30°C temperature, but the differences were in mobile phase which was 0.1 % formic acid and acetonitrile using linear gradient from 0-100% B in 12 min. However, it was deionized water: methanol (6:3:1, V/V). The area under a peak is used for calculating the concentration of a sample as the following formula:

$$\text{Conc. of sample } \mu g/\text{ ml} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{conc. of standard} \times \text{dilution factor.}$$

Analysis of the chemical composition was made by injecting 20 µl of the extract of each sample in FLC for identification. The procedure that used outlined by Hartley and Buchan was followed. The peaks were detected by UV detector. The analysis was carried out in the laboratories of ministry of science and technology, Baghdad, Iraq [17].

Identification of bacterial isolates

Test Microorganisms

Six isolates of resistant *S. aureus* were obtained from laboratory of Biology Department, College of Science, University of Baghdad (**Table 1**). For the purpose of antimicrobial evaluation, the microorganisms were cultured into tryptic soya broth (TSB) at room temperature for 24 h and number of bacteria was adjusted to be 10^7 CFU/ ml with sterile saline.

Table 1. Bacteria isolates used in the study.

Microorgar	Type of the isolates
S. aureus	3 Blood
S. aureus	3 Wound

Activation and maintenance of isolates

Bacterial cultures were activated in test tubes containing 5 ml of brain heart infusion broth and then incubated at 35-37°C for 24 h until the inoculum turbidity equivalent to 0.5 McFarland standard. Nutrient agar was used for bacterial strains storage at 4°C.

Determination of minimum inhibitory concentration (MIC)

The MIC is considered the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after 24 h incubation [18]. MIC of plant extracts was determined by micro dilution method in sterile 96-wells microtiter plates according to the protocol described previously [19]. Different plant extracts concentrations (100%, 50%, 25%, 12.5%, 6.25%, and 3.125%) (v/v) were prepared containing bacterial cells comparable to McFarland standard no. 0.5 in a final volume of 200 µl. Sterile distilled water, broth and plant extracts was used as a negative control while sterile distilled water, broth and bacteria was used as positive control. After 24 h at 37°C, the MIC of each sample was determined.

Statistical Analysis

Complete randomized design (CRD) was used as experimental design. Data were analyzed by using statistical analysis system- SAS [20] to study the effect of different factors on the diameters of inhibition zones. Least significant difference (LSD) was used to compare the significant difference between means at P≤ 0.05.

RESULTS AND DISCUSSION

MIC against *S. aureus* isolates

The MIC tests in **fig 1** shows clear inhibitory action against *S. aureus* isolate. The lowest active concentration before and after mixing with Ag-NPs was 12.5% for *T. vulgairs* and *C. zeylanicum*, respectively. While the highest concentration was 25% for *Z. officinale* before and after mixing with Ag-NPs. Also, the antimicrobial activity of Ag-NPs appears no change in concentration against *S. aureus* isolate No.1 Before and after mixing with the three phenolic plant extracts.

The MIC result in **fig 2** indicated that the lowest active concentration was 3.125 for *T. vulgairs* extract after mixing with Ag-NPs, while the highest concentration was 100% for *C. zeylanicum* before mixing with Ag-NPs. Also, the reducing in Ag-NPs concentration to 6.25%

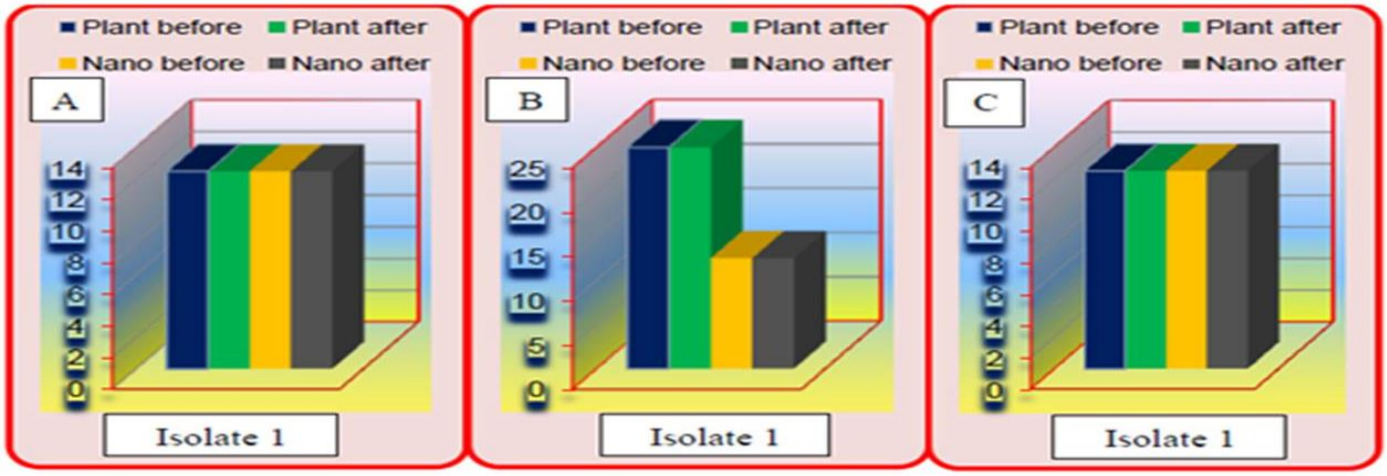

Fig 1. MIC of plant extracts against *S. aureus* (isolate 1) that isolated from wound . A, *T. vulgaris*; B, *Z. officinale; C, C. zeylanicum.* Y *axis*, percentage of concentration.

was significant inhibitory action after mixing with *T. vulgaris* and *Z. officinale* extract, respectively. According to MIC result in **fig 3** against *S. aureus* isolate No.3. The lowest active concentration was 6.25 for *T. vulgairs* after mixing with Ag-NPs. Another result showed that the highest concentration was 50% for *C. zeylanicum* before

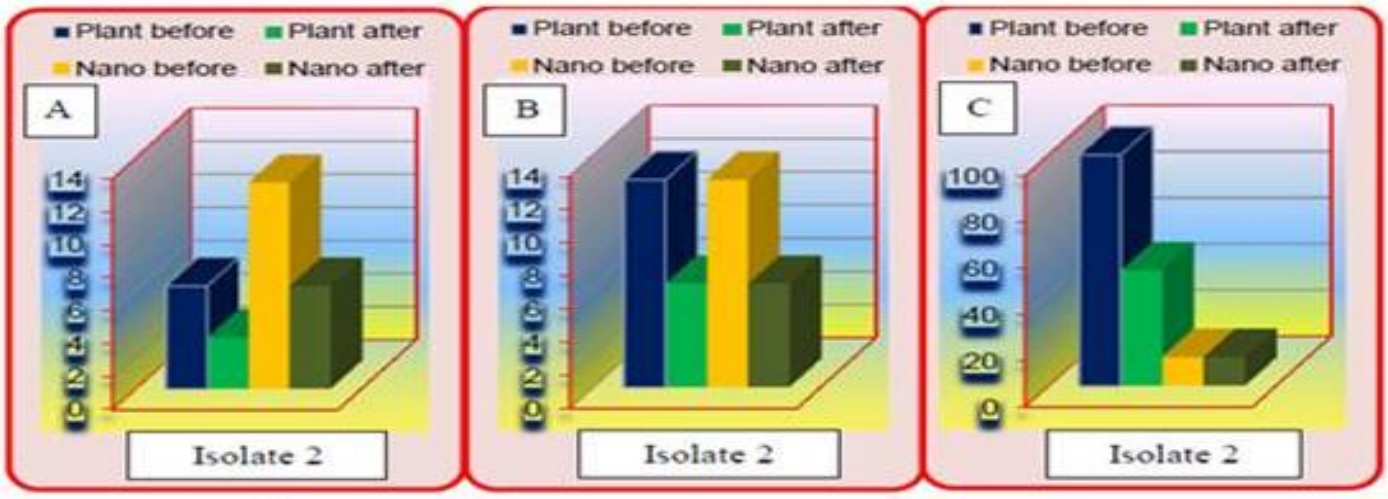

Fig 2. MIC of plant extracts against *S. aureus* isolate (isolate 2) that isolated from wound. A, *T. vulgaris;* B, Z. officinale*; C, C. zeylanicum.* Y *axis*, percentage of concentration.

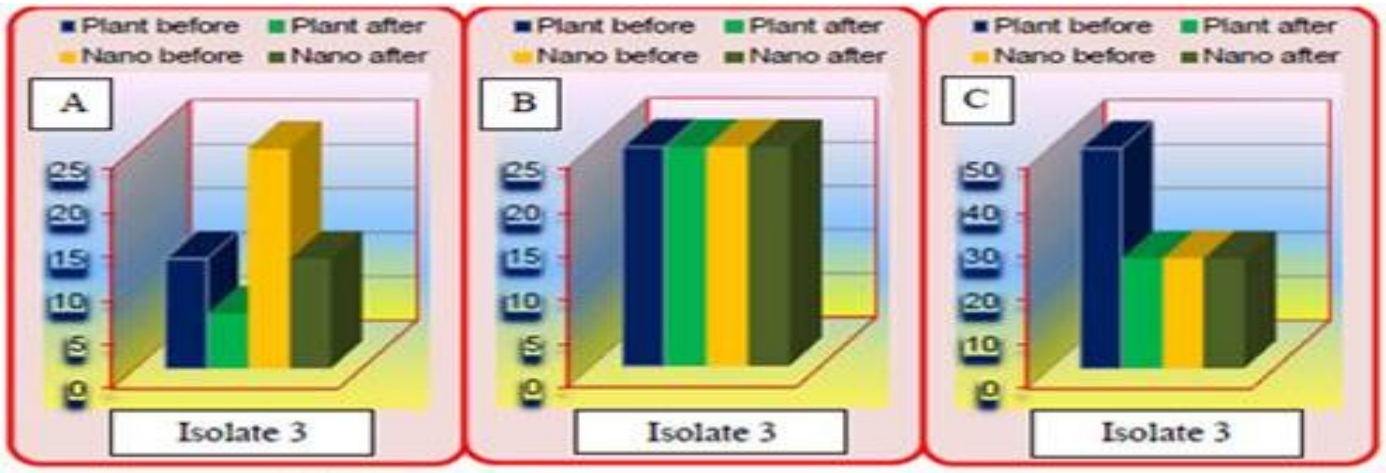

Fig 3. MIC of plant extracts against *S. aureus* isolate (isolate 3) that isolated from wound. A, *T. vulgaris;* B, Z. officinale*; C, C. zeylanicum.* Y *axis*, percentage of concentration.

mixing with Ag-NPs. Besides, the antimicrobial activity of Ag-NPs appears significantly high against *S. aureus* isolate No.3. The reducing in Ag-NPs concentration was 6.25 after mixing with *T. vulgaris*. But, there was not changing in Ag-NPs concentration before or after mixing with *Z. officinale* extracts. The MIC values in **fig 4** shows clear inhibitory action against *S. aureus* isolate No.4. The lowest concentration after mixing with Ag-NPs was 6.25% for *T. vulgairs*, while the highest concentration was 50% for *Z. officinale* before mixing with Ag-NPs.

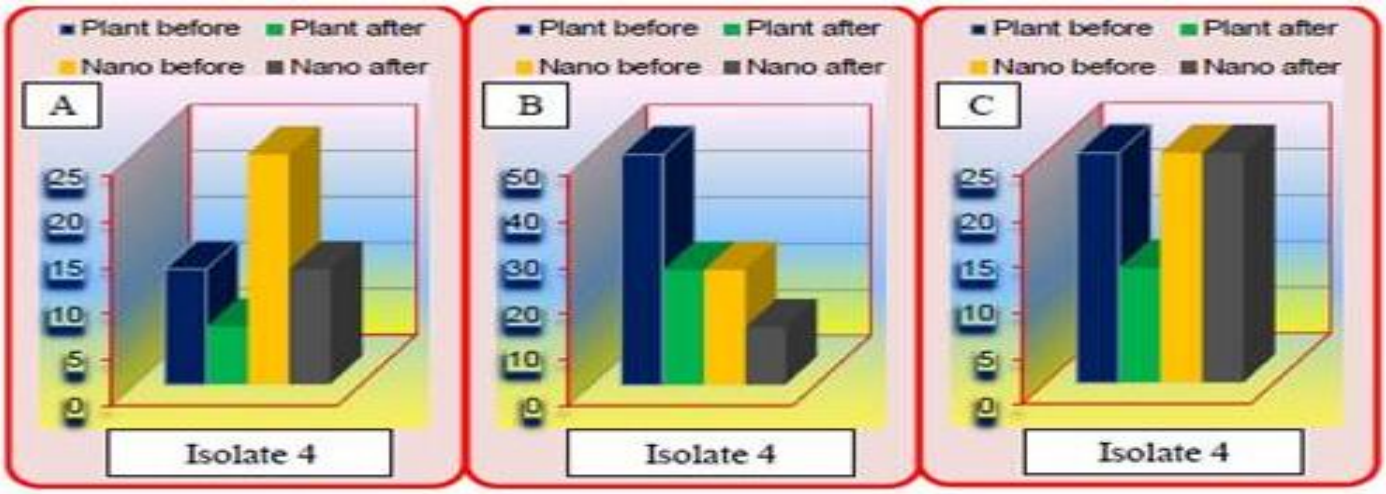

Fig 4. MIC of plant extracts against *S. aureus* isolate (isolate 4) that isolated from wound. A, *T. vulgaris;* B, Z. officinale*; C, C. zeylanicum.* Y *axis*, percentage of concentration.

Also, the antimicrobial activity of Ag-NPs appears significantly high against *S. aureus* isolate No.1.The reducing in Ag-NPs concentration was significant clear after mixing with *T. vulgairs* and *Z. officinale* extract from 25 to 12.5 %. While for *C. zeylanicum* extract from 50 to 25 %. The results in **fig 5** indicated that *T. vulgairs* extract was most active against *S. aureus* isolate No.5. The lowest concentration was 3.125% for *T. vulgairs* after mixing with Ag-NPs, while the highest concentration was 100 % for *C. zeylanicum*

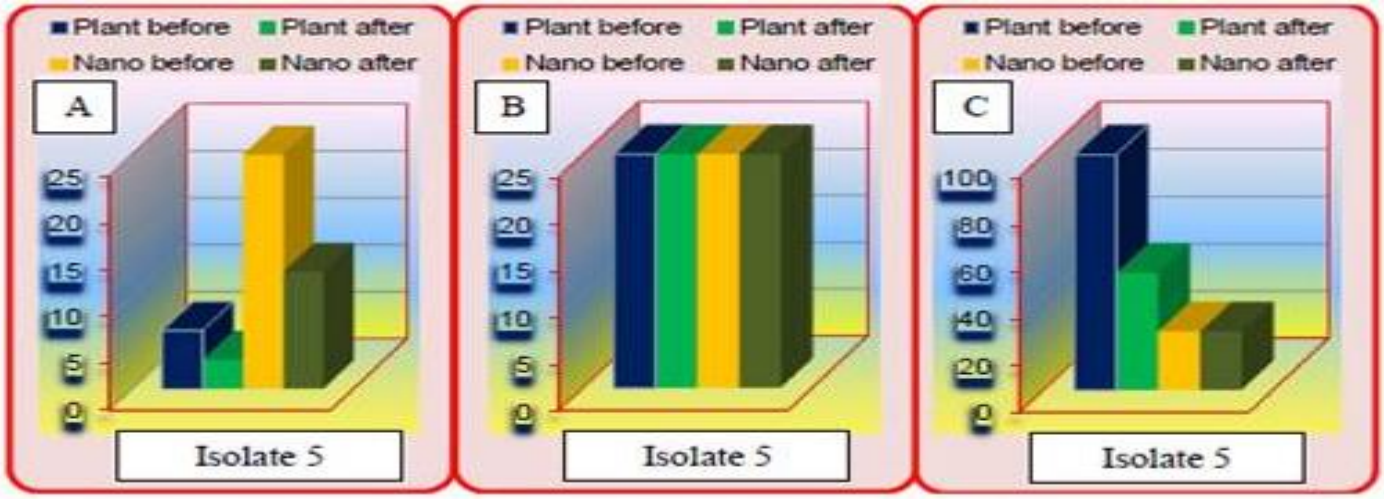

Fig 5. MIC of plant extracts against *S. aureus* isolate (isolate 5) that isolated from wound. A, *T. vulgaris;* B, Z. officinale*; C, C. zeylanicum.* Y *axis*, percentage of concentration.

mixing with Ag-NPs. Also, the reducing in Ag-NPs concentration was significant clear after mixing with *T. vulgairs* extract from 25 to 12.5 % and from 50 to 25 % for *C. zeylanicum* extract. But, there wasn't any significant change in *Z. officinale* concentration before and after mixing with Ag-NPs. The highest antibacterial activity was exhibited by *T. vulgairs* extract against *S. aureus* isolate No.6. The MIC tests in **fig 6** shows the lowest concentration after mixing with Ag-NPs was 6.25 % for *T. vulgairs*, while the high extract concentration was 50 % for *Z. officinale* before mixing with Ag-NPs. Also, the reducing in Ag-NPs concentration was significant clear after mixing with *T. vulgairs* extract from 12.5 to 6.25 %.

Chemical constituents of the phenolic plants extracts

FLC results are shown important differences between the concentrations of each secondary metabolic compound among the phenolic plants extracts, whether

it is phenol compounds or even between their total concentrations. However, the total concentration of phenol in the extracts of *T. vulgaris, C. zeylanicum* and *Z. Officinale* were 968.95 µg/ml, 446.612 and 49.143, respectively. Thymol (453,613 µg/ml) was the major phenolic compound, while gallic acid (21.505 µg/ml) was the minor in the *T. vulgaris,* respectively. Carvacrol

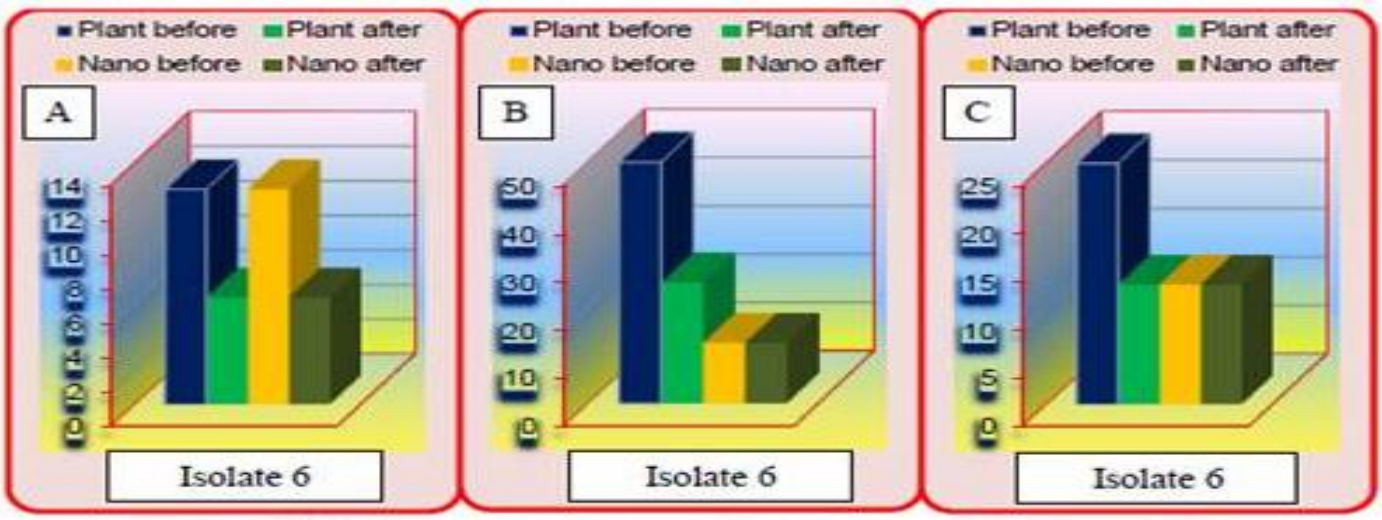

Fig 6. MIC of plant extracts against *S. aureus* isolate (isolate 6) that isolated from wound. A, *T. vulgaris;* B, Z. officinale*; C, C. zeylanicum.* Y *axis,* percentage of concentration

(145.050 µg/ml) was the major phenolic compound, while P-coumaric acid (4.436 µg/ml) was the minor in the *C. zeylanicum* extract. Gingerol (34.664 µg/ml) was the major phenolic compounds, while shogoal (6.169 µg /ml) was the minor in the *Z. officinale* extract, respectively (**Table 2**).

Table 2. types and concentrations of phenols in plant extracts after running in FLC test technique.

No.	Phenolic compound µg/ml	*T. vulgaris*	*C. zeylanicum*	*Z. officinale*
1	Gallic acid	21.505	11.112	-
2	Caffeic acid	76.208	8.748	-
3	Vanillic acid	152.533	50.130	-
4	p- coumaric acid	-	4.436	-
5	Rosmaric acid	108.435	61.375	-
6	Carvacrol	26.593	145.050	-
7	Thymol	453.613	75.602	-
8	Carnasol	96.496	20.974	-
9	Eugenol	33.567	69.185	8.310
10	Shogoal	-	-	6.169
11	Gingerol	-	-	34.664
	Total concentration µg/ml	968.95	446.612	49.143

DISCUSSION

A wide variety of synthetic compounds exert antibacterial effect, but just some of them can be used as biocides to develop drugs or coatings. The primary impediment for their use is their toxicity compared with their bactericidal effect; some of them are so toxic for eukaryotic cells that cannot be proposed as antibiotics. In this study, the bacterial isolates were chosen because the importance of these isolates in the hospital environment and their outbreak in the community. The study aimed to determine the *in vitro* antibacterial activity of extracts from some selected medicinal plants against the most common bacterial pathogens including multidrug-resistant (MDR) bacteria.

Among these materials, silver compounds (salts and colloids) rise as potent bactericidal agents whose application is restricted to topical creams used to reduce the risk of wound infection and to treat infected wounds. In order to challenge silver nanoparticles as novel antimicrobial agents, the principal aim of this research was to assess, by in vitro assays, the bactericidal properties of silver nanoparticles against a clinical isolate of MRSA.

Results of FLC of the extracts referred to present of phenols in the *T. vulgaris, Z. officinale* and *C. zeylanicum*. Antimicrobial activity of plants extracts depends upon their active components. However, the biological effect is often due to a synergy between the compounds [21]. Thus, the extracted in this study were analyzed in a screening for the most active compounds (thymol, carvacrol, P-coumaric acid, gallic acid, eugenol …….etc.]. In the case of *T. vulgaris* and *C. zeylanicum* were antimicrobial abilities are mainly attributable to the presence of phenolic components in their extracts [22]. Alves-Silva *et al.* [23] showed that due to their Thymol and carvacrol are the main constitutes and responsible for their disinfection potential through a variety of inhibition and killing mechanisms, which target on multiple sites of the bacterial cell will preferentially partition from an aqueous phase into membrane struct-ures. This results in membrane expansion, increased membrane fluidity and inhibition of a membrane embedded enzyme. Nadia *et al.* [24] found many bioactive compounds such as phenolics, flavonoids, thymol, carvacrol, biphenyl's and aliphatic phenols in thymus species. They inducted that phenolic acids are present in thymus species which contribute to their therapeutic potential. While Aghsaghali *et al.* [25] notes that caffeic acid is present in thyme which has antibacterial, antiviral and antifungal activity.

However, according to FLC results all those active components were actually found in the extracts in this study. The results of the antibacterial activity of ginger against the *S. aureus* isolates were given in table [4]. The antimicrobial activity of ginger may be attributed to the fact that it contains antimicrobial substances such as zingiberol, zingiberine and bisabolene [26]. The rhizome of ginger contains pungent vanillyl ketones including gingerol and paradole, etc. [27, 28]. Gingerol is a mixture of crystal ginger one and it is the major cause of acidity of ginger and plays a role in inhibiting bacteria such as *S. aureus*, *Trichomons vaginalis* and help to cure bacterial vaginitis and skin diseases [26]. Silver ions are known to bind to sulfhydryl groups, which lead to protein denaturation by the reduction of disulfide bonds [S–S →S–H + H–S] [29]. Besides, silver ions can complex with electron donor groups containing sulfur, oxygen, or nitrogen that are normally present as thiols or phosphates on amino acids and nucleic acids [30]. Thus, silver nanoparticles would not bind to specific proteins or structures of the bacterial cell *S. aureus* but to a broad spectrum of targets that would include membrane and cytoplasmic proteins and genomic or plasmid DNA. Indeed, silver nanoparticles have been found to attach to the surface of the cell membrane and disturb its function, penetrate bacteria, and release silver ions [31]. Werner, *et al.,* [31]; Siskova, *et al.,* [32] also found that nano-Ag target the bacterial membrane, leading to a dissipation of the proton motive force. More study need to understand many issues like uptake potential of various species, process of uptake and translocation and the activities of the Ag-NPs at the cellular and molecular levels. Also, most of the extracted in this study were active against contaminant isolates which most of them actually are pathogenic for human, thus, further studies are required to test their activity against other pathogenic bacteria and fungi and study the possibility of using these active components by drugs companies. Finally, further studies using *in vivo* models are needed to study the impact of nanoparticles on reproductive health.

Conflict of interest

The authors declare that they have no conflict of interests.

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