Egyptian Journal of Medical Microbiology Volume 33 / No.2 / April 2024 91-97 Online ISSN: 2537-0979

ORIGINAL ARTICLE

The effect of Thymus Vulgaris extracted silver nanoparticles on multi-drug resistant Microorganisms. (Student project)

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Key words:
Multi drug resistant bacteria, Thymus Vulgaris extracted silver nanoparticles, Antibiotic sensitivity

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ABSTRACT

Background: *Nanoparticles provide the hope in solving the problem of anti-biotic resistance. Thymus Vulgaris extracted silver nanoparticles gives a promising results as antibacterial, and antifungal agent.* ***Objectives:*** *We aimed at testing Thymus Vulgaris extracted silver nanoparticles antimicrobial activity against multi drug resistant microorganisms (MDR).* ***Methodology:*** *The study was implemented by fourth year students of faculty of Applied Medical Sciences, Medical Laboratory Department, Menoufia University with Microbiology laboratory of the National Liver Institute, Menoufia University cooperation. Bacterial isolates were examined for antibiotic sensitivity and confirmed as MDR by VITEK2 system. Thymus Vulgaris extracted silver nanoparticles were utilized to the tested strain in order to determine its antimicrobial properties.* ***Results:*** *Thymus vulgaris extracted silver nanoparticles produce inhibition zones of 20mm against Pseudomonas aeruginosa and 30mm against C. albicans.* ***Conclusions:*** *From our study we concluded that silver nanoparticle (AgNPs) extracted from Thymus vulgaris, and Thymus vulgaris essential oil extract has shown promising results as an alternative treatment for multidrug -resistant bacterial and fungal infections (MDROs)*

INTRODUCTION

Antimicrobial resistance is dangerous to global public health. Recently, the Strains of Multidrug-resistant organisms have become quadrupled worldwide, presently antimicrobial resistance poses a major danger to patient treatment that led to increase mortality, morbidity, and hospital stay.

Previously, nanotechnology and nanoparticles were developed to combat and reduce bacterial resistance and multidrug resistance (MDR).

There has been an evolution in nanotechnology throughout the last few years in the treatment of MDR infection[1]

Nanotechnology is a rapidly growing field of science with the potential to revolutionize various scientific fields.

It made it possible for nanoparticles to improve new properties based on particular characteristics such as size, morphology, and distribution[2]

Nanotechnology is an emerging area of technology and science that will revolutionize materials use in the 21st century. The relatively crude and unsophisticated technologies on which we currently will be replaced with environmentally friendly nanotechnology[3]

The advances of Nanotechnology open new horizons in life in biomedical devices and biotechnology. That has shown excellent bactericidal properties against microorganisms.

The significance of nanotechnology is increasingly important in our society. For that, scientists are endeavoring to comprehend the structure of the scale atoms, molecules and material's properties[4].

There are many biological activities of Sliver nanoparticles such as antioxidant, anti-cancer activities, and antimicrobial[5].

These particles have excellent medical and non-medical properties when compared with other metal nanoparticles. It usually decreases the number of multi-drug resistant bacteria due to the variability of environmental circumstances and genetic mutation[6].

Silver salts are utilized in the human system to impede the growth of various bacteria[7]. It is used in burns, catheters, and wounds. (to defend them from infection)[8]

Silver is usually used for antimicrobial activity in the form of silver nitrate (NO3−).

Medical applications of AgNPs, focusing on antimicrobial, potential mechanisms, anticancer properties, and other medical applications, including wound repair, dental applications, vaccine adjuvant, bone healing and biosensing, and antidiabetic agent[9].

In this work, we will test and discuss the effect of antibacterial effect of plant-extracted silver

nanoparticles against multi-drug resistant organisms (MDROs).

METHODOLOGY

Microorganisms:

Our study was conducted within the Microbiology laboratory of the National Liver Institute, Menoufia University on six microorganisms, including *Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Klebsiella pneumoniae, C. albicans and Pseudomonas aeruginosa.*

Antibiotics discs were purchased from (Hi Media, India):

For gram-positive bacteria, we used Cefoxitin, Methicillin, Vancomycin, Erythromycin, and Piperacillin. For gram-negative bacteria, we used Piperacillin/tazobactam (TZP) (100/10 µg), ceftazidime (FEP) (30 µg), meropenem (MEM) (10 µg), imipenem (IPM) (10 µg), amikacin (AK) (30 µg), gentamicin (GM) (10µg), ciprofloxacin (CIP) (5 µg), Norfloxacin (NOR), levofloxacin (LEV), Cefepime (FEP) (30µg), Ceftazidime-avibactam (CZA)(50/30µg), Ofloxacin(OF) (5µg), Gemifloxacin (GAT) (5 µg).

Antifungal discs were bought from (Hi Media, India):

For *Candida albicans,* we used Flucytosine (10µg), Ketoconazole (10µg), Fluconazole (10µg) and Itraconazole (10 µg).

Steps of antimicrobial sensitivity testing: according to microbiology standards and (CLSI, 2023), with the disc diffusion method.

- Using a sterile loop, 3-5 well-isolated colonies from the pure culture of the organism were emulsified in sterile saline
- The turbidity of the suspension was matched to the turbidity of 0.5 McFarland standards ***(BioMérieux, France)***
- A sterile swab was dipped in the inoculum suspension; excess fluid was removed by squeezing the swab against the inner side of the tube and then rubbed over a plate of Muller-Hinton (MH) **(HiMedia, India)** agar in three several different directions to ensure even distribution.
- Antibiotic disks were placed on the inoculated plate, disks should be 15 mm from the edge of the plate, 25 mm from disk to disk, and no more than 6 disks in each plate. Each disk should be lightly pressed deep to ensure its touched with the agar and ought not to be moved once in place[10].

After 24 hours at 37°C in the incubator, results were reported by measuring the inhibition zone in mm. The results obtained were validated using the **VITEK 2 System *(BioMerieux, France)***

Preparation of Thymus Vulgaris Extracted Silver Nanoparticles:

Plant Material[11]:

The thyme leaves were collected from Egypt. The thyme leaves were separated at the Botany Department of the Faculty of Pharmacy at Menoufia University in Shebin El-Kom, the plant was rinsed with distilled water, weighed, and frozen at -20°C. After freeze drying (Dura Dry TM µpfreeze-drier; -45°C and 250 mTorr), it was properly stored till used for extraction.

Preparation of Thymus Extract[12]:

The stored plant leaves were soaked in 70% ethanol at room temperature for one week after being washed with tap water and finally with deionized water. It was then filtered by utilizing the Whatman filter paper. The extract was then warmed to 4°C and stored for future processing.

Synthesis of Plant-Extracted Nano Material[13]:

Using a hydrothermal method, Thyme-covered silver nanoparticles were synthesized with slight modifications. A 50 mL of 0.25M silver nitrate solution was prepared and stirred with 50 mL of leave extract of Thymus at 35°C for 1 h. The solution mixture was transferred into Teflon-lined sealed stainless-steel autoclaves and placed in a hydrothermal oven at a temperature set to 150°C for 1.5 hours. The contents were allowed to cool to room temperature upon completion of the reaction. The resultant dark brown T/AgNPs were obtained through centrifugation at 5000 pm for 10 minutes, resulting in the collection of pure and stable T/AgNPs. The stable T/AgNPs were subsequently dried in an oven at 60°C for 4 hours, ground, and preserved in an airtight bottle.

Silver nanoparticles characterization.

The National Research Centre in Cairo, Egypt conducted an analysis to characterize silver nanoparticles. To record the UV-vis spectroscopy estimates, a Jasco dual-beam spectrophotometer was utilized (model UV-VIS—NIR 570) at a resolution of 2 nm[13]. The biological study was conducted at the National Liver Institute, Menoufia University.

Procedure:

Once bacteria had been identified following microbiological culture, susceptibility testing is conducted using the disk diffusion technique with thymus and T/AgNPs to select appropriate antibiotics and antifungals.

1. First, we inoculated nutrient agar with bacteria using the swabbing technique and divided it into 2 halves. We used one half for antibiotic sensitivity and the second half for nanomaterial or thymus essential oil. We applied this method to all microorganisms we used.

2. We applied antibiotics on their special half and applied 100 micros of Thymus Vulgaris extracted nanoparticles (T/AgNP) on the other side by punching a hole using sterile blue TIPS in the current study. As shown in Fig No.1

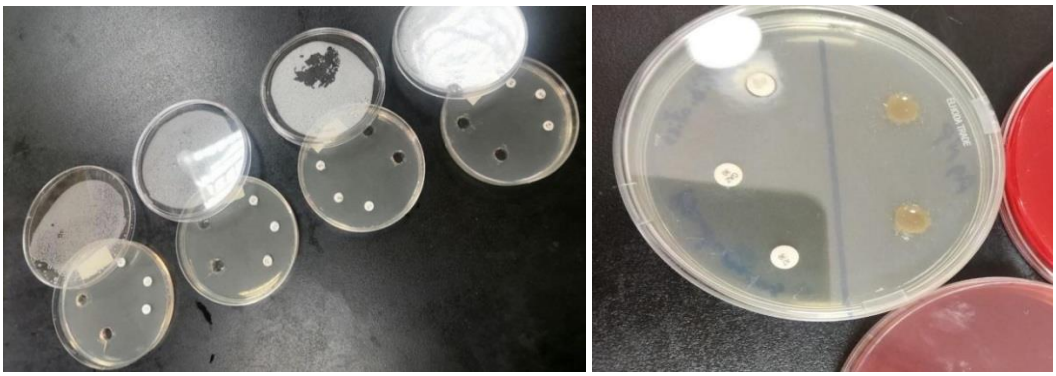


Fig. 1: Preparation of anti-microbial sensitivity testing plates

3. We incubated the plate at 37°C for 24 hours and then recorded the result.
4. In cases where the antibiotic or thymus vulgaris extracted silver nanoparticles exhibit inhibitory effects on microbial growth, a discernible zone of inhibition can be observed around the disc or the slot. Such instances are categorized as effective treatment measures.

   Bacteria were classified as resistant, intermediate, or sensitive by comparing the diameter of the inhibition zone to defined thresholds that correlate with MICs.
5. To calculate the inhibitory zone, we placed the plate on a non-reflective surface and took a ruler that measured in millimeters. We placed the "Zero" ruler in the middle of the antimicrobial disk or a slot and measured from the middle of the disk to the end of the area with zero growth. Then we took measurements in millimeters.

Statistical analysis:

Information was gathered, tabulated, and analyzed by statistical package for the social sciences (SPSS, version 22; SPSS Inc., Chicago, Illinois, USA) software. Mean and standard deviation, number, and percentage were done at a 5% level of significance.

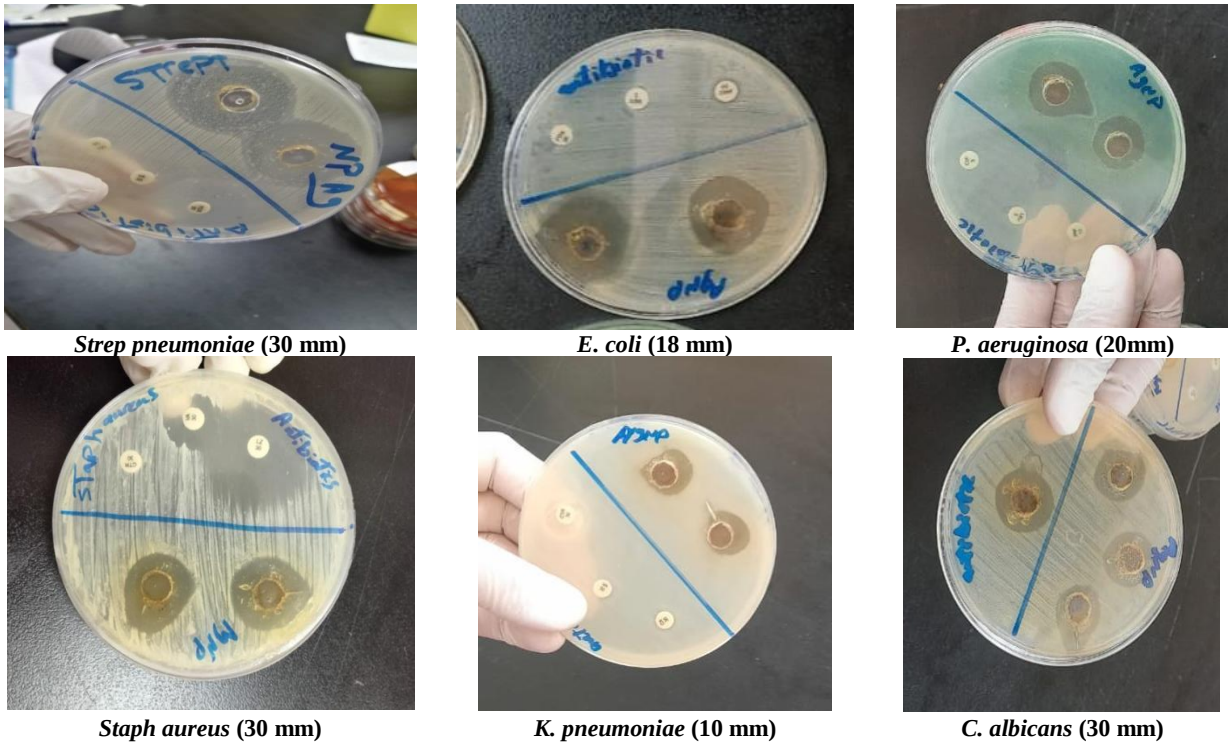
RESULTS

The present study was carried out by Applied Medical science students in cooperation with the National Liver Institute Microbiology Department, Menoufia University between February 2023 and June 2023. It aimed to assess the antimicrobial effectiveness of thymus vulgaris-extracted silver nanoparticles. The examination involves bacterial strains resistant to multiple drugs. As shown in table 1 from table 1 and Fig. 2.

Table 1: Antimicrobial activity of thymus Vulgaris extracted silver nanoparticles (AgNPs)

Microorganism	MET	FOX	AMS	HLG	CAZ	AT	ETP	CIP	IMI	LEV	AUG	VA	PIP	AFY	KCA	ITC	AgNPs
Staph aureus (MRSA)	R	R	R	-	-	-	-	-	-	-	-	-	-	-	-	-	S 30 mm
Strep pneumoniae	-	S 25mm	-	-	-	-	-	-	-	-	-	S 20mm	R	-	-	-	S 30 mm
Klebsiella pneumoniae 1	-	-	R	R	-	-	R	-	-	-	-	-	-	-	-	-	S 10 mm
Klebsiella pneumoniae 2	-	-	-	-	R	R	R	-	-	-	-	-	-	-	-	-	S 10 mm
Pseudomonas aurigonosa 1	-	-	-	-	R	R	R	-	-	-	-	-	-	-	-	-	S 20 mm
Pseudomonas aurigonosa 2	-	-	-	-	-	-	-	R	R	R	-	-	-	-	-	-	S 20 mm
E.coli	-	-	S 20mm	-	-	-	S 25mm	-	-	-	S 15 mm	-	-	-	-	-	S 18 mm
Candida albicans	-	-	-	-	-	-	-	-	-	-	-	-	-	S 10mm	S 20mm	R	S 30 mm

S : Sensitive, R : Resistance, MET: Methicillin, FOX :Cefoxitin, AMS : Ampicillin Sulbactam, HLG: Gentamicin, CAZ: Ceftazidime, AT: Aztreonam, ETP : Ertapenem, CIP : Ciprofloxacin, IMI : Imipenem, LEV : Levofloxacin, AUG : Amoxicillin, VA :Vancomycin.



***Strep pneumoniae* (30 mm)** ***E. coli* (18 mm)** ***P. aeruginosa* (20mm)**

***Staph aureus* (30 mm)** ***K. pneumoniae* (10 mm)** ***C. albicans* (30 mm)**

Fig. 2: The diameter of the inhibition zone caused by Thymus vulgaris extracted silver nanoparticles from the current study

Thymus vulgaris extracted silver nanoparticles showed an antimicrobial effect on all microorganisms incorporated in this research. Also, the significant effect was with *Staph aureus (MRSA), Strept pneumoniae*, and *Candida albicans* with a (30 mm) inhibition zone. Finally, different strains of both *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* displayed antimicrobial resistance, whereas the sensitivity was to AgNPs. As shown in Table 2.

Table 2: The antibacterial effect Of Thymus Vulgaris essential Oil extract compared to antibiotics

Microorganisms	ITC	FLU	AFY	KCA	AUG	IMI	CAZ	ETP	PI	AK	CPZ	VA	E	FOX	Thymus
Staph aureus ‘ MRSA’	-	-	-	-	-	-	-	-	-	-	-	S 15mm	R 15mm	R 20mm	S 15 mm
Strept pneumoniae	-	-	-	-	-	-	-	-	S 10mm	-	-	S 20mm		S 30mm	S 15 mm
Klebsiella pneumonia	-	-	-	-	R	R	R	-	-	-	-	-	-	-	R
Klebsiella pneumonia	-	-	-	-	-	-	R	R	R	-	-	-	-	-	S 11 mm
E-coli	-	-	-	-	-	R 16mm	-	-	-	R 12mm	R 20mm	-	-	-	R
Pseudomonas	-	-	-	-	-	R	-	-	-	R	R	-	-	-	S 17-20 mm
Candida	R	R	R	-	-	-	-	-	-	-	-	-	-	-	S 15-19 mm
Candida	R	R	-	R	-	-	-	-	-	-	-	-	-	-	S 20 mm

S: Sensitive, R: Resistance, VA: Vancomycin, PIP: Piperacillin, FLU: Flucytosine, KCA : Ketoconazole, ITC = Itraconazole, AgNPs : Silver nanoparticles, ITC = Itraconazole: FLU= Fluconazole, AFY: Flucytosine KCA: Ketoconazole, AUG: Amoxicillin, IMI: Imipenem, CAZ: Ceftazidime: ETP: Ertapenem, PI: Piperacillin, AK: Amikacin, CPZ: Cefoperazone, E: Erythromycin, FOX: Cefoxitin.

Comments:

• Thymus vulgaris essential oil extract shows antimicrobial activity on both bacterial and fungal isolates and able to prevent the growth of microorganisms (gram positive, gram negative and fungi).
Except for E. coli and some strains of klebsiella.

• Klebsiella pneumonia taken from two different patients show different in their sensitivity to thymus vulgaris essential oil extract.

• Thymus vulgaris show antimicrobial effect on two candida isolates taken from different patients.

Thymus vulgaris essential oil extract demonstrated antimicrobial activity on both bacterial and fungal isolates and successfully inhibited the growth of microorganisms (gram-positive, gram-negative, and fungi), Except for *E. coli* and some strains of *K. Pneumoniae.* Two distinct strains of *K. pneumoniae,* isolated from separate patients, exhibit variability in their susceptibility to thymus vulgaris essential oil extract. Thymus vulgaris demonstrated antimicrobial efficacy against two strains of *candida* isolated from distinct patients.

DISCUSSION

The escalating rate of antimicrobial resistance has become a serious public health concern worldwide recently. This phenomenon not only poses a significant threat to the effectiveness of antimicrobial agents but also to the treatment of various infectious diseases. The rise in harming effect of emerging microorganisms with high resistance to current antimicrobials highlighting the urgent need for new therapeutic approaches. So, exploring alternative therapeutic options to treat this type of infection is a must. To treat persistent MDR bacterial, fungal, and biofilm infections nonmaterial offers an unconventional approach[1]. Many researchers employed more than one method as the plant material (bark, leaves, stem, roots) for the synthesis of silver nanoparticles and testing its antimicrobial activity[14,15]. This study aimed at studying the influence of Thymus Vulgaris extracted silver nanoparticles T/AgNP on MDR.

In the present work, we reported that Thymus Vulgaris extracted silver nanoparticles/AgNP has antimicrobial activity on Multidrug-resistant bacterial and fungal isolates with variable potencyas and were able to suppress the growth of microorganisms including gram-positive bacteria (*S.aureus and streptococcus pneumoniae*), gram-negative bacteria (*E.coli, K. pneumoniae, and pseudomonas aeruginosa)* compared with a set of traditional antibiotics used in this study. These results agreed with Aldosarya et al [16].

The findings indicated that significant microbial inhibition of T/AgNP was detected against *S. aureus* with a (30mm) inhibitory zone, which was strongly resistant to known antibiotics such as methicillin, Cefoxitin, and ampicillin sulbactam. It also revealed the significant effect of T/AgNP on suppressing the growth of *Streptococcus pneumoniae* where the clean zone was (30 mm). Other researchers obtained similar results against *S. aureus* with an inhibition zone of 18.3 ± 0.4 [17]. According to our study, the growth of two different clinically identified strains of *K.pneumoniae* 1,2 and P. aeruginosa1,2 taken from two different patients was suppressed by the potent of T/AgNP.

As per the research conducted by Dhara N. Shah, et al[18], numerous scholars have demonstrated a strong association between lower susceptibility to candida and previous exposure to antifungal agents, as well as to inappropriate antifungal therapy in the past. According to Khalil et al[11], Candida isolates demonstrated resistance to miconazole and clotrimazole in 51.2% of the isolates, and all Aspergillus isolates showed resistance. Flucytosine resistance was observed in 76.7% of the isolates, nystatin resistance in 37.2%, and fluconazole resistance in 46.5% of the isolates.

The present work reports a noteworthy antifungal effect of silver nanoparticles derived from Thymus vulgaris. The findings indicate that the nanoparticles exhibit significant efficacy against fungal infections. This caused a reduction in the number of isolates that were resistant, contrasted to the known antifungal Itraconazole, Ketoconazole and Flucytosine with (30 mm) of inhibitory zone. That agreed with study by Mohsen Mohammadi [20]. So, it's highly recommended as an antifungal treatment. As per the research conducted by Mohammadi [20], the cytotoxicity analysis of ANPs derived from thyme extract concluded that these nanoparticles are a possible alternative to Fluconazole for treating superficial fungal infections. The study showed that the ANPs showed no toxicity at concentrations below 3.5 ppm. This finding establishes the future of thyme extract-derived ANPs as a potent antifungal agent[19].

In this investigation Thymus vulgaris oil extract was tested against MDR as argued in methodology and its outcomes were contrasted with that of nanomaterial T/AGNP with the intention of knowing which is more potent and effective to treat these infections. Current research shows that Thymus vulgaris essential oil extract has antimicrobial activity on gram-positive bacteria including *S. aureus (MRSA)* and *Streptococcus pneumoniae* with an inhibitory zone of (15 mm). This result agreed with Aziz et al[21]. It also showed an antimicrobial effect on *P.aeruginosa* with an average inhibition zone of around (17-20) mm as in Table 2, in contrast, it displayed no effect against *K. pneumoniae* strains and E. coli. Based on recent findings, the extract of Thymus vulgaris has been demonstrated to display antifungal properties against Candida albicans, with an inhibitory zone of around (15-20) mm. This agreed with Rahimifard et al[22], but it was less effective on *C.albicans* compared with silver nanoparticle essential oil extract.

However, T/AgNPs were determined to be more effective in suppressing the growth of (MDROs) compared to thymus vulgaris essential oil extract which was not active against some bacteria as discussed before. Consequently, it ought to be highly recommended to treat this kind of infection. According to Fahimirad et al[23], the utilization of plant-mediated

silver nanoparticles has gained significant interest among various research groups due to their excellent antibacterial and anticancer activities. This finding aligns with Fahimirad's study, which suggests that the considerable potential of these nanoparticles has led to an increase in research efforts seeking to apply them in various fields.

CONCLUSIONS

The use of AgNPs extracted from Thymus vulgaris represents a promising area during the creation of novel antimicrobial substances for the addressing of multidrug-resistant infections. Furthermore, additional in vivo cytotoxicity and biocompatibility studies must be conducted before utilizing the material for biomedical purposes.

Declarations:
Consent for publication: Not applicable
Availability of data and material: Data are available upon request.
Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
This manuscript has not been previously published and is not under consideration in another journal.
Funding: Authors did not receive any grants from funding agencies.

Ethical approval:
The study was approved by the Faculty of Medical Science, Menoufia University, Egypt.

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