ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Microdilution Broth Method for Determination of Minimum Inhibitors Concentration of Thyme and Clove Essential Oil against Methicillin Resistant *Staphylococcus aureus*

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Abstract

The essential oil extracted from air-dried Thyme leaves (*Thyme vulgaris*) and Clove buds (Eugenia *caryophyllata*) by distillation method. The results showed lowest MICs value when exposure to natural bioactive products and demonstrated high activity against MRSA isolates. The MICs value of Thyme and Clove EOs and the standards (thymol and eugenol) were ranged from (0.195-0.39) µg/ml, (0.39-0.78) µg/ml and (0.097-0.048) µg/ml respectively, to enhance microdilution method described in our study improved through adding redox indicator which was Resazurin dye.

Keywords: *Clove Essential Oil; Inhibitors; Staphylococcus aureus.*

Introduction

The emergence and spread of antimicrobial resistant *S. aureus* isolates, particularly methicillin resistant *S. aureus* (MRSA), constitutes a global challenge for the treatment of infections caused by these bacteria. Infections caused by resistant bacteria extend the duration of stay at the hospital, increase the cost of health care services, and most importantly, lead to a significant increase in both morbidity and mortality rates [1]. Resistance to therapeutic drugs encompasses a diverse range of biological systems, which all have a human impact.

One of the most frequently employed strategies in biological systems that demonstrate resistance to cytotoxic drugs is the extrusion or efflux of these compounds from the cell via membrane proteins. Stated in its simplest terms, drug resistance decreases the chance of proving successful treatment against a plethora of diseases and need to develop new and effective antibacterial agents [2].Plant-derived compounds have historically been recognized as effective antimicrobial agents. Essential oils (EOs) derived from plant are known for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties, EOs contain tow biosynthetically related groups. These main groups include terpenes, terpenoids and aromatic, aliphatic constituents and some hydrocarbons exhibit antimicrobial effects [3,4].The genus *Thymus* (thyme) consists of about 215 species of herbaceous perennials. This is one of the most widely used genera in folk medicine. Thyme herb (*T. herba*) is produced from the dried leaves and flowering tops of *T. vulgaris*, and it contains tannins, flavonoids, triterpene compounds, and up to 2.5 % of essential oils [5].

The essential oils of *T. vulgaris* are known to have antiseptic, antiviral and antimicrobial activities. The main components are thymol and *p*-cymene, while other thyme species also contain carvacrol, a-terpinyl acetate, and *cis*-myrtanol [6].The buds of *E. caryophyllata* commonly named as clove, it is avowed to possess certain pharmacological properties including an aesthetic activity, antioxidant potential antimicrobial role, anti-inflammatory action, anti-carcinogenic effects, neuroprotective ability, hypolipidemic

efficiency and anti-diabetic effectiveness. Eugenol is declared as GRAS (generally recognized as safe) by World Health Organization (WHO) and is considered as non-mutagenic [7] . Clove has been reported to target both the bacterial membrane, leading to a non-specific increase in antibiotic penetration, and different metabolic pathways [8].

The minimal inhibitory concentration (MIC), is defined as the concentration (mg l^{-1}) at which visible growth of bacteria is prevented under defined growth conditions [9]. Well- and disc-diffusion methods have frequently been reported as qualitative indicators for testing the antimicrobial activity of natural products [10]. The improved microdilution method described in this report was enhanced through the addition of resazurin dye as a redox indicator. Active bacterial cells reduce the non-fluorescent resazurin (blue) to the fluorescent resorufin (pink) which can be further reduced to hydroresorufin [11].

Materials and Methods

Standard strain used in this study was *staphylococcus aureus* ATCC- 25923 obtained from central public health laboratory.Determinations of Minimal Inhibitory Concentration (MIC) of natural bioactive products by broth micro dilution method [9].

Preparation of Natural Products Stock Solutions

Essential oil was extracted from air-dried Thyme leaves (*T. vulgaris*) and Clove buds (E. *caryophyllata*) by the distillation method described in the [12]. The dried Clove buds and Thyme leaves were identified in department of biology; College of Science University of Baghdad .The samples (50 g) were added with distilled water (500 ml) in round bottom flask. They were distilled in Clevenger apparatus for 2-3 hours according to the plants, EOs were dried by Na2So4 and kept in (4 ºC) for sup sequent experiments.

To prepare, each EO was dissolved in dimethyl sulfoxide (DMSO) and were prepared in stock solution, which were subsequently diluted in Mueller Hinton Broth (MHB) test medium for further dilution preparations. A 2 ml of essential oils in the medium was seeded with the bacterial broth culture overnight (0.5 McF units).

The samples were incubated 18h at 37°C. After incubation the last tube without any visible growth of bacteria was taken to represent the minimum inhibitory concentration (MIC). The final concentration of EOs in the medium ranged from 25 %-0.05 % (v/v).

Stock solutions were prepared by using the formula: V1 C1= V2 C2

Bacterial Inoculums Preparation

Bacterial inoculums was prepared by colony suspension method with Mueller Hinton Broth (MHB), turbid solutions visually compared with the McFarland standard for turbidity vs. cell concentration, and verified by measuring the absorbance of the suspension spectrophotometrically. The absorbance should be range between 0.08-0.13 which is equal to $1*10^8$ CFU/ml.

Preparation of Resazurin

Resazurin (sigma, USA) was prepared at 0.015 % by dissolving 0.015g in 100 ml of sterile distilled water, vortexed and sterilized (0.22 µm filter) and stored at 4 ºC for a maximum of 2 weeks after preparation [13].

MIC Plate Preparation

A sterile plastic, disposable microdilution plate with 96 wells was taken. Into the well in each column (from 1-10), 50 µl from each tube containing the corresponding concentration of target EO was dispensed from the stock. The final well concentrations reached were 25 to 0.05 µg/ml for EOs, after the addition of inoculum (50µl). For each test plate two drugs free controls were kept, one with the 100 µl medium alone (sterility control, column 12) and the other with 50µl of medium plus 50µl of inoculum suspension (growth control, column 11). After the addition of inoculum, the microdilution plates were incubated at 37 ºC with low humidity for 18 hours. After incubation, resazurin (0.015 %) was added to all wells (30 µl per well), and further incubated for 2-4 h for the observation of colour change.

Reading and Interpretation

On completion of the incubation, columns with no colour change (blue resazurin colour remained unchanged) were scored as above the MIC value.

Results and Discussion

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation [14]. Well and disc diffusion methods have frequently been reported as qualitative indicators for testing the antimicrobial activity of natural products. The extracted Thyme EO and Clove EO were screened for their antibacterial activity and their MIC against the tested strain and two standards Thymole (Naissance, U.k) and Eugenol (Supelco, Germeni) were purchased to compare the MIC value with our extracted EOs Table (1) the results of the essential oils were interpreted after resazurin was added to all wells, and further incubated for 2-4 h at 37 °C.

Table 1: Minimum inhibitory concentrations (MICs) of antimicrobial essential oil for methicillin resistant S.aureus isolates

Natural products	MIC range (µg/mL)
Thyme EO	0.195 – 0.39
Clove EO	0.39 – 0.78
Thymol	0.045 - 0.097
Eugenol	0.045 - 0.097

Depending on break point of EOs for each isolate, if the value was equal or more than breakpoint means the bacterium was resistant to that EO. The results of MIC confirmed the previous results of natural bioactive products diffusion test. In our results it was found that thyme EO and clove EO showed the highest antibacterial activity at low MIC value against methicillin resistant *S.aureus* isolates. MIC value ranged from 0.195 µg/ml to 0.39 for thyme EO and from 0.39 µg/ml to 0. 781 for Clove EO, while using standard Thymol and Eugenol showed very low MIC value which ranged from 0.045 µg/ml to 0.097 µg/ml as showed in Figure (1).

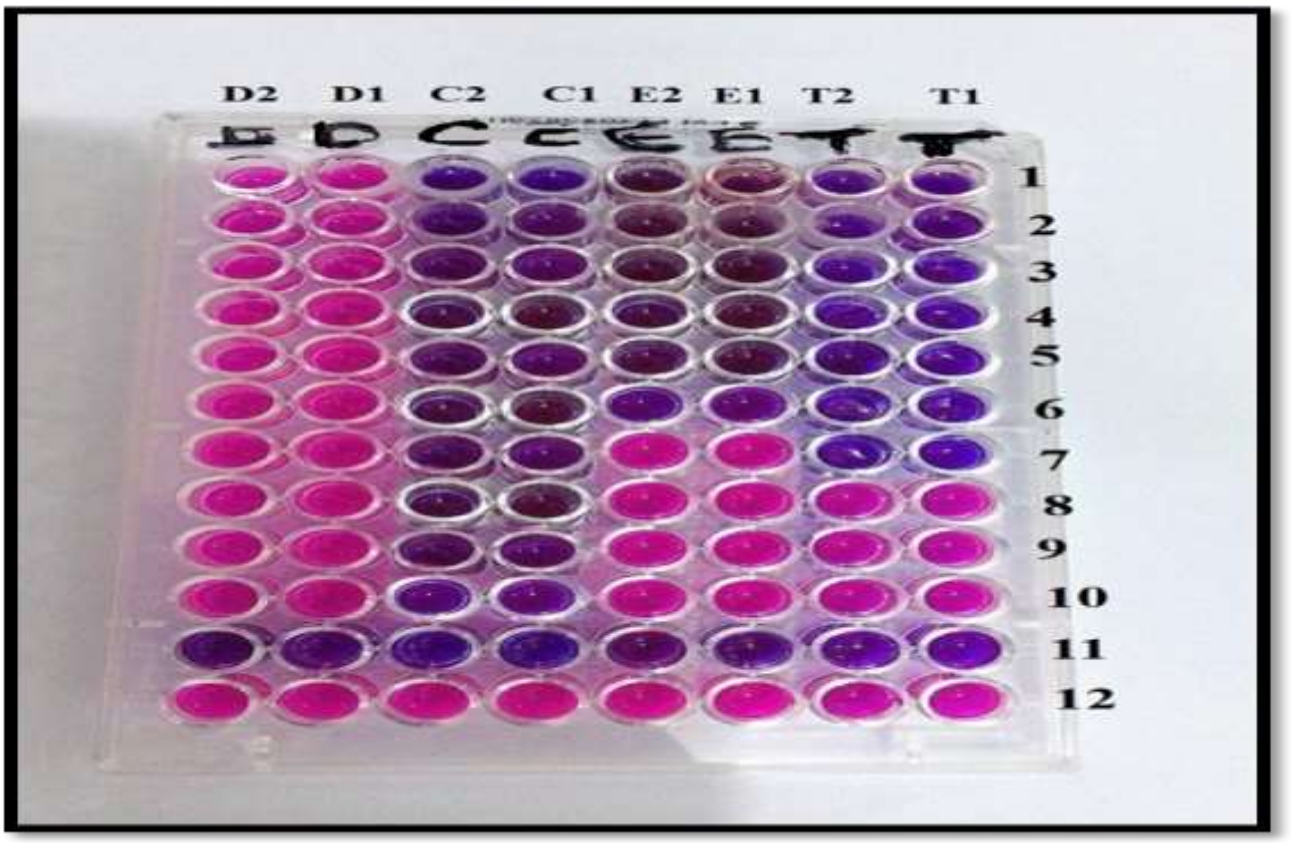

Figure 1: Determination of MIC for natural products and DMSO. Column 11 negative control confirms no contamination occurred while preparing the plate. Column 12, positive control shows a change of resazurin natural colour (blue/purple) to the reduced form (pink-colourless). The highest concentration incorporated into the plate is 25 µg/mL and the lowest achieved through double serial dilution is 0.05 µg/Ml to each of natural products, thyme EO (column T1 and T2), clove EO (column E1 and E2), standard (column C1= thymol and Eugenol = C2). The range of DMSO concentration in the wells of columns D1 and D2 was 50% - 0.05%

Because of the excessive use of antibiotics, bacteria become more resistant to drugs. On the other hand, bacteria have changed a lot over time, and antibiotics cannot treat most of the diseases they cause. Even if bacteria adapt and become resistant to antibiotics, medical and pharmaceutical specialists are trying to find new products that can be used to develop medicines to fight back against pathogens, in this context, researchers are continuously trying to find new molecules to fight against bacterial strains [15].

Essential oils were using in medicine and biomedical applications due to their antibacterial, antifungal and antiviral properties and their ability to prevent the growth of different pathogens [16].Was reported that the Clove EO have high activity against MRSA isolates and it had low MIC value (0.08 µg/Ml**)** which agree with our results and demonstrated that the clove EO does not pose a risk of resistance development in a continuous mode of use. Numerous studies in micro dilution broth model systems, have revealed the bacteriostatic or bactericidal activity of this essential oil on pathogens such as *S. aureus, Salmonella typhimurium, Escherichia coli, Ps. aeruginosa, Bacillus subtilis, spoilage bacteria Brochothrix thermosphacta, P. uorescens, Serratia liquefaciens* and *Lactobacillus carvatus* [17].

A study in Iran demonstrated that thyme EO had good activity against MRSA isolates and showed antimicrobial, and anti-inflammatory properties [18]. According to study an interesting result showed that Thyme EO had strong antibacterial activity against MRSA tested by microdilution method [19] which agrees with our results and these could be caused by inhibition of efflux pumps activity.

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