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Original Article

Antibacterial activity and mechanism of action of *Monarda punctata* essential oil and its main components against common bacterial pathogens in respiratory tract

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Abstract: The aim of the current research work was to study the chemical composition of the essential oil of *Monarda punctata* along with evaluating the essential oil and its major components for their antibacterial effects against some frequently encountered respiratory infection causing pathogens. Gas chromatographic mass spectrometric analysis revealed the presence of 13 chemical constituents with thymol (75.2%), p-cymene (6.7%), limonene (5.4), and carvacrol (3.5%) as the major constituents. The oil composition was dominated by the oxygenated monoterpenes. Antibacterial activity of the essential oil and its major constituents (thymol, p-cymene, limonene) was evaluated against *Streptococcus pyogenes, methicillin-resistant Staphylococcus aureus (MRSA), Streptococcus pneumoniae, Haemophilus influenzae and Escherichia coli.* The study revealed that the essential oil and its constituents exhibited a broad spectrum and variable degree of antibacterial activity against different strains. Among the tested strains, *Streptococcus pyogenes, Escherichia coli* and *Streptococcus pneumoniae* were the most susceptible bacterial strain showing lowest MIC and MBC values. *Methicillin-resistant Staphylococcus aureus* was the most resistant bacterial strain to the essential oil treatment showing relatively higher MIC and MBC values. Scanning electron microscopy revealed that the essential oil induced potent and dose-dependent membrane damage in S. pyogenes and MRSA bacterial strains. The reactive oxygen species generated by the *Monarda punctata* essential oil were identified using 2', 7'-dichlorofluorescein diacetate (DCFDA).This study indicated that the *Monarda punctata* essential oil to a great extent and thymol to a lower extent triggered a substantial increase in the ROS levels in *S. pyogenes* bacterial cultures which ultimately cause membrane damage as revealed by SEM results.

Keywords: *Monarda punctata*, essential oil, thymol, p-cymene, *Streptococcus pyogenes*, *methicillin-resistant Staphylococcus aureus*

Introduction

Upper respiratory tract infections (URTIs) are the ailments triggered by an acute infection involving nose, sinuses, pharynx, or larynx. The various URTIs include common cold, sinusitis, laryngitis, pharyngitis etc [1]. These infections are frequently caused by viruses like rhinovirus, corona virus, *parainfluenza* virus, *adenovirus* etc [2]. Many of the URTIs are also caused by bacteria like *Streptococcus pyogenes, Streptococcus pneumoneae, Heamophilus influenzae, Corynebacterium diptheriae, Bordetela pertursis and Bacillus anthracis.* Bronchitis, which is an inflammation of the mucous membranes of the bronchi, is also caused by certain types of bacteria. About 10% of the cases are caused by bacteria like *Mycoplasma pneumoniae, Chlamydophila pneumoniae, Bordetella perturis, Streptococcus pneumoniae* etc [3, 4].

Essential oils (which are composed of an odoriferous mixture of monoterpenes and Sesquiterpenes) have a long history of use in combating infection and many are marketed for their antiseptic properties. Essential oils are nature's antiseptics and their ability to kill microorganisms has been well documented over the centuries.Hippocrates, the founder of medicine, used aromatic plants as early as 500BC. For thousands of years, aromatic oils have been used to relieve a wide variety of

human maladies including bronchitis, pneumonia, pharyngitis, diarrhoea, periodontal disease, wounds, and numerous other illnesses. Many traditions surrounding the use of these oils are buried in antiquity, passed down orally from master to student until the origin of specific treatments were lost to the ages. In antiquity, medicinal oils were derived from aromatic plants and resins by extraction into other fatty oils such as olive oil and used as a mixture [5-7]. Many of the scientists consider essential oils as alternative to antibiotics. There has been an increase in the wide spread development of drug resistant microorganisms such *MRSA*, multidrug resistant strain of *Klebsiella pneumonia* and *P. Aeruginosa.* The essential oils target bacterial cell wall and cytoplasmic membrane and cause membrane permeabilization, which is followed by leakage of essential ions, drop in membrane potential, breakdown of the proton pump and depletion of the ATP pool [8, 9]. Tea tree oil, as an example, disrupts the cell membrane of the bacterium, causing a loss of potassium ions from the cell. The lipophilic (lipid loving) monoterpenes integrate with the phospholipids in the membrane and leakage occurs. Antimicrobial essential oils have also been known to cause inhibition of glucose dependant respiration in *S. aureus*, *E. coli* and *C. albicans*. Some clinical research in 2003 set out to compare tea tree oil with the standard topical antibiotic mupirocin. The purpose of the trial was to compare two methods of eliminating MRSA - from the nose.

Due to their multifunctionality and diverse chemical composition, essential oils and their components find a huge application in medicine and aroma therapy. Essential oils exhibit potent antimicrobial actions against a wide range of Gram-positive and Gram-negative bacteria [10]. Essential oils and their components have also been used traditionally for treating respiratory tract infections and are used these days as ethical medicines for colds [11-13]. Inhalation therapy using essential oils has been used to treat acute sinusitis and acute and chronic bronchitis. It has been reported that inhalation therapy using volatile essential oil vapours enhanced the respiratory tract fluid output [14], maintained the ventilation and drainage of the sinuses, reduced asthma and the inflammation of the trachea [15-17].

The objective of the present study was to study the chemical composition of the essential oil of *Monarda punctata* along with evaluating the essential oil and its major components for their antibacterial effects against some frequently encountered respiratory infection causing pathogens. Scanning Electron Microscopy (SEM) revealed that the essential oil of *Monarda punctata* induced substantial and dose-dependent morphological changes in the *Streptococcus pyogenes* bacterial strain killing more than 85% of the bacteria at higher concentration.

Materials and methods

Materials

The major essential oil components viz; thymol, p-cymene, limonene, and carvacrol were purchased from Sigma-Aldrich Chemical Company.

Plant material and essential oil isolation

The flowers of *Monarda punctata* were collected from a local region, China, during June-July 2013. The plant was identified by a well-known taxonomist. The extraction of the essential oil from the dried flowers was carried out by hydrodistillation for 4 hours using Clevenger apparatus as recommended in the European pharmacopoeia. The dried flower samples (500 gm) were subjected to hydrodistillation at separate times. The essential oil was collected and then dehydrated with anhydrous Na_2SO_4 (Sigma-Aldrich) and stored at -4°C before use.

GC-MS analysis and identification of components

GC-MS analysis was carried on a Varian Gas Chromatograph series 3800 fitted with a RTX-5 ms fused silica capillary column (30 m × 0.25 mm, film thickness 0.25 µm) using split/splitless injection, coupled with a 4000 series mass detector under the following conditions: injection volume 1.2 µl with split ratio 1:60, helium as carrier gas at 1.2 ml/min constant flow mode, injector temperature 240°C, oven temperature was programmed from 50 to 280°C at 5°C/min. Mass spectra: electron impact (EI+) mode, 70 ev and ion source temperature 250°C. Mass spectra were recorded over 50-500 a.m.u range. Identification of the essential oil constituents was done on the basis of Retention Index [RI, determined with respect to homologous series of n-alkanes (C_5-C_{28}, Polyscience Corp., Niles IL) under the same

Table 1. Chemical composition of the essential oil from the flowers of *Monarda punctata*

S. No	RT	Compound	% Peak area	Methods of identification
1		α-Thujene	1.0	MS, RI
2		α-Pinene	0.2	MS, RI
3		β-Pinene	0.9	MS, RI
4		α-Phellandrene	0.1	MS, RI
5		α-Terpinene	1.5	MS, RI
6		P-Cymene	6.7	MS, RI, Std
7		Carene	0.2	MS, RI
8		Limonene	5.4	MS, RI, Std
9		4-Terpineol	0.1	MS, RI
10		Methyl carvacrol	0.4	MS, RI
11		Thymol	75.2	MS, RI, Std
12		Carvacrol	3.5	MS, RI, Std
13		β-Caryophyllene	0.1	MS, RI
		Total (%)	95.3	
		Monoterpene hydrocarbons	16.0	
		Oxygenated monoterpenes	79.2	
		Sesquiterpene hydrocarbons	0.1	
		Oxygenated sesquiterpenes	nil	

experimental conditions], co-injection with standards (Sigma Aldrich and standard isolates), MS Library search (NIST 05 and Wiley), by comparing with the MS literature data [18].

Microorganism strains and culture media

Streptococcus pyogenes (ATCC 12344), Methicillin-resistant Staphylococcus aureus (MRSA) (ATCC 43300), Streptococcus pneumoniae (ATCC 2730), Haemophilus influenzae (ATCC 33391), Escherichia coli (Clinical strain) were used in the present study. All these bacteria strains were obtained from the State Key Laboratory of Microbial Resources (SKLMR), the institute of microbiology, Chinese academy of Sciences, China. Bacterial strains were grown on nutrient agar plates at 37°C and maintained on nutrient agar slants. Cell suspension of microorganisms in 0.5% NaCl was adjusted at 0.5 Mcfarland to obtain approximately 10^5 cfu/ml.

Determination of MIC and MBC values

The MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration) tests were carried out by the broth microdilution method [19]. The essential oil and the major components were dissolved in sterilized physiological saline solution (0.9%) supplemented with Tween-80 (Sigma) at final concentration of 0.5% (v/v). Serial doubling dilutions of the essential oils were prepared in a 96-well microtiter plate ranging from 10% to 0.125%. Each dilution (100 µL) was dispensed into the wells, then inoculated with (100 µL) of the bacterial suspension. The final concentration of each strain was adjusted to 105-106 CFU/mL. The MIC is defined as the lowest concentration of the essential oils, at which the microorganism does not show visible growth. The MBC is the lowest concentration at which the microorganism is killed. All experiments were performed in triplicate.

Time-kill dynamic curves

Time-kill dynamic were performed as described already [20] with slight alterations. The final concentration of suspension of the strain was adjusted to 105-106 CFU/mL. MICs, 2 MICs and MBCs of *Monarda punctata* essential oil and its main components were chosen for use in the time-kill dynamic protocols. After incubating for 0, 1, 2, 4, 8, 12, 24 and 30 h with the broth microdilution method, liquids (50 µL) were removed from the test solution for tenfold serial dilution. Thereafter, a 50 µL liquid from each dilution was spread on the surface of the agar plates and incubated at 37°C for 24 h, and the number of CFU/mL was counted. The solution with no essential oil was used as a control. Time-kill curves were created by plotting the log number of CFU/Ml against time (h).

Scanning electron microscopy (SEM) study of the bacteria after oil exposure

The SEM studies of the bacterial cultures were carried out as under. The overnight culture of *Streptococcus pyogenes* grown on MH agar at 37°C was put in a saline solution comprising of 0.2% Tween-80. Three different concentrations of the essential oil (30, 60, 60 and 90 µg/ml) were prepared and added into this suspension and was incubated at room temperature. After 24 hours, the bacterial cells were centrifuged at 8000 × g for 15 minutes. The bacterial cells were then washed with 0.1 mol/l Tris-acetate

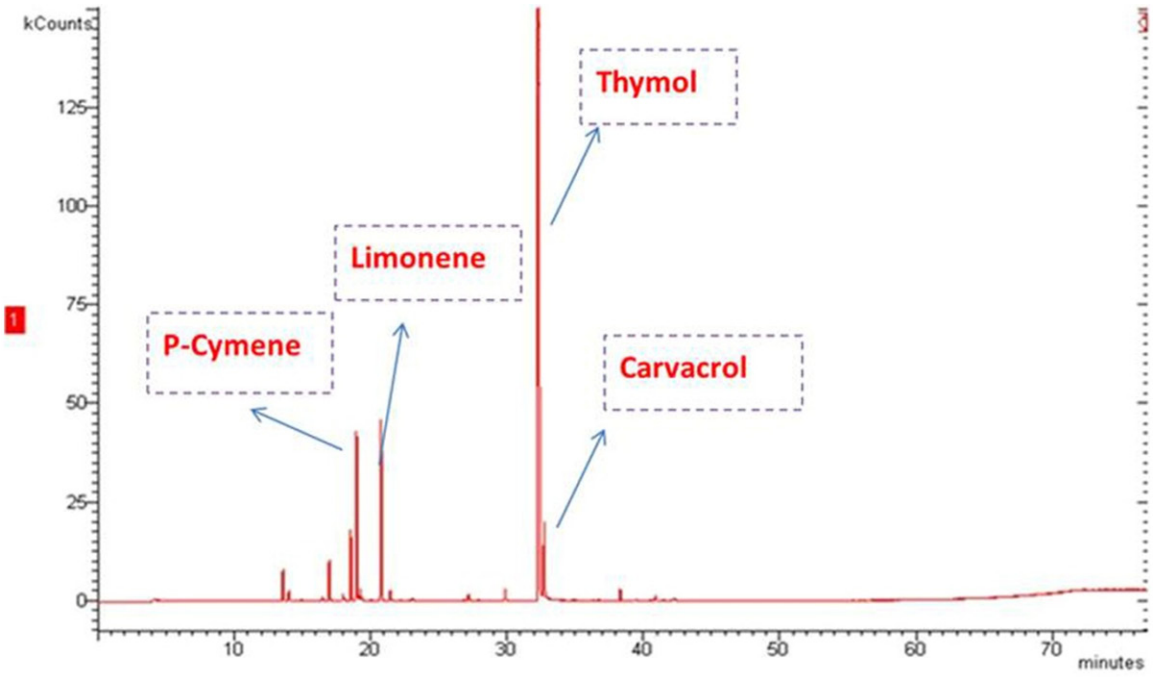

Figure 1. GC-MS Total Ion Chromatogram (TIC) of the essential oil of *Monarda punctata*.

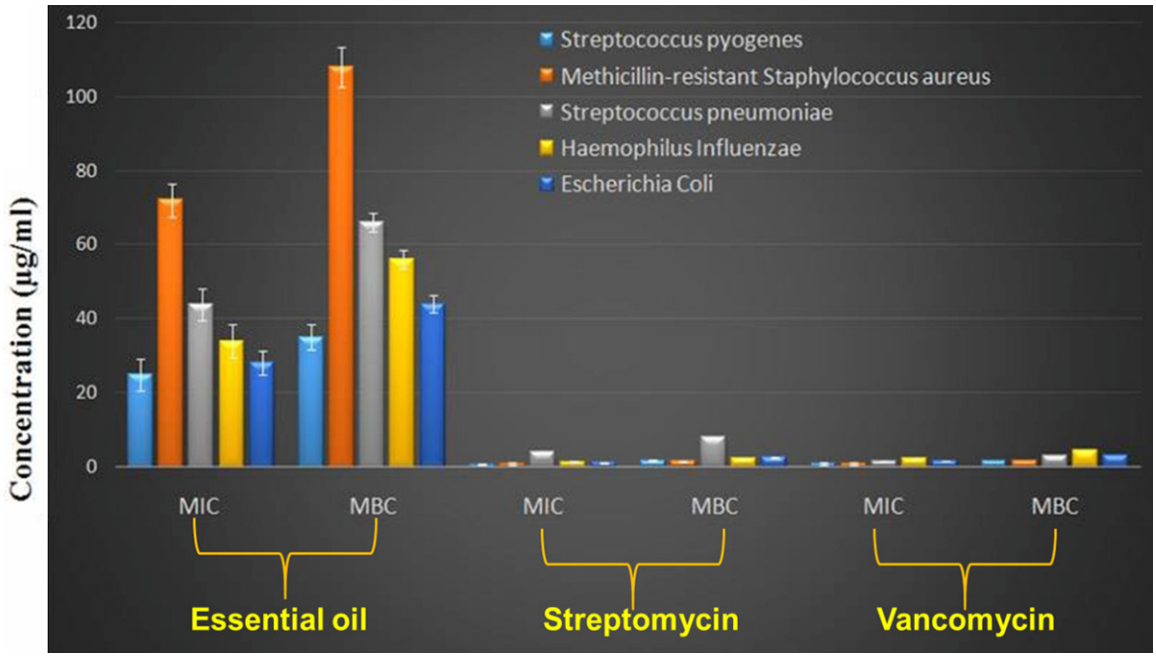

Figure 2. MIC and MBC values of the essential oil and two known antibiotics against different respiratory pathogens.

buffer (PH 7.1), fixed in tris-acetate buffer containing 1.5% glutaraldehyde, and then freeze-dried. Each of the bacterial culture was observed by SEM (Hitachi, Japan) at magnifications of 10,000 ×. The bacterial cell suspension in saline with no essential oil treatment served as a negative positive control.

Detection of reactive oxygen species (ROS) generated by essential oil

The reactive oxygen species generated by the *Monarda punctata* essential oil was identified using 2', 7'-dichlorofluorescein diacetate (DC-FDA) [21]. The concentration of essential oil,

thymol, limonene and p-cymene used was 90 µg/ml each and the number of bacterial cells used was adjusted to 105 CFU/ml. After all cultures were incubated at 37°C for 4 h, they were centrifuged at 4°C for 20 min at 300 × g, and then each supernatant was treated with 50 µM DCFDA for 1.5 h. The ROS formed in the sample were detected using Fluorescence Multi-Detection Reader (BIOTEK, U.S.A.) at 485 nm of fluorescence excitation wavelength, and 528 nm of emission wavelength.

Results

Chemical composition of the essential oil of Monarda punctata

The chemical compounds of the essential oil of *Monarda punctata* identified by GC-MS are given in (**Table 1**) and the GC-MS total ion chromatogram (TIC) is shown in (**Figure 1**). The yield of the essential oil obtained from the flowers was about 0.9% w/v. A total of 13 components were identified constituting 97.2% of the total oil composition. Thymol, p-cymene, limonene and carvacrol were found to be the major components. The oil composition was dominated by the presence of oxygenated monoterpenes constituting 83.2% of the total oil composition.

Antibacterial activity

The antibacterial activity of the *Monarda punctata* essential oil and its major constituents (Thymol, p-cymene, limonene and carvacrol) was assessed against some respiratory infection causing bacterial strains including *Streptococcus pyogenes, methicillin-resistant Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae and Escherichia coli.* The effectiveness of the *Monarda punctata* essential oil and the chemical constituents was assessed by determining inhibition zones, MIC and MBC values. The results are given in (**Figure 2**), (MIC and MBC values are given). *Streptococcus pyogenes, Escherichia coli* and *Streptococcus pneumonia* were the most susceptible bacterial strain showing lowest MIC and MBC values. *Methicillin-resistant Staphylococcus aureus* was the most resistant bacterial strain to the essential oil treatment showing relatively higher MIC and MBC values. With the aim to identify which of the chemical compounds of the *Monarda punctata* essential oil were active against these respiratory bacteria, further in-vitro experiment was carried out evaluating the antibacterial effects of the major constituents (thymol, p-cymene and limonene). Almost all the tested essential oil constituents exhibited reasonable to potent antibacterial effect against various bacterial strains. The major compounds of the essential oil viz., thymol, p-cymene and limonene exhibited higher values of MIC and MBC indicating that the whole essential oil is much more potent antibacterial agent as compared to the individual constituents. **Figure 3** shows the MIC and MBC values of thymol, p-cymene and limonene which constitute the bulk of the essential oil of *Monarda punctata*.

Time kill curve studies

The time-kill curve of *Monarda punctata* essential oil, towards *Streptococcus pyogenes* is shown in (**Figure 4**). MIC had a bacteriostatic effect on the bacterial population of *Streptococcus pyogenes* within the first 6 h, while MBC had a deadly effect on bacteria within 12 h by *Monarda punctata* essential oil. Time kill curve studies give an idea whether the essential oil has bacteriostatic or bactericidal effects with regard to the time span the microbes are exposed to the treatment of the drug. By this study, it can be concluded that within 12 h duration, the essential oil of *Monarda punctata* kills almost all the *Streptococcus pyogenes* bacterial strains.

Scanning electron microscopy (SEM) studies of Monarda punctata essential oil

In order to determine whether the essential oil of Monarda punctata essential oil induced serious morphological/structural changes in *S. pyogenes*, scanning electron microscopy was used which revealed that the essential oil induced a remarkable serious morphological change in the bacterial architecture of S. pyogenes and *Methicillin-resistant Staphylococcus aureus* (MRSA). **Figures 5** and **6** show the effect of the essential oil of *Monarda punctata* on the morphology of *S. pyogenes* and MRSA respectively. The bacterial cells of *S. aureus* were swollen after 12 hours of essential oil treatment. Three concentrations (30, 60, and 90 µg/ml) of the essential oil were used. SEM results showed that the essential oil induced dose-dependent cell morphological changes in the *S. pyogenes* and MRSA bacterial cells. It

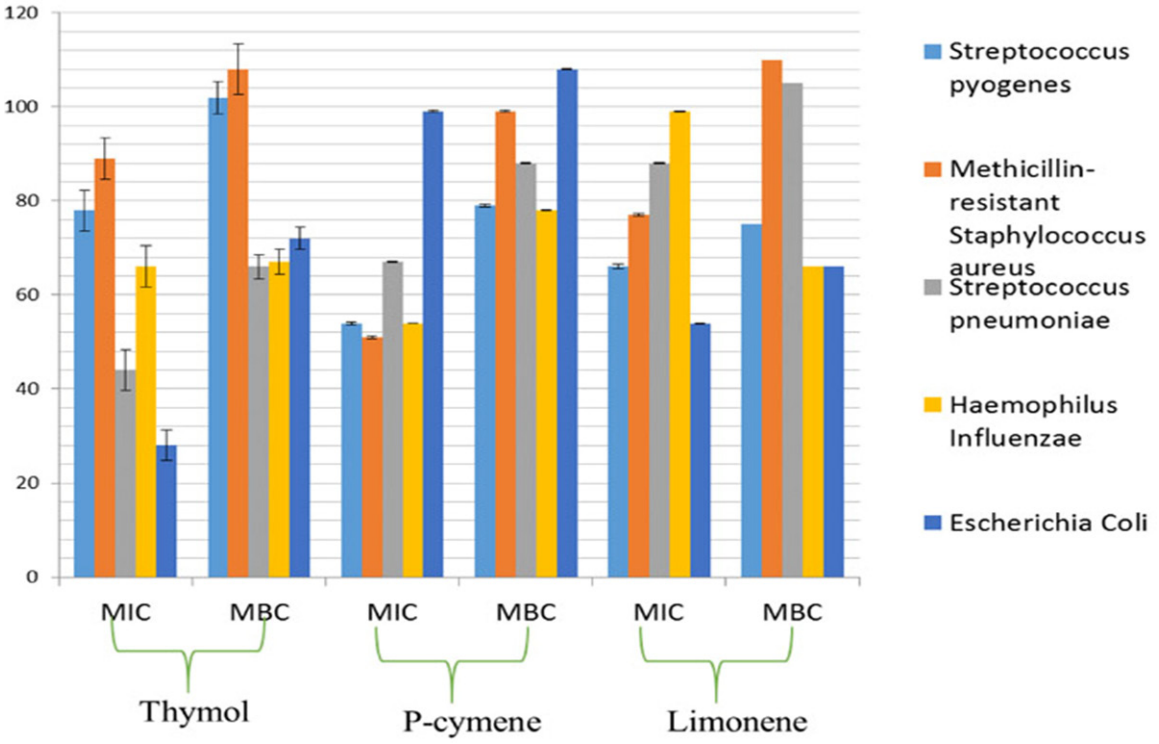

Figure 3. MIC and MBC values of thymol, p-cymene and limonene against different respiratory pathogens.

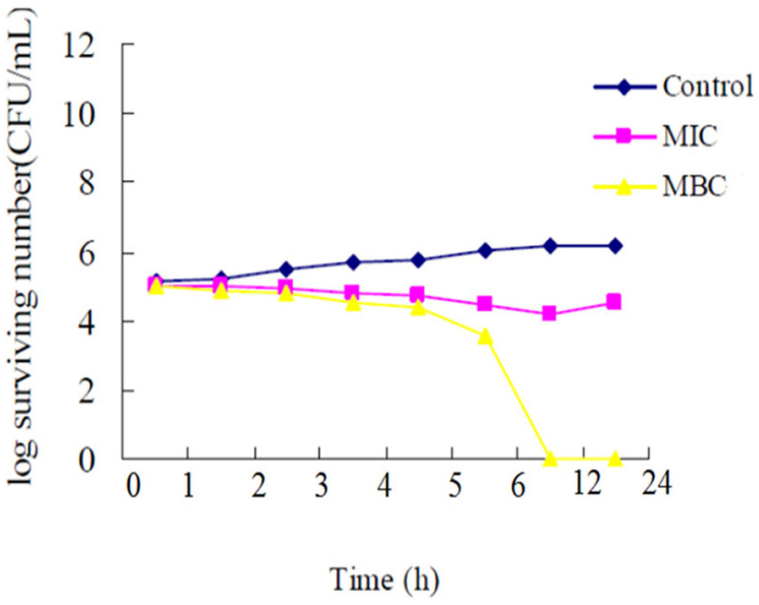

Figure 4. Time-kill curves of *Monarda punctata* essential oil against *Streptococcus pyogenes*. The control did not contain essential oil.

was observed that at a concentration of 90 μg/ml of the essential oil treatment to *S. pyogenes* and MRSA bacterial cells for 12 hours killed more than 95% of the bacterial cells indicating that the *Monarda punctata* essential oil produces bactericidal effects on these microbes. Alternatively, the untreated control cells did not show any changes in the cell morphology (**Figures 5** and **6**).

Generation of ROS from bacterial cells treated with Monarda punctata essential oil and three major constituents (thymol, p-cymene and limonene)

In several studies, it has been reported that various essential oils could trigger the formation of free radicals within bacterial cells, which eventually cause serious membrane damage. Increased levels of ROS could induce oxidative stress in bacterial cells which actually cause damage to cell membrane, vital proteins, DNA and bacterial respiratory system. In the current study, ROS was measured in S. pyogenes cultures using DCFDA. After 4 h incubation, elevated levels of ROS were detected

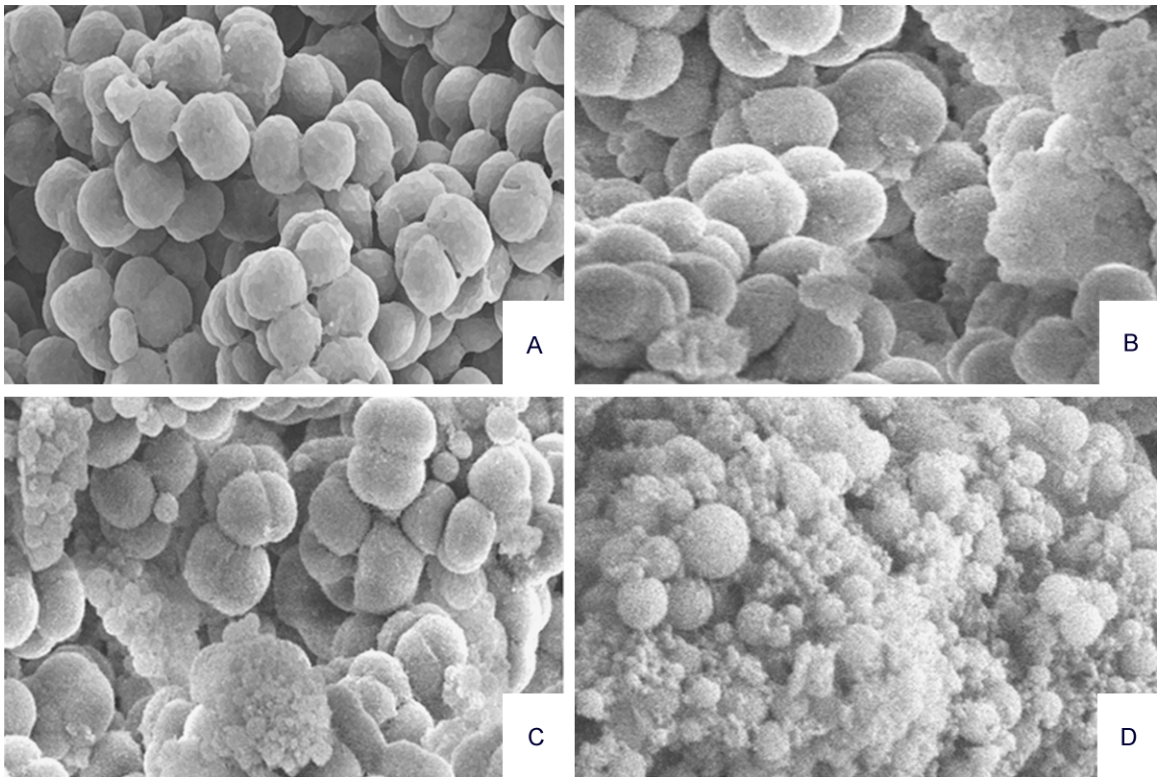

Figure 5. Electron micrographs of *S. pyogenes* exposed to different concentrations of the *Monarda punctata* essential oil. The images were taken at a magnification of 10,000 × (A, *S. pyogenes* 12 h in saline; B-D. scanning electron micrographs of *S. Pyogenes* treated for 12 h with 30, 60, and 90 µg/ml of the *Monarda punctata* essential oil.

and measured in the *Monarda punctata* essential oil treated bacterial group. Thymol treated group also showed moderate increase in the levels of ROS followed by p-cymene and limonene-treated groups (**Figure 7**).

Discussion

Since early times, essential oils have been used for the management of infections caused by different bacteria all over the world for centuries in the traditional medicine system. Today, essential oils have a wide range of applications and are used in alternative and holistic medicine for similar purposes. They are administered orally, topically or via aromatherapy procedures. An increasing number of scientific studies have initiated the process of explaining the specific mechanism (s) of action of essential oils and their components. Emerging evidence has shown that many essential oils have both non-specific and specific mechanisms of action which differs based on the comparative abundance and chemical composition of the components. Explanation of the mode of action of these essential oils/compounds may facilitate identification of new antibiotic targets and exploitation of novel biochemical pathways; pathways not currently targeted by existing antibiotics. Since antibiotic resistance is currently overtaking scientific research to find new drugs, medical scientists are confronting a return to the 'pre-antibiotic era'. Essential oils or some of their components are used in perfumes and make-up products, in sanitary products, in dentistry, in agriculture, as food preservatives and additives, and as natural remedies [8]. In recent years, due to the new interest in natural products, plant essential oils have come more into the focus of phytomedicine [22, 23]. It is important to develop a better understanding in basic research applications, especially, of the anti-microbial activity. The essential oils exhibit a broad spectrum of biological activity which has led to an increased interest among scientists [24].

In the current study, we evaluated the antibacterial potential of Monarda punctata essential oil as well as its three major constituents (thy-

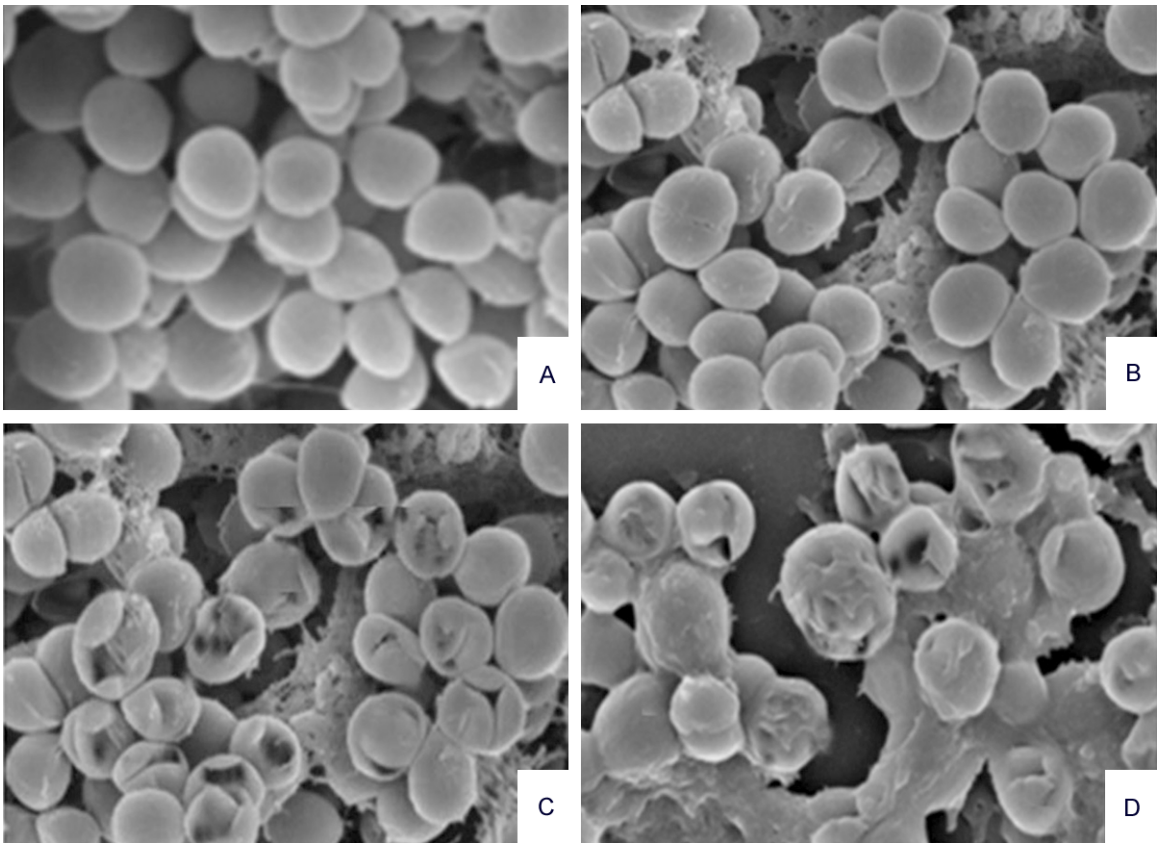

Figure 6. Electron micrographs of *MRSA* exposed to different concentrations of the *Monarda punctata* essential oil. The images were taken at a magnification of 10,000 × (A. MRSA12 h in saline; B-D. scanning electron micrographs of MRSA treated for 12 h with 30, 60 and 90 µg/ml of the *Monarda punctata* essential oil.

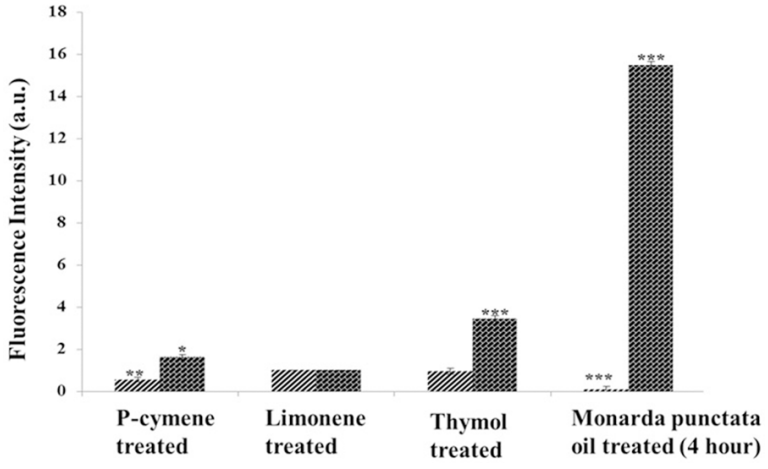

Figure 7. Formation of ROS in *S. Pyogenes* cells exposed to 90 µg/ml each of p-cymene, limonene, thymol and essential oil. Bacterial cultures were treated with 90 µg/ml each of these compounds and whole M.P essential oil for 4 h. ***Statistical significance at $P < 0.01$. **Statistical significance at $P < 0.05$.

mol, p-cymene and limonene) against a panel of respiratory tract infection causing bacterial bugs including *Streptococcus pyogenes, methicillin-resistant Staphylococcus aureus (MRSA), Streptococcus pneumoniae, Haemophilus influenzae and Escherichia coli.* The mode of action of the essential oil and its components was evaluated using scanning electron microscopy and ROS generation protocol in the bacterial cultures. Time kill curve studies were done to elucidate whether the essential oil has bacteriostatic or bactericidal effects. The results showed that at the essential oil is very active against all tested microbes especially against *S. pyogenes*. *S. pyogenes* was further studied whether the essential oil induced morphological damage in it, which was finally confirmed by SEM results. The essential oil to a large

extent and thymol to a lower extent triggered ROS generation in these bacterial cultures.

In conclusion, this study concludes that the essential oil of *Monarda punctata* is a potent source of antibacterial agents against various respiratory tract infection causing microbes. The antibacterial effects of the essential oil are facilitated through inducing significant morphological changes in bacterial cells along with generating reactive oxygen species within the bacterial cultures. The essential oil even caused serious morphological damage to the drug resistant Staphylococcus aureus which is very encouraging. These essential oils could be used as a safe and natural alternative to antibiotics with less incidence of showing multidrug resistance.

Disclosure of conflict of interest

None.

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