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Antibacterial activity of *Artemisia asiatica* essential oil against some common respiratory infection causing bacterial strains and its mechanism of action in *Haemophilus influenzae*

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**A R T I C L E   I N F O**

**Keywords:**
*Artemisia asiatica*
Essential oil
Gas chromatography
Antibacterial activity
*Haemophilus influenzae*

**A B S T R A C T**

The main objective of the current study was to investigate the chemical composition of the essential oil of *Artemisia asiatica* together with investigating the antibacterial effects it exerts on several common respiratory infection causing bacteria including *Haemophilus influenzae*. Its mechanism of action was studied using various state-of-the-art assays like scanning electron microscopy, DNA, RNA and protein leakage assays, growth curve assays etc. The essential oil was extracted from the leaves of *A. asiatica* by supercritical CO$_2$ fluid extraction technology. Chemical composition of essential oils was analyzed by gas chromatography-mass-spectrometry (GC-MS). The antibacterial activity was evaluated against 6 bacteria by the paper disc diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) values of the essential oil were estimated by agar dilution method. The antibacterial mechanism was evaluated by growth curve, the integrity of cell membrane and scanning electron microscope (SEM). Gas chromatographic analysis of the *A. asiatica* essential oil led to the identification of 16 chemical constituents accounting for 97.2% of the total oil composition. The major components were found to be Piperitone, (z)-davanone, p-cymene and 1, 8-cineole. The essential oil showed maximum growth inhibition against *Haemophilus influenzae* with a zone of inhibition of 24.5 mm and MIC/MBC values of 1.9/4.5 mg/mL respectively. Bacteria treated with the essential oil led to a rapid decrease in the number of viable cells. On adding the essential oil of *A. asiatica* to the bacterial culture, the constituents of the bacterial cell got released into the medium and this cell constituent release increased with increasing doses of the essential oil. SEM showed that the bacterial cells treated with the essential oil showed damaged cell wall, deformed cell morphology and shrunken cells.

1. Introduction

Plant essential oils are known for their multiple uses in perfumery, as preservatives and antimicrobials, which are commonly extracted from leaves, branches, roots, stems, fruits and seeds of aromatic plants [1]. The plant essential oil is a mixture of various components from plants. According to the different chemical structures of the essential oil, the composition can be divided into monoterpene, sesquiterpene, alcohols, esters, aldehydes and ketones. The constituents are very complex which are involved in the defense of the plant against pests, herbivores, fungi, and bacteria [2,3]. Essential oils have many applications in indigenous medicines, food flavouring and preservation as well as in drug and cosmetic industries [4]. It also has antibacterial, anti-inflammatory, anti-oxidation, anti-tumor and other functions [5,6]. Due to the toxicological effect of the synthetic products, renewed efforts were provided in respect of the use of essential oils as natural antioxidants and preservatives in the food processing, food supplement production and pharmaceutical industry [7].

The genus *Artemisia* L. (*Asteraceae*) comprises a variable number of species (from 200 to over 400, depending on the authors) found throughout the northern half of the world. *Artemisia* popularly known as sage brush or wormwood is a source of valuable drugs and essential oils. Because of medicinal importance and intricate chemical composition of several species and chemotypes, *Artemisia* continues to be a subject of wide interest for chemists and taxonomists [8–10]. It has been reported that most of the phytocompounds of *A. asiatica* comprise of terpenes and terpene alcohols, 1, 8-cineole, camphor, borneol, bornyl acetate, artemisolide, alkaloids, flavone eupatilin etc [11–14].

It has been reported that a standardized extract of *A. asiatica* exhibited hepatoprotective activity on liver damage induced by acetaminophen and carbon tetrachloride [15]. In another study, it was reported that this standardized extract of *A. asiatica* (which was called as...
DA-9601) showed chemopreventive effects against azoxymethane-initiated and dextran sulphate sodium-promoted mouse colon cancer. DA-9601 also led to the suppression of COX-2 expression and also inhibited nuclear factor (NF)-kappa-B DNA binding in the colonic tissues [16]. The essential oil composition of A. asiatica has already been reported as well as its antibacterial and antifungal activity against a range of microbial strains [12]. However, its mode of action with regard to its antibacterial action has not been studies so far. Therefore, the aim of the study was to investigate the chemical composition and antibacterial activity of the essential oil on several respiratory infection causing bacteria and to further evaluate the antibacterial mechanism against Haemophilus influenzae growth curve, the integrity of cell membrane and to determine the amounts of the DNA and RNA released from the cytoplasm, the supernatant was used to measure the optical density at 260 nm.

2.7. Experiment involving leakage of proteins through the bacterial membrane

The cell integrity was further examined by determining the release of proteins into the supernatant. The concentrations of proteins in supernatants were determined by Bradford's method [19]. Logarithmic growth phase cells of bacteria were treated with the essential oil at 1×MIC, 2×MIC value except the control. Then the samples were incubated at 37 °C for 1, 3, 9 and 24 h respectively. The samples were then immediately filtered with 0.2 μm organic membrane. To determine the amounts of the DNA and RNA released from the cytoplasm, the supernatant was used to measure the optical density at 260 nm.

2.8. Scanning electron microscope (SEM) study of bacterial ultrastructure

Scanning electron microscopy was used to observe the morphological changes of bacteria as per the method already described in literature [20]. Haemophilus influenzae bacteria were incubated in NB medium at 37 °C for 12 h. Logarithmic growth phase cells of K. pneumoniae was treated with the essential oil at 2×MIC value except the control. The samples were incubated at 37 °C for 3, 9 and 24 h.
was 0.4%. Gas chromatographic analysis of the lyzed by GC-MS. The yield of essential oil from the leaves of the technology. The chemical compositions of the essential oil were ana-
sential oil led to the identi-
Oxygenated monoterpenes were found to be the dominant class of were found to be Piperitone, (z)-davanone, p-cymene and 1, 8-cineole. counting for 97.2% of the total oil composition. The major components of inhibition of 24.5 mm and MIC/MBC values of 1.9/4.5 mg/mL respectively. Then the bacteria were centrifuged (12,000 g, 10 min, 4 °C), fixed with 2.0% (v/v) glutaraldehyde (4 °C, 3 h) and washed with 0.1 M PBS (pH 7.2, 2 times). After centrifugation, the cells were fixed with 1% osmic acid (4 °C, 1.5 h), then washed 0.1 M PBS (pH 7.2). Then the cells were dehydrated in a graded series of ethanol (50%, 70%, 80%, 90% and 100%). Finally, the samples were sputter-coated with gold 157 under vacuum, followed by microscopicexaminations using a SEM (Hitachi S-3000H; Hitachi, Ltd., Tokyo, Japan).

2.9. Statistical analysis

The experiments were carried out in triplicates and expressed as mean ± SD. Student's test was used for statistical analysis and p values were considered significant at p < .01.

3. Results

3.1. Chemical compositions of the essential oil of Artemisia asiatica

The essential oil was obtained by supercritical CO2 fluid extraction technology. The chemical compositions of the essential oil were analyzed by GC-MS. The yield of essential oil from the leaves of the A. asiatica was 0.4%Gas chromatographic analysis of the A. asiatica essential oil led to the identification of 16 chemical constituents accounting for 97.2% of the total oil composition. The major components were found to be Piperitone, (z)-davanone, p-cymene and 1, 8-cineole. Oxygenated monoterpenes were found to be the dominant class of terpenes, followed by the monoterpane hydrocarbons. Fig. 1 shows the GC-MS Total Ion Chromatogram (TIC) of the essential oil of A. asiatica while as Table 1 lists the identified chemical components.

3.2. Antibacterial activity of the essential oil

The essential oil was tested for its antibacterial activity against 6 kinds of bacteria. The zone of inhibition values of the essential oil from A. Asiatica are presented in Table 2. The results showed that the essential oil showed some antibacterial effect against all the tested bacteria including both Gram-positive and Gram-negative bacteria. In order to determine the antibacterial spectrum of the essential oil, 3 well-known standard drugs viz., Penicillin, chloramphenicol and streptomycin were used as positive controls. The results revealed that essential oil of A. asiatica exhibited a broad spectrum of antibacterial activity against the various microbial strains. The essential oil showed maximum growth inhibition against Haemophilus influenzae with a zone of inhibition of 24.5 mm and MIC/MBC values of 1.9/4.5 mg/mL. respectively. However, the essential oil showed lowest growth inhibi-against Pseudomonas aeruginosawith a zone of inhibition of 9.2 mm and MIC/MBC values of 5.8/9.4 mg/mL respectively. This is not un-
pected because Pseudomonas aeruginosais a Gram negativebacteria which usually are resistant to most of the drugs used. The MIC/MBC values of the essential oil indicated that the antibacterial activity of the oil is somehow comparable to the standard positive controls. MIC was the minimum essential oil concentration that can prevent the bacteria from obvious growth. MBC was the lowest concentration of the essential oil that could kill the inoculum bacteria [21]. Thus from zone of inhibition values and MIC/MBC values, it was shown that Haemophilus influenzae was the most affected bacteria, therefore further mechanistic studies were done on this bacterial strain.

3.3. Growth curve studies

According to the sensitivity of the tested bacteria, Haemophilus influenzae was selected as the model bacteria for further study to confirm the antibacterial mechanism of the essential oil from A. asiatica. The results are shown in Fig. 2. Compared to the bacteria treated with the essential oil, the control showed a fast increase in bacterial number. On the contrary the bacteria treated with the essential oil led to a rapid decrease in the number of viable cells. The growth of bacteria was suppressed by the essential oil. In the treatments (1 × MIC) showed a slow decrease in bacterial number over the first 12 h period of the test.

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**Table 1**

| S. No | Compound      | % Peak Area | Methods of identification |
|-------|---------------|-------------|---------------------------|
| 1     | α-Pinene      | 3.5         | MS, RI                    |
| 2     | β-Pinene      | 0.2         | MS, RI                    |
| 3     | δ-3-Carene    | 0.5         | MS, RI                    |
| 4     | α-Terpinepene | 1.9         | MS, RI                    |
| 5     | P-Cymene      | 14.5        | MS, RI, Std               |
| 6     | 1,8-Cineole   | 23.4        | MS, RI, Std               |
| 7     | γ-Terpinepene | 0.9         | MS, RI                    |
| 8     | (Z)-β-Ocimene | 4.2         | MS, RI                    |
| 9     | 4-Terpineol   | 1.2         | MS, RI                    |
| 10    | (E)-Piperitol | 4.2         | MS, RI                    |
| 11    | Piperitone     | 21.2        | MS, RI, Std               |
| 12    | Ascaridole    | 1.3         | MS, RI                    |
| 13    | α-Curcumene   | 3.3         | MS, RI                    |
| 14    | Germacrene    | 5.2         | MS, RI                    |
| 15    | α-Bergamotene | 2.2         | MS, RI                    |
| 16    | (Z)-Davanone  | 9.7         | MS, RI, Std               |
| Total |               | 97.9%       |                           |

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**Fig. 1.** The GC-MS Total Ion Chromatogram (TIC) of the essential oil of A. asiatica.
Fig. 3 shows the results when Leakage of DNA and RNA through the membrane of these bacteria. The number of viable cells with the treatment of 2×MIC reduced significantly in the first three hours after cultivation. The results indicated that different incubation time and concentration of the essential oil of A. Asiatica had great effects on antibacterial activities.

3.4. The essential oil of A. asiatica led to the leakage of DNA and RNA through bacterial cell membrane

Further antibacterial mechanism of the essential oil of Artemisia asiatica against the Haemophilus influenzae was tested using the assay of Leakage of DNA and RNA through the membrane of these bacteria. Fig. 3 shows the results when Haemophilus influenzae were treated with different concentrations of the essential oil from A. Asiatica for 1, 4, 8 and 24 h, respectively. Results showed that on adding the essential oil of A. asiatica to the bacterial culture, the constituents of the bacterial cell got released into the medium and this cell constituent release increased with increasing doses of the essential oil. The optical density (OD260) value of supernatant from Haemophilus influenzaetreated with essential oil (1xMIC) was much higher as that of the untreated control for 1, 4, 8 and 24 h respectively. The optical density values were even much higher when the concentration of the essential oil was 2 x MIC. It is also important to mention here that the optical density values also increased with the incubation intervals of 1, 4, 8 and 24 h. This assay confirms that the essential oil of A. asiatica led to the damage of the cell membrane of Haemophilus influenzae which results in the leakage of the macromolecules including DNA and RNA.

3.5. The essential oil of A. asiatica led to the leakage of proteins through bacterial cell membrane

Proteins play vital roles within bacterial cell. In this assay, the effect of A. asiatica essential oil on the protein leakage from the Haemophilus influenzae bacteria was studied. The results which are shown in Fig. 4 indicate that the essential oil of A. asiatica resulted in the leakage of essential proteins from the Haemophilus influenzae bacterial cells by damaging the bacterial cell membrane. The leakage of proteins at the start in the untreated control was much lower as compared to the leakage of proteins treated with A. asiatica essential oil. The leakage of the proteins at 1 h incubation was 0.5 μg/mL in the untreated control, while as the leakage of proteins increased to 2.4 μg/mL (1 x MIC) and 3.8 μg/mL (2 x MIC). The leakage of proteins not only increased with essential oil dose but also increased with the treatment time. The results indicated that the essential oil of A. asiatica decreased the content of cellular proteins by permeating and disrupting cell membrane.

3.6. Morphological evaluation of the bacteria using scanning electron microscope (SEM)

In this assay, we evaluated the effects of the essential oil on the ultrastructural features of Haemophilus influenzaeusing SEM. The results of this assay are shown in Fig. 5 and reveal that as compared to the untreated control bacteria, the essential oil treated bacterial cells

| Bacterial strain (cat. no.) | Zone of inhibition (mm) |
|----------------------------|------------------------|
|                            | Essential oil (μg/mL) | Penicillin | Chloramphenicol | Streptomycin | MIC (mg/mL) | MBC (mg/mL) |
|-----------------------------|-----------------------|------------|----------------|--------------|--------------|--------------|
| Staphylococcus aureus (ATCC6538) | 12.7                  | 34.2       | 33.6           | 32.3         | 2.5          | 5.2          |
| Streptococcus pyogenes (ATCC12344) | 14.3                  | 34.7       | 32.7           | 33.7         | 3.1          | 6.7          |
| Listeria monocytogenes (ATCC19115) | 18.1                  | 36.2       | 28.5           | 26.5         | 4.2          | 8.9          |
| Pseudomonas aeruginosa (ATCC27853) | 9.2                   | 24.7       | 23.9           | 28.4         | 5.8          | 9.4          |
| Klebsiella pneumoniae ATCC46117 | 19.2                  | 34.9       | 31.1           | 34.5         | 2.3          | 5.8          |
| Haemophilus influenzae ATCC-33391 | 24.5                  | 26.4       | 29.4           | 25.6         | 1.9          | 4.5          |
revealed serious damage to the cell. The bacterial cells treated with the essential oil showed damaged cell wall, deformed cell morphology and shrunken cells. The damage to the cells seemed to enhance with increasing doses of the essential oil. In contrast, untreated cells showed rod shaped, regular, and intact morphology which were uniform in size and shape. This supported the results of the growth curve study and integrity of cell membrane assays, and showed that the non-reversible damage to the cell wall and membrane occurred.

4. Discussion

Respiratory tract infections are characterized by acute infection usually involving nose, larynx, sinuses and pharynx. The different kinds of upper respiratory tract infections include the cold, laryngitis, pharyngitis, sinusitis etc [22]. The main contributing factors towards these nasty infections are the infections caused by certain bacteria like Haemophilus influenzae, Streptococcus pneumoniae, Bacillus anthracis, Streptococcus pyogenes etc. Some viruses like coronavirus, para influenza virus etc can also cause certain types of upper respiratory tract infections [23,24]. Many of the bacterial strains have acquired resistance against the commonly used antibiotics resulting in limited efficacy of these drugs. As such there is an urgent need for novel, promising and cheap antimicrobial drugs which do not develop resistance easily against these microbes. Essential oils have wide range of applications in medicine and aromatherapy owing to their multiple chemical constituents in their mixtures giving rise to multifunctionality. Essential oils have been reported to exhibit antibacterial activity against a range of Gram-positive as well as Gram-negative bacterial strains [25]. It has been reported that traditionally essential oils and their constituents have been used for the treatment of various upper respiratory tract infections including colds. Inhalation therapy using essential oils has been successfully used to treat acute disorders like sinusitis, acute and chronic bronchitis and even for reducing the tracheal inflammation [26–29].

In the current study, we evaluated the antibacterial effects of Artemisia asiatica essential oil along with demonstrating its mechanism of action by using different state-of-the-art assays including DNA, RNA and protein leakage assays, dynamic curve assay and scanning electron microscopy assay. The mechanistic studies were performed on Haemophilus influenzae which was shown to be most susceptible to the essential oil treatment. The essential oil showed significant and broad spectrum antibacterial activity against the various microbial strains. The essential oil showed maximum growth inhibition against Haemophilus influenzae with a zone of inhibition of 24.5 mm and MIC/MBC values of 1.9/4.5 mg/mL respectively. However, the essential oil showed lowest growth inhibition against Pseudomonas aeruginosa with a zone of inhibition of 9.2 mm and MIC/MBC values of 5.8/9.4 mg/mL respectively. Growth curve studies revealed that as compared to the bacteria treated with the essential oil, the control showed a fast increase in bacterial number. On the contrary the bacteria treated with the essential oil led to a rapid decrease in the number of viable cells. Our results are well in agreement with previous which have reported the essential oil of other Artemisia species to exert significant antimicrobial activities [30]. The essential oil components observed in case of other Artemisia were more or less similar as observed in the present study [30]. Furthermore the results showed that on adding the essential oil of A. asiatica to the bacterial culture, the constituents of the bacterial cell got released into the medium and this cell constituent release increased with increasing doses of the essential oil. SEM investigations revealed that the essential oil treatment led to damaged cell wall, deformed cell morphology, shrunken cells and also caused DNA damage. The damage to the bacterial cells seemed to enhance with increasing doses of the essential oil. Our results are information with previous studies wherein essential oils have been reported to inhibit cell wall synthesis via interaction with a number of cellular enzymes [31,32]. Besides, essential oils have also been reported to induce DNA damage in bacterial cells and hence further confirming our results [33]. Given the promising results of the present study, we believe that further in vivo investigation should be carried out on the essential of A. asiatica to establish it as an important antimicrobial agent.

5. Conclusion

In brief, the current study reports the chemical composition of the essential oil of Artemisia asiatica along with its antibacterial activity against some common respiratory infection causing microbes. The essential oil exhibited significant antibacterial activity against Haemophilus influenzae. Firstly, the essential oil was seen to damage the cell membrane which further led to the leakage of DNA, RNA, and proteins. Further, the SEM experiment was performed to examine the effects of the essential oil of A. asiatica on cell morphology of Haemophilus influenzae. The SEM experiment showed that severe morphological changes appeared in the cell wall and membrane, and the microscopy of treated cells showed many irregularly deformed and shrunken bacteria.
Conflicts of interest

The authors declare that there is no conflict of interest to reveal.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2017.12.032.

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