CHEMICAL CONSTITUENTS FROM ALGERIAN FOeniculum vulgare AERIAL PARTS AND EVALUATION OF ANTIMICROBIAL ACTIVITY

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ABSTRACT

Phytochemical study of the aerial parts of Foeniculum vulgare led to the isolation of seven compounds isolated for the first time from this species. After isolation and purification, these metabolites were characterized on the basis of spectroscopic analyses using 1D and 2D NMR as well as mass spectrometry. Furthermore, antimicrobial activity of the crude extract was evaluated using agar diffusion method. The antimicrobial test results showed that the crude extract had a great potential as antimicrobial activity against all 9 microorganisms especially fungal strains.

Keywords: Foeniculum vulgare, Umbelliferae, methane monoterpenic derivative.

1. INTRODUCTION

There are growing interests, both in the industry and in the scientific research, for aromatic and medicinal plants because of their antimicrobial and antioxidant properties. These properties are due to many active phytochemicals including flavonoids, terpenoids, carotenoids, coumarins, curcumains, etc. These bioactive principles have also been confirmed using modern analytical techniques1-4. Hence, they are considered to be important in diets or medical therapies for biological tissue deterioration due to free radicals. Herbs and spices are amongst the most important targets to search for new antimicrobials and antioxidants from the point of view of safety5,6. So far, many investigations on antimicrobial8-12 and antioxidant properties of spices volatile oils and extracts have been carried out.

Foeniculum vulgare Mill (Fam. Umbelliferae), commonly known as fennel, is a small genus of annual, biennial or perennial herbs distributed in central Europe and Mediterranean region which is used in traditional medicine and as spice. Herbal drug preparations, from numerous wild types, are active for dyspeptic complaints, bloating and flatulence5,6. Diuretic, analgesic and antipyretic activity has also been found in the fennel fruit10 as well as antioxidant activity11. It is widely cultivated throughout the temperate and tropical regions of the world for its aromatic fruits, which are used as a culinary spice14,16,17. Steam distillation of dried fruits yields an essential oil referred as “Fennel oil”, used in western countries for flavouring purposes18. Although, the chemical constituents and antimicrobial properties of the fruit volatile oil of F. vulgare are well studied18-22, potential antimicrobial properties have not yet been studied for its aerial parts. The leaves and fruit are mainly used to flavour fish and meat, giving them a strong aroma and taste, and as an ingredient in cosmetics. The most frequently investigated was the essential oil which showed antioxidant, antimicrobial and hepatoprotective activity23,24. The chemical composition of the volatile oil fraction has been well described in the literature23,25. Earlier investigation of F. vulgare fruit led to the isolation of phenolic components with antihypertensive activity23,25. The target of this study was to isolate and characterize antimicrobial compounds from the earlier parts of F. vulgare.

2. MATERIALS AND METHODS

2.1 GENERAL

1H NMR (500 MHz, CDCl3), 13C NMR (125 MHz, CDCl3) and 2D spectra were recorded on a JEOL Lambda 500 spectrometer, with TMS as an internal standard. EIMS and HREIMS were recorded on a JEOL SX102A mass spectrometer. IR spectra were recorded on a JASCO FT/IR-5300 spectrometer.

2.2 PLANT MATERIAL

F. vulgare aerial part were collected in March 2003 Ouargla, southeast of Algeria, and identified by Dr. Chahma Abdel- Majid, Department of biology, Ouargla university. A voucher specimen was kept at the Herbarium of Laboratory of natural products, Department of chemistry, Constantine University under the code Number ZA 102.

2.3 EXTRACTION AND ISOLATION

The air-dried powdered earlier parts (1 Kg) of F. vulgare were extracted with CHCl3-MeOH (1:1) at room temperature. The obtained extract was concentrated in vacuo to give a residue (65 g) which was fractionated by silica gel CC (6 × 120 cm) and eluted with n-hexane (3 L), followed by a gradient of n-hexane-CH2Cl2 up to 100% CH2Cl2, then CH2Cl2-MeOH up to 15% MeOH (2 L of each solvent mixture) with increasing the polarity. The n-hexane-CH2Cl2 (1:1) was pre-fractionated by CC using Sephadex LH-20 (2×40 cm) and eluted with n-hexane-CH2Cl2 (7.4 mg) to give compound 8 (80 mg, 0.12 %). The n-hexane-CH2Cl2 (2.3 mg) fraction was chromatographed on a Sephadex LH-20 (1 × 50 cm) and eluted with n-hexane-CH2Cl2-MeOH (7:4:0.25) to afford compounds 4 (60 mg, 0.092 %) and 5 (40 mg, 0.062 %), respectively. The CH2Cl2 (100%) fraction, was pre-fractionated by CC on Sephadex LH-20 (1 × 30 cm) eluting with n-hexane-CH2Cl2-MeOH (7:4:0.5) to afford compounds 6 (30 mg, 0.046 %), 7 (45 mg, 0.069 %) and 2 (35 mg, 0.054 %). Further purification for the CH2Cl2-MeOH (1:1) fraction using CC on Sephadex LH-20 (1 × 30 cm) eluting with n-hexane-CH2Cl2-MeOH (7:4:0.25) led to the isolation of compounds 1 (65 mg, 0.1 %) and 3 (55 mg, 0.085 %), respectively.

2.4 MICROORGANISM

All of the bacteria (standard strains; Escherichi coli ATCC 25922, Staphylococcus blanc ATCC 27853) were obtained from Bacteriology Laboratory Constantine Hospital University (C.H.U), and clinical strains; Proteus merabilis, Proteus vulgaris, Staphylococcus epidemicids Staphylococcus saprophyticus) were obtained from Bacteriology Laboratory Constantine Hospital University (C.H.U), while the fungi Aspergillus versicolor, Aspergillus fumigatus and Penicillium camemberti strains were isolated in microbiology laboratory, department of biology, Oum el Bouaghi University.

2.5 ANTIMICROBIAL ASSAY

The Antimicrobial assay was carried out on crude extract (CH2Cl2 / MeOH 1:1) using agar diffusion method27,28 against six human pathogenic bacteria, including Gram positive (S. epidermidis, S. saprophyticus, S. blanc ATCC 27853) and three fungal strains A. versicolor, A. fumigatus and P. camemberti.
The bacterial strains were first grown on Muller Hinton medium (MHI) at 37 °C for 24 h prior to seeding on to the nutrient agar and the fungal strains at 30 °C for 48 h. The crude extract was mounted on sterile filter paper discs (6 mm in diameter) with the following concentrations (mg/mL) 8, 4, 2, 1, 0.5 and 0.25. The discs were placed on the inoculated agar media. The treated Petri dishes were kept at 4 °C for 1 h, and incubated at 37 °C for 24 h. The antibacterial activity was assessed by measuring the zone of growth inhibition surrounding the discs. Each experiment was carried out in triplicate.

3. RESULT AND DISCUSSION

From n-hexane extract, of the earlier parts and fruits, of F. vulgare Mill. Subsp. piperitum the fatty acids, hydrocarbons and sterols were identified. The furocoumarins imperatorin, psoralen, bergapten, xanthotoxin and isopimpinellin were isolated from the methylene chloride extract. The flavonoids isorhamnetin 3-O-arabinoside, quercetin and kaempferol were isolated from the ethyl acetate extract, whereas quercetin 3-O-β-glucoside were isolated from the methanol extract. The crude n-hexane, methylene chloride, ethyl acetate and methanol extracts from the earlier parts of F. vulgare were isolated for their antimicrobial activity and seven oxygenated monoterpenes were isolated for the first time.

3.1 Structural Elucidation of Compounds 1-7.

Compound 1 was obtained as colorless oil. Its HR-EI-MS showed a molecular-ion peak [M+H]+ at m/z 205.0536 ([C16H20O2]+), and the EI-MS gave a molecular-ion peak at m/z 204 and fragment-ion peaks at m/z 186 ([M-H2O]2), 168 ([M-2H2O]3), 153 ([M-2H2O-Me]2), 125 ([M-2H2O-isopropyl]), and 107 ([M-3H2O-isopropyl]), corresponding to a molecular formula of C16H20O2. The IR spectrum of 1 showed an obvious absorption band for OH groups at 3420 cm⁻¹. From further spectral data, the structure of 1 was deduced to be rel-(1R,2S,3R,4R,6S)-p-menthene-1,2,3,6-tetrol. The structure of 1 was determined from careful investigation of the 1D and 2D NMR measurements. 1H- and 13C-NMR spectra of 1 are shown in Tables 1 and 2, respectively. In its 13C-NMR spectrum, compound 1 shows ten carbon signals and the DEPT experiments indicated that these signals are corresponding to three methyl groups, one methylene group, five methine groups including three attached to oxygen appeared at δC 76.7, 68.9 and 72.9 for (C-2, C-3 and C-6), and one quaternary carbon atom. In the 1H-NMR spectrum of 1, the three methyl groups appeared at δH 1.39 (s), 0.92 (d, J = 7.2 Hz), and 0.78 (d, J = 7.2 Hz), and out of the five methine protons, three were OCH signals at δH 3.69 (d, J = 9.3 Hz), 3.95 (dd, J = 9.3, 11.4 Hz), and 3.77 (t, J = 2.4 Hz). The signal of the quaternary C-atom was at δH 7.46 (s) in the 1H-NMR spectrum. Thus, compound 1 was a menthane monoterpene derivative with four OH groups.35

The 'H-H COSY cross-peaks at δH 3.95 (H-3)/δH 3.69 (H-2), and δH 1.97 (H-4)/H-4/H-3, δH 1.59 and 1.77 (H-5) and δH 2.30 (H-8), and δH 3.77 (H-6) and δH 3.77 (H-6)/H-5, and the HMBC correlations (Fig. 1) of a sharp singlet methyl at δH 1.39 (H-7) with δC 74.6 (C-1), 76.7 (C-2), and 72.9 (C-6) as well as other HMBC long-range correlations indicated that the four OH groups were located at C-1, C-2, C-3, and C-6, and the Pr group at C-4, thus establishing the p-menthane-1,2,3,6-tetrol structure. The relative configuration of 1 was determined by the 1H, 1H-coupling pattern of the ring protons. The large Js of H-3 with H-2 and H-4 (J(3, 2) = 9.3 Hz, J(3, 4) = 11.4 Hz) showed that H-2, H-3, and H-4 were axial protons, and the small Js of H-6 with H-5 and H-7 (J(6, 5ax) = 4.08 (dd, J = 6.5 Hz), and 0.25 Hz) were characteristic for an equatorial H-6 (Fig.2). The relative configuration at C-1 was determined from NOE experiments: irradiation of the methyl signal at δH 1.39 (Me-7) enhanced the signal at δH 3.69 (H-2). Therefore, the configuration rel-(1R,2S,3R,4R,6S) was deduced.
Compound 3 was obtained as colorless needles. The molecular formula was assigned as \( \text{C}_{19}\text{H}_{20}\text{O}_2 \) by the [M+H]\(^+ \) peak at \( m/z \) 171.1324 in its HREI-MS and the NMR spectral data. The IR spectrum showed the presence of hydroxyl groups (3450 cm\(^{-1} \)) and the NMR spectral data. The IR spectrum showed the presence of an isopropyl at \( \delta \) 1.31 (3H, s) and a quaternary carbon at \( \delta \) 70.91, and H-9 and H-10 with C-6 (70.91) and C-3 (70.91) and C-2 (70.91), H-1 with C-8 (70.91) and C-3 (70.91), and H-9 and H-10 with C-6 (70.38) and C-8 (70.91) were shown. So the structure of 3 was elucidated as 3,4-dihydroxy-\( \alpha \)-menth-1-ene.\(^{25} \) The relative stereochemistry of 3 was determined by the coupling constants and NOE difference spectra.

Compounds 4 and 5 exhibited similar \( ^1\text{H} \) and \( ^1\text{C} \)-NMR spectra with those of compound 2. That revealed the presence of another methylene monoterpenone derivative but differ in only a few aspects to the one recorded for 2, these differences included absence of oxygenated proton signal at C-3.

### Table 1: \( ^1\text{H} \) NMR spectroscopic data for 1–7 (500 MHz, CDCl\(_3 \))

<table>
<thead>
<tr>
<th>Position</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tbody>
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<td>1</td>
<td>---</td>
<td>---</td>
<td>5.71 dd (10.1, 2.8)</td>
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<tr>
<td>2</td>
<td>3.69 d (9.3)</td>
<td>5.46 d (1.5)</td>
<td>5.62 dd (10.1, 1.6)</td>
<td>5.82 s</td>
<td>5.86 s</td>
<td>6.78 dq (5.9, 1.5)</td>
<td>6.66 q (1.7)</td>
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<tr>
<td>3</td>
<td>3.95 dd (11.4, 9.3)</td>
<td>4.08 dd (2.0, 9.0)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>4.43 dd (5.8, 3.2)</td>
<td>4.36 dt (9.7, 3.6)</td>
</tr>
<tr>
<td>4</td>
<td>1.97 ddt (11.7, 11.4, 3.0)</td>
<td>1.40 m</td>
<td>3.81 dd (7.5, 3.1)</td>
<td>2.37 dt (9.0, 5.3)</td>
<td>2.20 dt (14.0, 3.5)</td>
<td>1.66 ddt (13.0, 9.3, 3.7)</td>
<td>1.96 ddt (13.4, 9.7, 3.6)</td>
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<tr>
<td>5</td>
<td>1.75–1.79 m, 1.56–1.61 m</td>
<td>2.01 dd (2.5, 5.5)</td>
<td>1.21 dd (3.0, 13.0)</td>
<td>1.83 ddd (13.8, 6.9, 3.2)</td>
<td>1.76 ddd (……, 4.0)</td>
<td>2.05 dt (9.0, 5.1)</td>
<td>2.14 ddd (13.7, 9.0, 4.3)</td>
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<td>6</td>
<td>3.77 t (2.4)</td>
<td>4.19 dd (3.0, 8.0)</td>
<td>2.15 m</td>
<td>4.36 t (4.9)</td>
<td>4.48 dd (10.0, 3.5)</td>
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<tr>
<td>7</td>
<td>1.39 s</td>
<td>1.80 s</td>
<td>1.31 s</td>
<td>2.04 s</td>
<td>2.04 s</td>
<td>1.82 s</td>
<td>1.79 s</td>
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<tr>
<td>8</td>
<td>2.24–2.34 m</td>
<td>2.13 m</td>
<td>1.71 m</td>
<td>2.27 t (6.6)</td>
<td>2.55 m</td>
<td>1.80 overlapping</td>
<td>2.18 despt (6.9, 3.2)</td>
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<tr>
<td>9</td>
<td>0.92 d (7.2)</td>
<td>0.90 d (6.5)</td>
<td>0.94 d (6.8)</td>
<td>0.90 d (6.0)</td>
<td>0.83 d (7.0)</td>
<td>1.05 d (6.6)</td>
<td>0.91 d (6.9)</td>
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<tr>
<td>10</td>
<td>0.78 d (7.2)</td>
<td>0.99 d (6.5)</td>
<td>0.94 d (6.8)</td>
<td>0.95 d (7.0)</td>
<td>0.98 d (7.0)</td>
<td>0.98 d (6.5)</td>
<td>0.98 d (6.7)</td>
</tr>
</tbody>
</table>

* Multiplicity was determined by DEPT experiments (s, quaternary; d, methine; t, methylene; q, methyl).

### Table 2: \( ^1\text{C} \) NMR spectroscopic data for 1–7 (125 MHz, CDCl\(_3 \))

<table>
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<th>Position</th>
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<td>C-1</td>
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<td>139.13 s</td>
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<td>158.8 s</td>
<td>162.6 s</td>
<td>137.3 s</td>
<td>135.3 s</td>
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<tr>
<td>C-2</td>
<td>76.72 d</td>
<td>128.76 d</td>
<td>132.00 d</td>
<td>127.4 d</td>
<td>127.5 d</td>
<td>142.9 d</td>
<td>148.2 d</td>
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<td>C-3</td>
<td>68.86 s</td>
<td>69.40 d</td>
<td>70.91 s</td>
<td>200.6 s</td>
<td>199.4 s</td>
<td>64.3 d</td>
<td>69.2 d</td>
</tr>
<tr>
<td>C-4</td>
<td>41.43 d</td>
<td>48.13 d</td>
<td>73.53 d</td>
<td>48.3 d</td>
<td>51.0 d</td>
<td>46.0 d</td>
<td>50.1 d</td>
</tr>
<tr>
<td>C-5</td>
<td>26.77 t</td>
<td>31.81 t</td>
<td>28.79 t</td>
<td>32.4 t</td>
<td>32.7 t</td>
<td>36.9 t</td>
<td>36.3 t</td>
</tr>
<tr>
<td>C-6</td>
<td>72.95 d</td>
<td>71.07 d</td>
<td>38.38 d</td>
<td>67.4 d</td>
<td>70.5 d</td>
<td>200.1 s</td>
<td>199.8 s</td>
</tr>
<tr>
<td>C-7</td>
<td>23.89 q</td>
<td>21.00 q</td>
<td>23.63 q</td>
<td>26.4 q</td>
<td>25.6 q</td>
<td>28.5 q</td>
<td>26.4 q</td>
</tr>
<tr>
<td>C-8</td>
<td>27.84 d</td>
<td>26.42 d</td>
<td>31.9 d</td>
<td>19.0 d</td>
<td>17.5 d</td>
<td>20.2 d</td>
<td>16.6 d</td>
</tr>
<tr>
<td>C-9</td>
<td>20.92 q</td>
<td>16.63 q</td>
<td>19.76 q</td>
<td>20.6 q</td>
<td>20.3 q</td>
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<td>C-10</td>
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<td>20.9 q</td>
<td>19.4 q</td>
<td>15.6 q</td>
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</tbody>
</table>
and appearance of carbon signal at δc 200.60 and 199.40, which revealed the presence of a keto-group at C-3.

The relative stereochemistry of 4 and 5 was determined by the coupling constants and NOE difference spectra were shown. So the structure of 4 and 5 were elucidated as (4R, 6S)-6-hydroxypiperitone (4) and (4R, 6R)-6-hydroxypiperitone (5).\textsuperscript{37, 38}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{images}
\caption{Oxygenated monoterpenes (1-7) from \textit{F. vulgare}}
\end{figure}

Also Compounds 6 and 7 exhibited similar \textsuperscript{1}H and \textsuperscript{13}C-NMR spectra with those of compound 2. That revealed the presence of another methane monoterpane derivative but differ in only a few aspects to the one recorded for 2, these differences included absence of oxygenated proton signal at C-2 and appearance of carbon signals at δc 200.10 and 199.80 due to the presence of a keto-group at C-3.

The relative stereochemistry of 4, 5 was determined by the coupling constants and NOE difference spectra were shown. So the structure of 4 and 5 were elucidated as (4R, 3S)-3-hydroxypiperitone 6 and (4R, 3R)-3-hydroxypiperitone 7.

3.2 Antimicrobial Activity.

The antimicrobial activities and toxicity of terpenes have been documented, but their modes of action are complex and still in some cases unknown, considering the large number of different groups of chemical compounds present. It is most likely that their antimicrobial properties are not attributable to one specific mechanism, because of other targets in the cell. Oxygenated monoterpenes such as menthol and aliphatic alcohols were reported to possess strong to moderate activities against several bacteria.\textsuperscript{39} The CHCl\textsubscript{3} extracts of the stems of \textit{F. vulgare} showed significant antimicrobial activity against the bacteria and fungi.\textsuperscript{40} In addition, it was observed that the essential oil and seed extracts of \textit{F. vulgare} exhibited different degree of antimicrobial activities depending on the dose applied.\textsuperscript{41} Therefore, fennel oil could be a source of pharmaceutical materials required for the preparation of new therapeutic and antimicrobial agents. Due to these data we were interested to study antibacterial activity of the crude extract (CH\textsubscript{3}Cl/MeOH 1: 1) containing monoterpenes compounds from \textit{F. vulgare} against both standards and isolated strains bacteria together with some fungi using the disc diffusion method.

The diffusion test was applied to nine, six bacterial and three fungal, strains. The results are summarized in Table 3 which showed that the crude extract (CH\textsubscript{3}Cl/MeOH 1: 1) from \textit{F. vulgaris} prevented the growth of all the tested microorganisms with an inhibition zone medium diameter increasing proportionally with the concentration of the tested samples. The obtained inhibition varied from 6.00 to 15.00 mm with a highest inhibition zone recorded with \textit{P. vulgaris} (11-15 mm). Nevertheless the fungi \textit{Aspergillus Sp.} displayed very high inhibition diameter even with low concentration of 0.25 mg/mL (6 mm and 11.6 mm). It should be mentioned that there are no background study on \textit{F. vulgare} terpenes except for some coumarin compounds such as Scopoletin, bergapten, psolaren on \textit{A. niger} and \textit{E. coli}.\textsuperscript{42}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
\textbf{Microorganism} & \textbf{Crude extract conc. (mg/mL)} & \multicolumn{5}{|c|}{\textbf{Inhibition zone (mm) ± standard deviation}} \\
\hline
 & \textbf{0.25} & \textbf{0.5} & \textbf{1.0} & \textbf{2.0} & \textbf{4.0} & \textbf{8.0} \\
\hline
\textit{E. coli} (ATCC 25922) & 10.0 ± 10 & 10.0 ± 0.0 & 10.33 ± 0.57 & 10.66 ± 0.57 & 12.5 ± 1.80 & 12.5 ± 0.86 \\
\textit{P. merabilis} & 6.0 ± 0.0 & 6.0 ± 0.0 & 7.50 ± 0.86 & 7.50 ± 1.80 & 10.0 ± 2.0 & 10.66 ± 1.15 \\
\textit{P. vulgaris} & 11.0 ± 2.64 & 11.20 ± 1.0 & 11.5 ± 2.17 & 13.0 ± 1.0 & 13.66 ± 2.30 & 15.0 ± 2.00 \\
\textit{S. epidemidis} & 6.0 ± 0.0 & 6.0 ± 0.0 & 6.66 ± 1.52 & 10.0 ± 0.0 & 12.50 ± 1.47 & 12.50 ± 1.47 \\
\textit{S. saprophyticus} & 6.0 ± 0.0 & 7.33 ± 0.81 & 10.0 ± 0.0 & 10.0 ± 1.0 & 10.66 ± 1.15 & 10.66 ± 1.15 \\
\textit{S. blanc} (ATCC 27853) & 7.50 ± 0.86 & 7.50 ± 0.86 & 7.50 ± 0.86 & 8.0 ± 1.00 & 8.0 ± 1.00 & 1.66 ± 1.15 \\
\textit{A. versicolor} & 6.66 ± 1.15 & 8.0 ± 0.0 & 11.33 ± 0.81 & 11.33 ± 0.81 & 11.33 ± 1.80 & 21.0 ± 0.86 \\
\textit{A. fumigatus} & 11.66 ± 1.15 & 15.33 ± 0.57 & 15.33 ± 0.86 & 15.33 ± 0.57 & 16.66 ± 2.0 & 20.0 ± 1.15 \\
\textit{P. camemberti} & 9.0 ± 1.00 & 14.0 ± 0.0 & 14.33 ± 0.81 & 14.33 ± 0.81 & 14.33 ± 1.00 & 14.33 ± 0.57 \\
\hline
\end{tabular}
\caption{Antimicrobial activity of crude extract (CH\textsubscript{3}Cl/MeOH 1: 1) at different concentrations on six bacterial strains.}
\end{table}
In general, crude extract containing this monoterpenes exhibited stronger antifungal activity than bacteria strains. The antimicrobial activity of the crude extract of *F. vulgare* can be attributed to the content of oxygenated monoterpenes constituents, since there is a relationship between the chemical structures and their antimicrobial activities. Although the mechanism of action of terpenes is not fully understood, it is thought to involve membrane disruption by the lipophilic compounds 40, 41.

4. CONCLUSIONS

Our results of antimicrobial assays justified and supported partly the popular usage of the all organs especially seeds as traditional remedies for some infections. It was of interest to note that the strong antimicrobial activity of the crude extract of *F. vulgare* against clinical and standard microorganisms, especially *S. saprophyticus* and *E. coli* which were established as major pathogens responsible for a wide variety of infection, suggested that the crude extract containing monoterpenes could be a new medicinal resource for antibacterial agents.

REFERENCES