Chemical Composition and Biological Activities of the Essential Oil from the Leaves of Vaccinium Myrtillus L.

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Abstract

The chemical composition, antimicrobial and antioxidant properties of the essential oil (EO), obtained from the leaves of Vaccinium myrtillus naturally grown in the northernmost of Turkey were determined by GC and GC-MS and chemical differences were discussed with the help of chemotaxonomy. The leaves of the plant samples were hydro-distilled to produce oil in the yields of 1%. Nineteen components were identified representing 96.4% of the oil. The main compounds in the EO of V. myrtillus were; 1,8-cineole (38.6%), α-pinene (21%), linalool (19.5%), α-terpinol (5.8%). The EO extract was screened for their antimicrobial activities against the 9 bacteria and 3 yeast species by using disc-diffusion and MIC procedure. The EO extract displayed more effective against all the tested bacteria (especially, S. aureus ATCC 6538 and MRSA) and yeast (only C. krusei). The MIC values of sample against tested microorganisms were found to be in the range of 320 to ≥1280 µg/ml. The most effective MIC values were observed against the S. aureus and MRSA (320 µg/ml). In vitro the antioxidant activity based on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was evaluated for the EO extract, and it was found that the extract had good antioxidant activity in the range of the IC50 = 583.4 ±11 µg ml. Antibacterial and antioxidant activities of the EO from the leaves of V. myrtillus has been reported for the first time.

Introduction

Bilberry (Vaccinium myrtillus L.) is a deciduous shrub growing to 50 cm, with elliptical leaves. The flowers are single on short stems. The fruits are berries, globular, dark purple, juicy and sour. In many European countries, the bilberry is one of the most economically important wild berry species (Vučić et al. 2013). Turkey is a very important area for plant diversity. Many fruit species are grown and so many different local or native fruit, berry species and varieties are known. North part of Turkey from east to west is the gene center of heathers (Ericaceae) and several Vaccinium species like Caucasian whortleberry, bilberry, lingonberry and bog blueberry, bog whortleberry or bog bilberry (Celik and Koca 2013).

The aim of this study was to determine and compare the antibacterial, antifungal, antioxidant activity of EO of leaves of this plant collected from Turkey and to carry out the chemical composition of V. myrtillus EO from the leaves. The results were also discussed with the genus patterns of Vaccinium by means of chemotaxonomy in the current study.

Materials and Methods

Aeriel parts of V. myrtillus were collected (1000 g) in Turkey, Sinop; Ayancık, between Sinop-Ayancık, at an altitude of 50 m. (41°56'38" N, 34°41'15" E), April, 2016 by O. Elkiran. Plant materials were identified by taxonomist O. Elkiran with volume 6 of Flora of Turkey and East Aegean Islands (Davis 1978).

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The EO was analyzed using HP 6890 GC equipped with a FID detector and an HP-5 MS column (30 m × 0.25 mm i.d., film thickness 0.25 µm) and the capillary column was used. The column and analysis conditions were the same as in GC-MS. Relative percentage amounts of the separated compounds were calculated from the FID chromatograms.

The oil samples were analyzed by GC-MS, using a Hewlett Packard system. HP-Agilent 5973N GC-MS system with 6890 GC is in Çankırı Karatekin University. HP-5 MS column (30 m × 0.25 mm i.d., film thickness 0.25 µm) was used with helium as the carrier gas. Injector temperature was 250°C, split flow was 1ml/min. The GC oven temperature was kept at 70°C for 2 min and programmed to 150°C at a rate of 10°C / min and then kept constant at 150°C for 15 min to 240°C at a rate of 5°C/min. Alkanes were used as reference points in the calculation of relative retention indices (RRI). MS were taken at 70 EV and at a mass range of 35 - 425. Component identification was carried out using spectrometries electronic libraries (WILEY NIST). The identified constituents of the EO are listed in Table 1.

The test organisms, six Gram positive bacteria, namely Bacillus cereus 7064, Bacillus subtilis (C), Enterococcus faecalis ATCC 51299, Staphylococcus aureus ATCC 6538, Vancomycin Resistant Enterococcus (VRE), methicillin resistant Staphylococcus aureus (MRSA); 2 Gram negative bacteria Escherichia coli ATCC 11293, Pseudomonas aeruginosa ATCC27853 and 3 yeast species namely, Candida krusei ATCC 6258, Candida albicans ATCC 14053 and Candida parapsilosis ATCC 22019 collected as pure cultures from the Molecular Biology and Microbiology Laboratory, Department of Biology, Faculty of Arts and Science, Sinop University, Turkey.

The antibacterial and antifungal activity of the EO of V. myrtillus was evaluated by using disc diffusion method (Bauer et al. 1966). All the microorganisms were maintained at −80°C in Muller Hinton Agar (MHA) for bacteria and Sabouraud Dextrose Agar (SDA) for fungus (Difco) containing 15% (v/v) glycerol. Before testing, the microorganisms were transferred to Muller Hinton Broth (MHB) and Sabouraud Dextrose Broth (SDB) (Difco) and cultured overnight at 37°C (28°C for fungus). Then, the turbidity was adjusted equivalent to 0.5 McFarland standards (1.5 × 10⁸ cfu/ml). Then, 100 µl of microorganisms was spread over the surface of an agar plate. The filter paper discs (6 mm) were loaded with 50 µl EO and were allowed to dry completely. Then, it was placed on the surface of the freshly inoculated medium. The media were incubated for 24 hrs at 37°C (28°C for fungus). Antibiotic susceptibility discs including imipenem (10 µg), novobiocin (5 µg), bacitracin (0.04 U), ceftazidime (30 µg), ampicillin (10 µg), tetracycline (30 µg), polymyxin B (300 U) and cycloheximide were used as control, and negative control was to DMSO (12.5%). The antimicrobial activity was evaluated by measuring the diameter of inhibition zone (Avşar et al. 2016).

The minimum inhibitory concentration (MIC) was determined by the serial tube dilution method. EO of sample (100 µl) was used as initial stock solution. The samples were mixed into 0.9 ml of MHB and SDB in tubes in order to adjust to the concentrations of 1280 - 80 µg/ml. All tubes were inoculated with 100 µl standardized inoculums of each organism and incubated for 24 hrs at 37°C (for bacteria) and 48 - 72 hrs at 28 ± 1°C (for fungus). The MIC of the EO was taken as the lowest concentration that showed no growth (Bagci and Yuce 2011, Avşar et al. 2016).

The antioxidant activities of the samples based on DPPH were performed according to Blois (1958) and Kumar et al. (2011) methods. The EOs at different concentrations such as 1000, 500, 250, 125 and 62.5 µg/mL were obtained using serial dilution technique in ethanol. One ml of an ethanol solution of the EO of each concentration was mixed with 4 ml of a DPPH-ethanol solution (0.1 mM). These samples were shaken well and kept in dark for 30 min at room temperature. The
absorbance was measured at 517 nm. The scavenging activity on the DPPH radical was calculated by using the following equation:

\[
\% \text{ inhibition} = \left( \frac{A_B - A_S}{A_B} \right) \times 100
\]

The \( A_B \) is the absorbance of the control reaction and \( A_S \) is the absorbance of the test compound in this equation. Ascorbic acid was used as a standard. The compound without a sample or standard was used as a control. Scavenging activity was expressed as median inhibitory concentration (IC\(_{50}\)), which represents the concentration of the EO (µg/ml) required to inhibit 50% of the free radical scavenging activity.

All tests were done in triplicates and values are expressed as means with standard deviations (±Sd). Graphics were drawn using MS Office Excel 2013.

**Results and Discussion**

Using hydro-distillation of the leaves of *Vaccinium myrtillus*, oil yield of 1% (wt./wt.), was obtained. EO of dried leaves of *V. myrtillus* was analyzed in terms of their chemical composition via GC and GC-MS. The results of the analysis of EO of *V. myrtillus* are presented in Table 1. A total of 19 compounds were identified, representing 96.4% of the total oil. The primary compounds detected in EO were 1,8-cineole (38.6%), \( \alpha \)-pinene (21%), linalool (19.5%) and \( \alpha \)-terpineol (5.8%). The compounds in the EO of *V. myrtillus* may be grouped in three main classes: oxygenated monoterpenes (69.9%), monoterpene hydrocarbons (22.9%), and sesquiterpenes hydrocarbons (3.6%) as shown in Table 1.

**Table 1. Chemical composition of essential oil of Vaccinium myrtillus.**

| No. | Compounds               | RT  | RRI  | Percent % |
|-----|-------------------------|-----|------|-----------|
| 1   | Limonene                | 11.17 | 1094 | 0.8       |
| 2   | \( \alpha \)-pinene     | 12.05 | 1114 | 21        |
| 3   | \( \beta \)-pinene      | 13.57 | 1147 | 0.6       |
| 4   | 1,8-cineole             | 15.23 | 1184 | 38.6      |
| 5   | Linalool                | 17.39 | 1302 | 19.5      |
| 6   | \( \alpha \)-terpineol  | 20.61 | 1303 | 5.8       |
| 7   | Estragole               | 20.75 | 1305 | 0.9       |
| 8   | Geraniol                | 21.63 | 1324 | 0.3       |
| 9   | Linalyl acetate         | 22.49 | 1343 | 2.5       |
| 10  | Eugenol                 | 22.70 | 1348 | 0.1       |
| 11  | \( \alpha \)-terpinyl acetate | 25.73 | 1414 | 0.7       |
| 12  | Myrcene                 | 26.00 | 1420 | 0.5       |
| 13  | Geranyl acetate         | 26.65 | 1435 | 2.8       |
| 14  | Methyl eugenol          | 27.38 | 1451 | 1         |
| 15  | Humulene                | 29.36 | 1494 | 0.2       |
| 16  | \( \alpha \)-gurjunene  | 31.14 | 1533 | 0.3       |
| 17  | Caryophyllene oxide     | 33.30 | 1581 | 0.3       |
| 18  | Calarene epoxide        | 34.05 | 1597 | 0.4       |
| 19  | \( \beta \)-guaiene     | 35.27 | 1624 | 0.1       |

|                  |                  |      |      | Total     |
|------------------|------------------|------|------|----------|
| Monoterpenes     |                  |      |      | 28.2     |
| Oxygenated monoterpenes |              |      |      | 66.9     |
| Sesquiterpenes hydrocarbons |          |      |      | 1.3      |

RRI: Relative retention indices and RT: Retention time.
Table 2. Inhibition zones (mm) of the plant essential oil extracts against tested microorganisms using disc diffusion method.

|            | C. kruzei | C. parapsilosis | C. albicans | P. aeruginosa | E. coli | E. faecalis | S. aureus | MRSA | M. luteus | R. subtilis | R. cereus | VRE |
|------------|-----------|-----------------|-------------|---------------|---------|-------------|-----------|------|-----------|-------------|-----------|-----|
| Extract    | 9 ± 0.1   | -               | -           | 12 ± 0.4      | 9 ± 0.2 | 9 ± 0.01    | 15 ± 0.9  | 15 ± 0.4 | 10 ± 0.4  | 11 ± 0.6    | 10 ± 0.8  | 10 ± 0.2 |
| DMSO       | -         | -               | -           | -             | -       | -           | -         | -     | -         | -           | -         | 8   |
| Bac        | *         | *               | *           | -             | -       | -           | -         | -     | -         | -           | -         |     |
| Nov        | *         | *               | *           | -             | -       | 15 ± 0.2    | 29 ± 0.4  | 24 ± 0.7 | 24 ± 1.4  | 20 ± 1.4    | 10 ± 0.1  | 10 ± 0.5 |
| Tet        | *         | *               | *           | 17 ± 0.1      | 26 ± 1.3 | 23 ± 0.5    | 40 ± 1.4  | 10 ± 0.2 | 32 ± 1.8  | 34 ± 2.1    | 32        | 13 ± 0.9 |
| Amp        | *         | *               | *           | -             | 35 ± 1.2 | 42 ± 1.6    | 16 ± 0.5  | *     | 32 ± 1.3  | *           | 24 ± 0.5  |     |
| Imp        | *         | *               | *           | 14 ± 0.3      | 28 ± 0.5 | 34 ± 1.2    | 50 ± 1.6  | 50 ± 2.3 | 44 ± 2.3  | 50 ± 4.1    | 36        | 30 ± 1.3 |
| Poly B     | *         | *               | *           | 22 ± 1.0      | 11 ± 0.4 | -           | 11 ± 0.1  | 11 ± 0.2 | 24 ± 1.1  | 10 ± 0.3    | 9         |     |
| Cef        | *         | *               | *           | 26 ± 1.4      | 19 ± 0.2 | 20 ± 1.1    | 25 ± 0.6  | 23 ± 0.8 | 26 ± 1.3  | 23 ± 1.2    | 9         | 8 ± 0.10 |
| Cyc        | 43 ± 1.2  | 40 ± 1.1        | 42 ± 1.6    | *             | *       | *           | *         | *     | *         | *           | *         |     |

- Not effect and * Not tested.

Table 3. MIC values (µl/ml) of the plant essential oil extracts against tested microorganisms using microdilution procedure.

| Plant extract | C. kruzei | C. parapsilosis | C. albicans | P. aeruginosa | E. coli | E. faecalis | S. aureus | MRSA | M. luteus | B. subtilis | B. cereus | VRE |
|---------------|-----------|-----------------|-------------|---------------|---------|-------------|-----------|------|-----------|-------------|-----------|-----|
| Extract       | 1280      | *               | *           | 1280          | *       | *           | 320       | 320  | *         | 1280         | *         | 1280|
There is one study in the literature on the chemical composition of *V. myrtillus* EO and similar results have been previously reported (Bayar et al. 2017). At the same time, among the several commercial liquid preparations, those derived from *V. myrtillus* L. (bilberry) and *V. vitis-idaea* L. (lingonberry) foliar tissues are widely used, but not well known in terms of composition and chemical stability. Therefore, it is crucial to improve the knowledge of these extracts to assure their final quality (Ieri et al. 2013).

The antimicrobial activities of EO detected against 9 bacteria and 3 yeasts using disc-diffusion and MIC techniques are shown in Tables 2 and 3. The tested sample inhibited the growth of bacteria and yeast according to the results of the disc-diffusion method. The EO in the present study especially showed higher activity against MRSA and *S. aureus* ATCC 6538 (15 mm). The findings also show that EO possesses a strong antibacterial activity in comparison with some antibiotics as standard (Table 2). In addition, our results showed that there was a correlation between disc-diffusion and MIC. The MIC values of sample against tested microorganisms were found to be in the range of 320 to ≥1280 μg/ml. The most effective MIC values were observed against the *S. aureus* and MRSA (320μg/ml). In addition, the sample showed only low antiyeast activity against *C. krusei* (1280 μg/ml < Table 3). Vučić et al. (2013) observed a high effect of *V. myrtillus* extracts on *E. coli* and *E. faecalis*, but this did not match the present data. In agreement with the present study, Burdulis et al. reported that *V. myrtillus* had an antibacterial effect but less or no antifungal effect (Burdulis et al. 2009). Chu et al. reported that *V. myrtillus* extracts showed a direct effect against MRSA (Chu et al. 2011). Puupponen-Pimiä et al. also determined that *V. myrtillus* extracts and other berry fruits showed direct antimicrobial effects against human pathogens (Puupponen-Pimiä et al. 2005), including *S. aureus*. Miljković et al. stated that the most sensitive strain was *S. aureus* against *V. myrtillus* antimicrobial activity (Miljković et al. 2018).

![Fig. 1. Antioxidant activity (IC_{50} value) of the extract of Vaccinium myrtillus.](image)

The IC_{50} value for the DPPH-radical-scavenging activity of the sample is shown in Fig. 1. The IC_{50} value of the sample was found to be 583.4 μg/ml and that of the control was determined to be 745 μg/ml. These results support the data reported by Uleberg et al. (Uleberg et al. 2012), who reported that the *V. myrtillus* clones they collected from different regions had a high antioxidant effect. Recent studies confirm the present findings also for antioxidant capacity of *V. myrtillus* leaves or fruit.

This report demonstrates the occurrence of 1,8-cineole, α-pinene, linalool and α-terpineol chemotypes of *V. myrtillus* in Central Black Sea region of Turkey. In addition, indicates that the EO of *V. myrtillus* has a potential with regard to antimicrobial, antifungal and antioxidant...
activities. It has also been determined as a result of the examination of the compositions of the EO of *V. myrtillus* samples that they can be used as raw material for medicinal, pharmaceutical purposes, cosmetics industries and as natural products.

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