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ASSESSMENT OF THE CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF ESSENTIAL OIL FROM BULGARIAN YARROW (*ACHILLEA MILLEFOLIUM L.*)

Krastena Nikolova[1], Natalina Panova[1], Anelia Gerasimova[2], Yulian Tumbarski[3], Ivan Ivanov[4], Ivayla Dincheva[5], Christina Tzvetkova[6,7], Alexander Pashev[8], Galia Gentscheva[6,8]

[1]*Medical University of Varna, Department of Physics and Biophysics Faculty of Pharmacy, 84 Tsar Osvoboditel Blvd., Varna 9000, Bulgaria, Krastena.Nikolova@mu-varna.bg (K. N.); panova@mu-varna.bg (N. P.)*
[2]*Medical University of Varna, Department of Chemistry, Faculty of Pharmacy, 84 Tzar Osvoboditel St., Varna 9000, Bulgaria, anelia.gerasimova@mu-varna.bg (A. G.)*
[3]*University of Food Technologies, Department of Microbiology and Biotechnology Technological Faculty, Plovdiv 4002, Bulgaria, tumbarski@abv.bg (Y.T.)*
[4]*University of Food Technologies Department of Organic Chemistry and Inorganic Chemistry 26 Maritsa Blvd., Plovdiv 4002, Bulgaria, ivanov_ivan.1979@yahoo.com (I.I.)*
[5]*AgroBioInstitute, Agricultural Academy, Department of Agrobiotechnologies 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria, ivadincheva@yahoo.com (Iv. D.)*
[6]*Institute of General and Inorganic Chemistry Bulgarian Academy of Sciences, "Acad. Georgi Bonchev" St., Bl.11 Sofia 1113, Bulgaria; hrisi@svr.igic.bas.bg (Ch. Tz.)*
[7]*National Centre of Excellence Mechatronics and Clean Technologies, 8 Kliment Ohridski Blvd., 1700 Sofia, Bulgaria*
[8]*Medical University-Pleven, Department of Chemistry and Biochemistry, 1 Saint Kliment Ohridski St., Pleven 5800, Bulgaria, galia.gentscheva@mu-pleven.bg (G. G); aleksandar.pashev@mu-pleven.bg (Al. P.)*

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ABSTRACT

The present study aimed to investigate the chemical composition and biological activity of yarrow essential oil (Achillea millefolium) from Bulgaria. Gas chromatography-mass spectrometry (GC-MS) analysis, total reflection X-ray fluorescence (TXRF) technique, antioxidant activity (ABTS and DPPH methods), antimicrobial activity tests were used. Through an optimized procedure with Ga as an internal standard, thirteen elements - S, Cl, K, Ca, Cr, Mn, Fe, Ni, Cu, Zn, Se, and Br - were quantified. The highest concentrations were observed for S, Ca, and Fe (12.35 - 7.26 mg kg^{-1}), followed by Cl, K, Ni, Cu, and Zn (2.23 - 0.53 mg kg^{-1}). The dominant compounds identified by GC-MS analysis are monoterpene hydrocarbons, of which α-pinene (41.29 %) and β-pinene (27.15 %), limonene and p-cymene are predominant. The ABTS method a moderate degree radical neutralization with an IC_{50} value of 9.15 ± 0.01 μg mL^{-1}, while the DPPH method had a significantly lower IC_{50} of 205.00 ± 0.03 μg mL^{-1}, indicating selective antioxidant activity depending on the type of radicals. The maximum inhibition observed at 100 μg mL^{-1} reached 95.13 % with the ABTS method and 29.82 % with the DPPH method. Bulgarian yarrow essential oil shows moderate activity against some Gram-negative bacteria, such as K. pneumoniae. and weak inhibitory activity against other pathogens, such as Staphylococcus aureus and Escherichia coli. It does not show activity against the molds Aspergillus niger and Aspergillus flavus.

Keywords: antioxidant activity, chemical composition, yarrow, Achillea millefolium, antimicrobial activity, essential oil.

INTRODUCTION

Yarrow is a perennial herb that is drought- and cold-resistant, found in Europe, Asia, and North America. Four morphotypes of *Achillea millefolium* L. are known: white, pink, deep pink, and red. In all of them, the flowers contain the highest amounts of essential oil, with the white yarrow morphotype having the highest content [1]. The healing effects of this essential oil are primarily due to the complex mixture of natural compounds, which results in a wide range of beneficial effects. Some authors have experimentally found that elements such as P, S, B or Mn can increase the amount of essential oil [2]. It has also been found that altitude can affect the chemical composition of the oil. For example, oil harvested from 2300 m above sea level contains 1,8-cineole, camphor, borneol, terpinolene, γ-terpinene, and thujone as its main components, while terpinolene, borneol, β-pinene, and chamazulene are the main components of oil from 700 m above sea level [3]. An essential oil obtained by hydrodistillation of yarrow from the Eastern Anatolia region (Van, Turkey), containing as its main components 1,8-cineole, α-phellandrene, p-eugenol, and camphor, showed moderate ABTS activity of 4.2 mM trolox equivalents [4]. Su-Tze Chou et al. identified 19 compounds in steam-distilled yarrow essential oil, with the main components being artemisinin ketone, camphor, linalyl acetate, and 1,8-cineole, and found that they inhibit inflammatory responses and oxidative stress in LPS-stimulated RAW 264.7 macrophages. [5]. The oil from the wild plant *A. Millefolium* from the Midi-Pyrénées region (France) has a unique chemical composition – camphor germacrene-D, (E)-nerolidol, sabinene, (E)-p-mentha-2,8-dien-1-ol and 1,8-cineole, and has shown an inhibitory effect against microbial organisms (bacteria and fungi) [6]. From the reviewed literary sources, it follows that the phytochemical composition of yarrow oil is diverse, therefore it is interesting to examine the composition of Bulgarian oil and draw some conclusions regarding its application.

The aim of the present study is to investigate the phytochemical composition, mineral content, antioxidant and antimicrobial activities of yarrow essential oil (*Achillea millefolium* L.) extracted from plants grown in Bulgaria.

EXPERIMENTAL

Sample

The essential oil from *Achillea millefolium* L. was purchased from the company Kateko Ltd., Plovdiv, Bulgaria. The essential oil was obtained by steam distillation. The plant material was densely packed into the distillation vessel, which was then sealed with a lid. Direct steam at a temperature of 160°C and a pressure of 4 - 5 atm was applied.

The start of the distillation process was defined as the moment the first distillate began to flow from the condenser. When using wild-harvested raw material, the process lasted for 3 h. During distillation process, at every 15 - 20 min, the distillate temperature was raised to 60 - 65°C, maintaining a distillation rate of approximately 10 %.

GC-MS analysis of the chemical composition of bulgarian yarrow (*Achillea millefolium*) Essential oil

Researchers analyzed the chemical composition of the essential oil from Bulgarian *Achillea millefolium* L. using gas chromatography-mass spectrometry (GC-MS). The analysis was performed on an Agilent 7890A GC system, equipped with an inert XL EI/CI MSD 5975C mass selective detector (Agilent Technologies, Santa Clara, CA, USA).

A volume of 20 µL of the essential oil was diluted in 380 µL of hexane. Subsequently, 1 µL of the resulting solution was injected into an HP-5MS capillary column (30 m × 0.32 mm i.d. × 0.25 µm film thickness). The researchers initially set the oven temperature at 40°C, then ramped it to 300°C at 5°C min^{-1}, and finally held it isothermally at that temperature for 10 min. The researchers set the injector temperature to 250°C, employing a 100:1 split ratio.

Volatile constituents were identified by comparing their retention indices (RI) and mass spectra with entries in the NIST 08 mass spectral library [7].

Elemental composition

The elemental composition of *A. millefolium* essential oil was analyzed using total reflection X-ray fluorescence (TXRF) spectrometry, which enables direct sample analysis without the need for prior digestion. Sample preparation followed a slightly modified version of the method validated by Shaltout, A. A. et al., using a certified reference material (SRM) of multielement organometallic oil [8]. An aliquot of 5 µL of a gallium (Ga) standard solution (100 ppm) was added to 50 µL of the essential oil, resulting in a final internal standard concentration of 10 ppm Ga. After homogenization on a vortex shaker for 1 min at 2500 rpm, a 5 µL aliquot of the mixture was deposited onto a pre-siliconized fused silica glass carrier and dried at room temperature (25 - 27 °C) for three weeks. TXRF measurements were carried out using a benchtop S2 PICOFOX 400 spectrometer (Bruker Nano GmbH, Germany) equipped with a molybdenum anode X-ray tube (50 W, max 1000 µA) and a multilayer monochromator (17.5 keV). Samples were prepared and analyzed in triplicates, with an acquisition time of 500 s per measurement to ensure reliable quantification.

Antioxidant activity: ABTS radical scavenging assay

The antioxidant activity of yarrow essential oil was evaluated using the $ABTS^{\bullet+}$radical scavenging method as described by Ivanov et al. [9]. A 7.0 mM ABTS (Sigma-Aldrich, Germany) solution was mixed with 2.45 mM potassium persulfate (Merck, Germany) to generate the $ABTS^{\bullet+}$radical cation and left in the dark for 16 h at room temperature. The solution was diluted with methanol (1:30 v/v) to achieve an appropriate absorbance (1.0 - 1.1) at 734 nm. For the assay, 2.85 mL of the $ABTS^{\bullet+}$solution was mixed with 0.15 mL of essential oil (EO) dissolved in methanol. The mixture was kept in the dark at 37°C for 15 min, then measured the absorbance relative to methanol at 734 nm. The antioxidant activity (AOA) was calculated using a calibration curve, expressed as Trolox equivalents per gram of essential oil (mM Trolox equivalents per gram of essential oil, abbreviated as (mM TE g^{-1} EO). The concentration of EO required to inhibit 50 % of $ABTS^{\bullet+}$radicals (IC_{50} value) was determined.

Antimicrobial activity

The antimicrobial activity of yarrow EO, pre-diluted in methanol at a concentration of 10 mg mL^{-1}, was evaluated using the agar diffusion method, previously described by Tumbarski et al. [10].

Infrared spectroscopy

Fourier-transform infrared (FT-IR) spectra were obtained using a Jasco FT-IR-4X FTIR spectrophotometer. The spectral range covered 4000 - 400 cm^{-1}. Each sample was scanned 32 times at a resolution of 2 cm^{-1} to minimize background noise generated by the instrument.

Statistical analysis

All measurements were repeated three times. The results are presented as the mean value plus or minus the mean standard deviation. For this purpose, Excel ANOVA software was used.

Twenty-eight compounds were identified in the essential oil of yarrow and classified into the following groups: monoterpenes, sesquiterpenes, and oxygenated monoterpenes (Table 1). Monoterpenes represent the main class of substances (93.57 %), followed by sesquiterpenes (3.49 %) and oxidized monoterpenes (2.64 %).

As seen from the results, the studied essential oil is predominantly composed of monoterpenes. It is particularly rich in α-pinene (41.29 %) and β-pinene (27.15 %). Other compounds that distinguish it from essential oils from other geographic regions include the presence of limonene (10.26 %) and p-cymene (10.09 %). The Bulgarian chemotype of yarrow differs from those found in Serbia and Estonia, in whichthe content of chamazulene is high, and their samples had a predominantly blue color. Regarding the color, the Bulgarian EO was light, almost transparent.

In the Bulgarian *A. millefolium* essential oil, the content of oxygenated monoterpenes was low. In contrast, oils obtained from plants growing in France, Belgium, Spain, Italy, Russia, and Armenia contained oxygenated monoterpenes ranging from 54 % to 76.1 %. Additionally, oils originating from Serbia, France, and Eastern Turkey contained 1,8-cineole and camphor [11 - 13].

The literature reveals substantial variation in the monoterpene composition of *A. millefolium* essential oils. Notable differences have been observed in compounds such as lavandulyl acetate, α-thujone, and β-thujone. Lavandulyl acetate has not been reported in the essential oil of *A. millefolium* [14, 15], whereas α- and β-thujone have been identified in oils from China and Chile [16].

The absence of α- and β-thujone in the Bulgarian sample indicates the high quality of the essential oil obtained from wild-growing *A. millefolium*, making it suitable for incorporation into various products due to the

Table 1. Chemical composition of bulgarian yarrow (*Achillea millefolium*).

Peak	RT	RI	Name	% of TIC
Monoterpenes				
1	9.84	930	α-Pinene	41.29
2	10.23	948	Camphene	1.06
3	11.0	969	Sabinene	1.21
4	11.14	977	β-Pinene	27.15
5	11.58	990	β-Myrcene	1.63
6	12.05	1001	δ-2-Carene	0.12
7	12.08	1003	α-Phellandrene	0.21
8	12.15	1010	3-Carene	0.17
9	12.46	1016	α-Terpinene	0.1
10	12.77	1022	p-Cymene	10.09
11	12.98	1026	Limonene	10.26
13	14.62	1088	Terpinolene	0.28
Oxygenated monoterpenes				
12	14.21	1067	Sabinene hydrate	0.13
14	15.03	1099	α-Pinene oxide	0.15
15	15.69	1121	exo-Fenchol	0.2
16	16.36	1136	L-Pinocarveol	0.24
17	16.67	1145	Camphor	0.99
18	17.58	1176	Terpinen-4-ol	0.13
19	18.0	1187	α-Terpineol	0.48
20	18.3	1203	Verbenone	0.18
21	20.52	1285	Bornyl acetate	0.14
Sesquiterpenes				
22	23.01	1376	α-Copaene	0.16
23	23.36	1390	β-Elemene	0.22
24	24.2	1420	β-Caryophyllene	1.83
25	25.07	1452	α-Caryophyllene	0.25
26	25.74	1486	Germacrene D	0.3
27	28.16	1585	Caryophyllene oxide	0.55
28	28.45	1592	Viridiflorol	0.18

lack of these toxic constituentsn [17]. It may be assumed that the plant material was collected from locations with altitude above 1000 m, where the ecological and climatic factors play a significant role in the absence of thujones. In Norway, besides α- and β-pinene, borneol, bornyl acetate, and sabinene dominated [18], while in Estonia, the prevailing components were 1,8-cineole and sabinene [19]. In Iran, camphor and 1,8-cineole were most abundant [20], and in Lithuania, 1,8-cineole and (E)-nerolidol were dominant [21].

In addition to the chemical composition, the elemental composition of the Bulgarian *Achillea millefolium* essential oil was investigated, and the results are presented in Table 2.

It should be noted that low atomic number (low-Z) elements such as Na, Mg, and Al cannot be determined by TXRF due to absorption of their fluorescence lines by air. Furthermore, elements like P, Cl, and S may be underestimated because of their lower fluorescence yield and overlapping spectral lines.

Table 2. Elemental composition of *Achillea millefolium* essential oil.

Elements	Concentration, mg kg^{-1}
S	4.57 ± 0.02
Cl	4.17 ± 0.07
K	0.68 ± 0.04
Ca	1.71 ± 0.01
Cr	0.025 ± 0.002
Mn	0.027 ± 0.001
Fe	0.526 ± 0.005
Ni	0.017 ± 0.001
Cu	0.075 ± 0.001
Zn	0.086 ± 0.002
Se	0.008 ± 0.001
Br	0.069 ± 0.002

In contrast, elements with atomic numbers Z > 20 can be detected with high sensitivity [22]. Toxic elements such as Cd, Pb, Hg, and As were not detected in the analyzed samples.

As no literature data were found regarding the mineral composition of *A. millefolium* essential oil, a comparative literature analysis was performed with various essential oils including those from *Thymus vulgaris, Lavandula angustifolia, Mentha piperita, Mentha longifolia, Pinus sylvestris, Rosmarinus officinalis, Ocimum basilicum, Juniperus communis*, and many others. They have been analyzed using different techniques such as ICP-MS after microwave-assisted digestion [22], ETAAS without decomposition [22], direct TXRF [8], ICP-OES following ultrasonic extraction, wet digestion, and the three-phase emulsion method [24].

As shown in Fig. 1, the elemental profile of the yarrow EO aligns well with the reported ranges for other essential oils, with trace element concentrations falling near the lower end of literature values. Elements such as K and Ca are essential macronutrients, while Fe, Zn, Mn, Cu, and Se are required in trace amounts as essential micronutrients [25]. The low levels of potentially toxic elements like Cr and Ni indicated a high degree of purity and quality of the analyzed oil.

As noted above, the composition of a plant's essential oils can vary considerably depending on factors such as habitat, geographic location, climate, and soil characteristics [22]. Extraction techniques

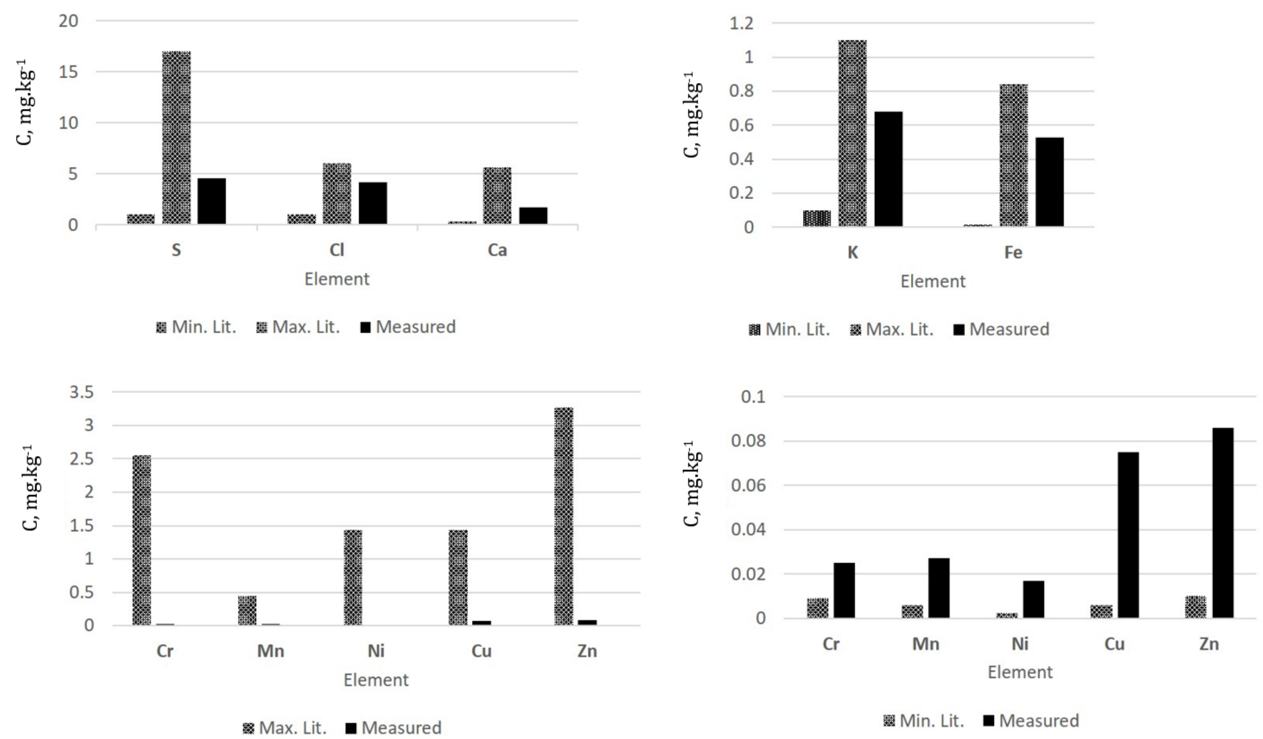

Fig. 1. Comparison of elemental concentrations, mg kg^{-1}, in yarrow essential oil with literature data for other essential oils

and contamination introduced during processing often have a greater impact than the botanical source itself. Essential oils typically exhibit low mineral content due to their hydrophobic and volatile nature, which limits the solubility and transfer of inorganic elements during distillation [8, 26, 27]. However, certain elements may be present in forms that volatilize at or below hydro-distillation temperatures (~100°C), allowing their co-distillation with the oil. For example, halogens such as Cl and Br, which have boiling points of 34.0°C and 58.8°C respectively, may transfer into the essential oil during the extraction process [8]. Similarly, the relatively high sulfur content could be attributed to the co-distillation of some of its soluble and volatile forms at elevated temperatures.

In conclusion, the elemental profile of *Achillea millefolium* essential oil reveals low overall mineral content and high purity, with trace element concentrations comparable to or lower than those reported for other essential oils.

The results obtained from the study of antioxidant activity according to the ABTS method μg mL^{-1} (9.47 %). The results are presented in Table 3 and Fig. 2.

The IC_{50} value for the ABTS method was 9.15 ± 0.01 μg mL^{-1}. In comparison, a sample from Serbia had an IC_{50} value of 26.03 μg mL^{-1} due to the dominance of 1,8-cineole and camphor [28]. Samples from Bosnia and Herzegovina and Iran, on the other hand, demonstrated an IC_{50} value of 0.34 μg mL^{-1}. These samples exhibited stronger antioxidant activity than the Bulgarian sample we studied, as they contained phenolic compounds such as thymol and carvacrol [29, 30]. The antioxidant activity is probably due to the terpene compounds α- and β-pinene. α-Pinene helps stop harmful radicals and boosts antioxidant enzymes, while β-pinene reduces the levels of inflammatory substances like TNF-α and IL-6 [31]. Due to the limitation of lipid peroxidation by the above-mentioned monoterpenes, Bulgarian yarrow oil shows moderate antioxidant activity by the ABTS method.

As seen from the results presented in Table 4, the *A. millefolium* EO at concentration of 10 mg mL^{-1} exhibited low antibacterial and antifungal activity (diameter of IZ < 12 mm), except the Gram-negative bacterium *K. pneumoniae* ATCC 13883 against which the inhibitory activity was moderate. The *A. millefolium* EO did not inhibit the fungi *A. niger* ATCC 1015 and *A. flavus*.

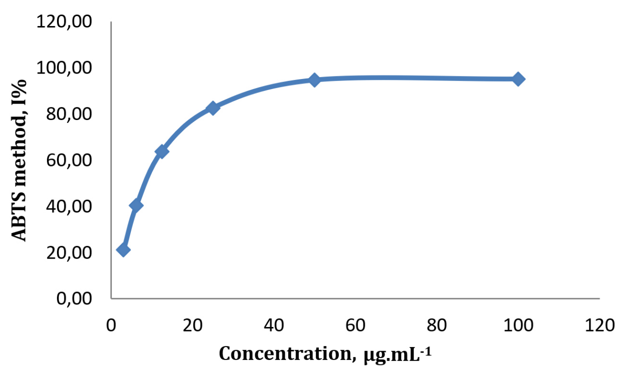

Fig. 2. IC_{50} of Bulgarian *Achillea millefolium* essential oil determined by the ABTS method.

Table 3. Antioxidant activity of Bulgarian yarrow (*Achillea millefolium*) EOs by ABTS method.

Concentration, μg mL^{-1}	Inhibition, %	mM TE g^{-1} EO
100	95.19 ± 0.01	6.40 ± 0.05
50	81.10 ± 0.48	12.74 ± 0.10
25	59.95 ± 0.20	22.24 ± 0.15
12.5	30.14 ± 0.20	34.35 ± 0.33
6.25	17.21 ± 0.14	43.29 ± 0.33
3.125	9.47 ± 0.34	45.35 ± 1.33

Table 4. Antimicrobial activity of Bulgarian yarrow (*Achillea millefolium*) EO compared to control - methanol.

Test microorganisms	Inhibition zone (IZ), mm*
B. subtilis ATCC 6633	9 ± 0.00
B. cereus NCTC 11145	9 ± 0.00
S. aureus ATCC 25923	8 ± 0.00
L. monocytogenes NBIMCC 8632	8 ± 0.00
E. faecalis ATCC 29212	8 ± 0.00
S. enteritidis ATCC 13076	8 ± 0.00
K. pneumoniae ATCC 13883	12 ± 1.41
E. coli ATCC 25922	8 ± 0.00
P. vulgaris ATCC 6380	8 ± 0.00
P. aeruginosa ATCC 9027	9 ± 0.00
C. albicans NBIMCC 74	8 ± 0.00
S. cerevisiae ATCC 9763	8 ± 0.00
A. niger ATCC 1015	-
A. flavus	-
P. chrysogenum	10 ± 0.71
F. moniliforme ATCC 38932	8 ± 0.00

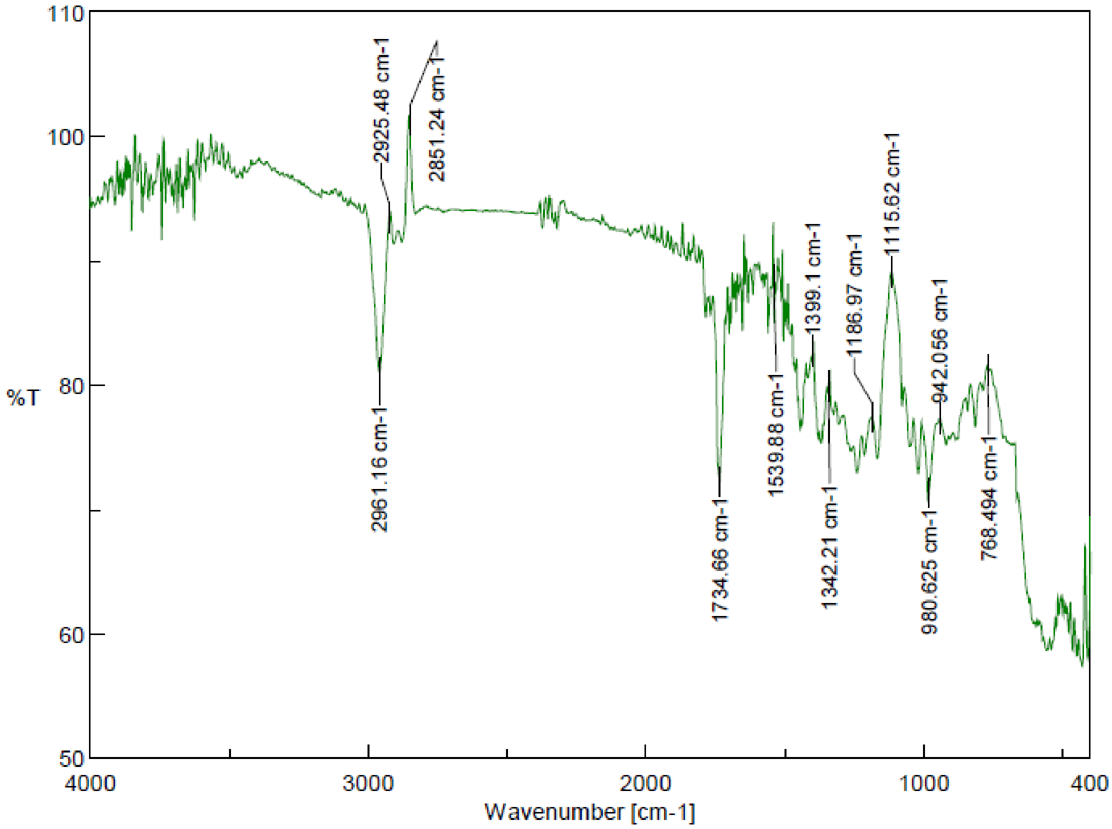

Fig. 3. IR spectrum of essential oil of *Achillea millefolium.*

The antimicrobial potential of *A. millefolium* EO is related to its chemical composition. Since it is rich in α-pinene (41 %), β-pinene (27 %), and limonene (10 %), it showed a moderate activity against some Gram-negative bacteria such as *K. pneumoniae*. Its weak inhibitory activity against other pathogens as *Staphylococcus aureus* and *Escherichia coli* may be explained by the absence of compounds such as chamazulene, thymol, carvacrol, or 1,8-cineole. Anyway, the yarrow essential oil can be used as a natural preservative in facial tonics, creams, and other products. When combined with other phenol-rich oils such as oregano and thyme, it may produce a synergistic effect against resistant bacterial strains. When impregnated into paper wraps or fabrics, it can be used to inhibit pathogens in food products.

The IR spectrum is presented on the Fig. 3. The analysis of the IR spectrum showed several distinct characteristic functional groups. The sharp peak around 2958-2952 cm^{-1} indicates the presence of volatile terpenes such as α- and β-pinenes, which can be seen in the GC-MS analysis [32]. The intense absorption band at 1740 cm^{-1} is likely caused by the presence of different ketones and esters in the essential oil, and has been observed by other authors [32]. The lack of a C-H band around 2720 cm^{-1} shows that no aldehyde components are present in the sample. The signals between 1458-1380 cm^{-1} are caused by CH bending vibrations typical for various terpenes, such as Sabinene and β-pinene. Alcohol C-O vibrations could be seen around 1160 - 1150 cm^{-1} typical for esters and alcohols like borneol and cineole.

CONCLUSIONS

Bulgarian *A. millefolium* essential oil showed a balanced profile, with a dominant presence of volatile monoterpenes with proven antioxidant and antibacterial properties, lack of toxic ketones (thujones), which ensures safety in its application. This makes *A. millefolium* EO suitable for therapeutic uses such as inhalations for respiratory diseases, a component of antiseptic agents for external use as well as for incorporation in various cosmetic products.

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Authors' contributions

K.N.: conceptualized the research idea, I.I., I.D., C.T., A.P., and Y.T.: conducted the experimental tests, N.R., A.G., and G.G.: contributed to the analysis of the obtained data, K.N. and G.G. discussed the results and wrote the text. All authors approved the final version.

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