PHYTOCHEMICAL CONSTITUENTS, ANTIOXIDANT, ANTIBACTERIAL AND ENZYME INHIBITION ACTIVITIES OF ESSENTIAL OIL FROM THE AERIAL PART OF *Achillea tomentosa* L. GROWN IN JORDAN

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ABSTRACT

This investigation aimed to evaluate the phytochemical constituents, antioxidant, antibacterial and α-glucosidase inhibitory activities of *A. tomentosa* essential oil. The essential oil extracted using steam distillation method possessed antibacterial activity for all tested bacteria. Comparing to cefaclor the percentage of relative inhibition zone diameter (RIZD) was 100.30 ± 4.73 % for *Staphylococcus aureus* and ranged between 79.67 ± 3.79 % and 84.33 ± 4.16 % for other bacteria. The essential oil showed considerable antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, ferric reducing antioxidant power (FRAP) assay and metal chelating methods, the EC50 of the essential oil were 72.06 ± 3.99, 54.69 ± 4.69 and 50.39 ± 2.86 µg/ml respectively, comparing 7.34 ± 2.43, 10.87 ± 3.34 and 14.50 ± 3.46 µg/ml for positive controls (Trolox, EDTA and Quercetin respectively). The essential oil also possessed concentration dependent α-glucosidase inhibitory activity with EC50 149.20 ± 7.63 µg/ml comparing to acarabose EC50 14.50 ± 3.46 µg/ml. Forty compounds were identified depending in retention index (RI) and mass spectra (MS) that form 96.31 % of the essential oil composition. The result showed that the ratio of the identified compounds range between 0.11 % and 23.72 %, 3-Carene (23.72 %), Limonene (12.19 %) and α-Terpinyl acetate (10.22%) were found to be the major components of the essential oil. m-Cyneme (7.11 %) and Di-n-octyl phthalate (4.50 %) present in moderate quantities. The phytochemical analyzes showed that the identified compound quantitatively and qualitatively differ from the previous study that investigate the composition of *A.tomentosa* L. essential oil.

Keywords: *Achillea tomentosa*, Antioxidant, α-glucosidase inhibitory, Antibacterial, DPPH assay, FRAP assay.

1. INTRODUCTION

Investigations were directed nowadays toward exploring medicinal plants for their biological activities. Medicinal plants produce verities of biologically active compounds that reported to possess antimicrobial, antioxidant [1], and enzyme inhibitory activities [2]. Jordan has amazing plants biodiversity profile with 2,543 species belong to 868 genera and 142 families from which 363 species belonging to 263 genera and 86 families have been recorded as medicinal plants used in traditional medicine and pharmaceuticals industry [3].

Chemically synthesis antioxidants such as Butylated hydroxyanisole (BHA), tert-Butylhydroquinone (TBHQ) and Butylated hydroxytoluene (BHT) are widely used in food production to reduce lipid peroxidation. Even though these compounds are more economically than natural product they have different side effect [4]. In addition, the resistant of some bacteria and fungi to some common antibiotics and antimicrobial synthetic agents have been increased [5]. Naturally occurring plant products have gained much important because they showed a wide range of activities such as antimicrobial, antimutagenic, antioxidant and anti-inflammatory activities [6]. Moreover, the essential oils mode of antimicrobial action targets multiple sites that cause reduction in the development of microbial resistance [7].

The species of the genus *Achillea* are well known aromatic medicinal plants that used traditionally for the treatment of variety of disease in different cultures. Nowadays, several *Achillea* species are grown commercially and used as source for the production of biologically active substances such as anti-inflammatory, spasmolytic, choleretic, treatment of wounds [8], essential oils and their constituents [9]. The infusion of *A. tomentosa* L. traditionally used in Jordan as carminative, muscular relaxant of uterus and arteries, for the treatment of intestinal colic [10] and stomachache [11].

The essential oil of *A. tomentosa* L. has been reported to have verities of biologically active compounds such 1,8-Cineol, α-pinene, linalool, α-terpineol, β-pinene and terpinen-4-ol [12,13]. Previous studies reported the anticancer [14], enzyme inhibitory [15], immunomodulatory [16], antimicrobial [17] antioxidant [18, 19] cytotoxic [20] and antiinflamatory [21] activities of the essential oils of *Achililea* spp other than *A. tomentosa* L. To the best of our knowledge there is no comprehensive study was conducted to investigate the biological activity of the essential oil of *A. tomentosa* L. Therefore, the aim of this study was to investigate the phytochemical constituents, antimicrobial, antioxidant, and the enzyme inhibition activity of essential oil of *A. tomentosa* L. in Jordan.

2. MATERIALS AND METHODS

2.1. Plant Material

The aerial parts of wild *A. tomentosa* plant were collected in May 2017 at the bloom stage from Irbid (32°30'11.6"N 35°56'10.1"E), Jordan. The plant sample was identified by plant taxonomist and voucher specimen was deposited in the Ibn Albitar herbarium, Faculty of Sciences, Al Al-Bayt University.

2.2. Essential oil Extraction

The essential oil was extracted by steam distillation method for 3 h. After distillation, the collected oil was separated from the water using separatory funnel and dried over sodium sulfate (water free) then stored at 4°C for further analysis [17].

2.3. Antibacterial Activity

Antimicrobial assay of *A. tomentosa* essential oil was carried out according to agar well diffusion method [22]. The activity was evaluated against two gram positive bacterial strains (*Bacillus subtilis* and *Staphylococcus aureus*); two gram negative bacterial strain (*Escherichiacoli* and *Pseudomonas aeruginosa*). The essential oil was prepared in 10% (v:v) dilution in dimethylsulphoxide (DMSO) which was also used as negative control. The active bacterial suspension was spread over the surface of Muller-Hinton medium using a sterile cotton swab to ensure the uniform growth of the organism. Using a sterile cork borer of 8 mm diameter, wells were made in each agar plate. Each agar well was filled with 100µL of the diluted essential oil, DMSO was used as a negative control in the same procedure.

The plates were left for 30 min at room temperature to allow the diffusion of essential oil and then plates were incubated at 37°C for 24 h. The zones of inhibition diameters were measured and recorded. Cefaclor (5 mg/ml) was used as standard control. Each test was performed in three replicates. The antibacterial activity was calculated by applying the following equation [22]:

$$\% \text{ RIZD} = \frac{\text{IZD sample} - \text{IZD negative control}}{\text{IZD antibiotic standard}} \times 100 \%$$

Where RIZD is the percentage of relative inhibition zone diameter and IZD is the inhibition zone diameter (mm).

2.4. α-Glucosidase Inhibitory Assay.

The α-glucosidase inhibitory activity was determined according to the method of **Ooi et al.**[23] The essential oil (25 μL) was mixed with 25 μL of 0.1 M sodium phosphate buffer (pH 7), 25 μL of 15 mM PNPG, and 25 μL of α- glucosidase enzyme solution (5 U/mL). The mixture was then incubated for 40 min at 37 °C. One hundred microliters of 0.2 M sodium carbonate solution was added to terminate the reaction. Acarbose was used as a positive control and the mixture without the essential oilwas used as negative control. The increase in absorbance due to hydrolysis of PNPG by α-glucosidase was measured using a Multiskan EX microplate reader (Thermo Fisher Scientific, Finland). The inhibition percentage was calculated as follows: % α-glucosidase inhibition = [(absorbance of negative control − absorbance of extract)/absorbance of negative control] × 100%. All measurements were done in triplicate.

2.5. Antioxidant activity

The antioxidant activities of the essential oil were evaluated using DPPH Free Radical Scavenging, Ferric reducing antioxidant power (FRAP) assay and chelating power assay as mentioned below:

2.5.1. DPPH Free Radical Scavenging Assay.

Free radical scavenging activity of the essential oils was measured using the method described by **Sulaiman et al.** [24]. The reaction mixture in each well of the 96-well plate used consisted of 50 μL of diluted (10% in DMSO) essential oil was serially diluted using 150 μL of 300 μM DPPH ethanolic solution to get 9.77 -1250 μg/ml essential oil concentration. The plates were wrapped and incubated at 37 °C for 30 min. The decrease in absorbance was measured at 515 nm using a Multiskan EX microplate reader (Thermo Fisher Scientific, Finland). Trolox was used as a reference for this assay. A standard curve was obtained using different concentrations of Trolox (from 9.77 to 1250 μg/ml). The absorbance of the reaction sample was compared to that of the Trolox standard curve.

EC50 value represents the effective concentration that could scaving 50% of DPPH, it derived from a nonlinear sigmoidal dose–response curve (GraphPadPrism 8, USA). All experiments were performed in triplicate.

2.5.2. Ferric reducing antioxidant power (FRAP) assay.

Reducing power of the essential oil was evaluated according to the method of **Ooi et al.** [23]. Using 96-well microplate, 50 μl of the diluted essential oil (10 % in DMSO) was serially diluted to get 9.77 - 1250 μg/ml essential oil concentrations using FRAP solution (10 ml of acetate buffer 300 mM (pH of 3.6), 1 ml of ferric chloride hexahydrate 20 mM (in distilled water) and 1.0 ml of TPTZ 10 mM (dissolved in 40 mM HCl). The solution temperature was raised to 37 ∘C. After 20 min of incubation the increase in absorbance at 593 nm was measured using Multiskan EX microplate reader (Thermo Fisher Scientific, Finland). The negative control was prepared similar to the essential oil by using DMSO instead of the essential oil. Trolox was used as a positive control. The FRAP percentage was calculated using the following equation:

% ferric reducing = [absorbance of essential oil/maximum absorbance (3.0)] × 100%. EC50 value represents the effective concentration that could reduce 50% of ferric (Fe+3) to ferrous (Fe+2) ions, it derived from a nonlinear sigmoidal dose–response, curve (GraphPadPrism 8, USA). All experiments were performed in triplicate).

2.5.3. Chelating Power Assay.

The chelating power activity was conducted according to the method of **Ooi et al.** [23]. 50 μL of the diluted essential oil (10% DMSO) was serially diluted to get concentrations mentioned previously using 5 μL of 2.0 mM ferrous chloride hexahydrate and 130 μL of methanol in a 96-well plate. The reaction was initiated by the addition of 15 μL of ferrozine (5.0 mM). The plate was incubated 10 min at room temperature, then the absorbance was measured at 562 nm using a Multiskan EX microplate reader (Thermo Fisher Scientific, Finland). The chelating power activity (%) was calculated according to the following equation: chelating power activity (%) = [(absorbance of negative control − absorbance of essential oil)/absorbance of negative control] × 100%. EC50 value, the concentration that chelate 50% of ferrous (Fe^{+2}) ions, it was derived from a nonlinear sigmoidal dose response curve (GraphPadPrism 8, San Diego, CA, USA).

2.5.4. Gas Chromatography Coupled with Mass Spectrometry Analysis (GC-MS)

The identification of different chemical compounds was realised by gas phase chromatography (TRACE GC-ULTRA, S/N 20062969, Thermo-Fischer) coupled with mass spectrometry (PolarisQ, S/N 210729, Thermo Fischer) (GC/MS). The utilized column was Varian capillary column Test Report CP 7770 (CP-SIL- 5 CB; 50m length, 0.32mm of Inside diameter, 0.45mm Outside diameter and Film thickness 1.20 μm). The column temperature was programmed from 40 to 260°C for 5°C/min. The temperature of the injector was fixed to 250°C and the one of the detector (PolarisQ) to 200°C.

Ionisation of the sample components was performed in electron impact mode (EI, 70 eV). The debit of gas vector (Helium) was fixed to 1ml/min. Transfer line temperature was 300°C. The mass range from 40 to 650 amu was scanned at a rate of 2.9scans/s. The volume of injected specimen was of 1μl of diluted oil in hexane solution (10%). The constituents of essential oils were identified in comparison with their retention indices, calculated in relation to the retention time of a series of linear alkanes (C4- C28) with those of reference products and in comparison with their spectra of mass with those gathered in a library (NIST-MS Search Version 2.0) and with those reported in the literature[17].

3. RESULT AND DISCUSSION

Antibacterial activity of *A. tomentosa* essential oil was tested against two gram positive and two gram negative bacterial species in vitro (Figure1). Gram-positive bacteria were more sensitive to the essential oil than Gram-negative bacteria. *S. aureus* was the most susceptible bacteria to *A. tomentosa* essential oil with RIZD 103 ± 4.73 %, while *E. coli* was the least susceptible bacteria with RIZD 79.67 ± 3.79 %. The essential oil of *A. tomentosa* was reported to contain varieties of compound such as 1,8-Cineol, germacrene-D, camphor and sabinene [12,13] which was found to have bacteriocidal activity [25].
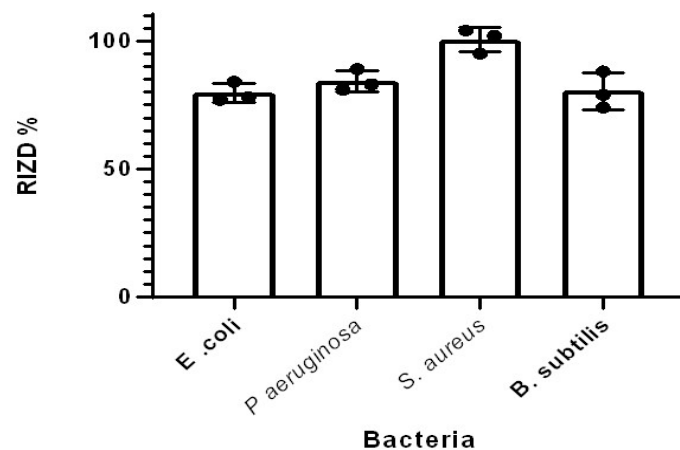
Figure 1. Antibacterial activity (RIZD %) of *A. tomentosa* essential oil comparing to the positive controls (Cefaclor 5 mg/ml). Values represented mean ± SD of triplicate analyses.

Using single antioxidant evaluation method did not give the complete picture about the antioxidant properties the essential oils [26]. Therefore, to evaluate the antioxidant activity of the essential oil DPPH, metal chelating, and FRAP methods were used. The purple colored DPPH compound act as electron acceptor and reduced to yellow colored diphenyl picrylhydrazine. Fig. 2A showed the DPPH radical scavenging activity of the essential oil of *A. tomentosa* essential oil and the positive control quercetin. The scavenging activity of the essential oil increased with concentration with EC 72.06 ± 3.99 μg/ml comparing to 7.34 ± 2.43 μg/ml for quercetin (table 1).

The ability of the essential oil to reduce ferric ion to ferrous ion (Fig. 2B) was tested using the FRAP method. The antioxidant properties of the essential oil convert ferric tripyridyltriazine complex to blue colored ferrous tripyridyltriazine at low pH at concentration dependent manner. The essential oil possessed lower ferric reducing activity (EC 54.69 ± 4.69 μg/ml) comparing to trolox (10.87 ± 3.34 μg/ml) (table 1). The reduction effect was found to be dose dependent and reach more than 85 % inhibition at high concentrations.

Table 1. EC50* values of essential oil and the positive controls (trolox, EDTA, quercetin and Acarabose) in different inhibitory assays.

Assay	Tested material	EC50 Value (µg/ml)
DPPH scavenging	Essential Oil	72.06 ± 3.99
	Trolox	7.34 ± 2.43
FRAP	Essential Oil	54.69 ± 4.69
	EDTA	10.87 ± 3.34
Metal chelating	Essential Oil	50.39 ± 2.86
	Quercetin	14.50 ± 3.46
α-Glucosidase inhibition	Essential Oil	149.20 ± 7.63
	Acarabose	14.50 ± 3.46

*EC50 values ± SD (µg/ml) were derived from the dose–response curve (variable) of triplicate analyses.

The result of the metal chelating properties of *A. tomentosa* essential oil is shown in Fig. 2C. These properties are based on the ability of essential oil to capture Fe^{+2} and disrupt the formation of intense red purplish in colored Fe^{+2}-ferrozine complex. The essential oil was found to disrupt the formation of Fe^{+2}-ferrozine complex in dose dependent manner with EC 50.39 ± 2.86 µg/ml comparing 14.50 ± 3.46 µg/ml for EDTA (table 1).

It has been reported that the antioxidant activity of essential oils related to the phytochemical composition of the oil that contain varieties of flavinoids and phenols, in addition to the synergistic effect of the constituents [27], *A. tomentosa* essential oil was found to contains varities of compound that belong to flavinoids and phenols [12,13]. **Carocho and Ferreira** [28] also reported that plant flavonoids may promote antioxidant activities via acting as reducing agents, hydrogen donors, metals chelating and/or free radicals scavenging.

The α-glucosidase inhibitory activity (Fig. 2D) is a preliminary approach for the development of medicines in treating type 2 diabetes mellitus. This assay measured the reduction of 4-nitrophenol from PNPG. In this assay, essential oil possessed α-glycosidase inhibitory effects in concentration dependent manner with more than 55 % of inhibition at high concentrations. The EC50 was found to be more than eight fold higher than acarbose, 149.20 ± 7.63 µg/ml comparing to 17.35 ± 3.57 µg/ml for acarbose (table 1). Antihyperglycemic drugs like α-glucosidase inhibitors (acarbose) that are often administered to type II diabetic patients before a meal to control postprandial glucose levels are effective to prolong the digestion of carbohydrates and cause the delay in glucose absorption into bloodstream [29].

The essential oil of A. tomentosa L. aerial parts collected form Irbid (Jordan) was extracted using steam distillation method, the yield of the oil was 0.93 %. The phytochemical composition of the *A. tomentosa* L. essential oil analyzed by GC-MS and identified by comparing mass spectra of the essential oil with the Wiley library, or with the published mass spectra is showed in table (2) A total of 40 compound were identified representing 96.31 % of the essential oil. The result showed 3-Carene (2372 %), Limonene (12.19 %) and α-Terpinyl acetate (10.22%) were found to be the main constituents of the essential oil. The identified compound qualitatively and quantitatively differ from the result of the only single study conducted by Chizzola[30] in which 1,8-cineol (eucalyptol) (56.1 %) and α-pinene (15.3 %) were main component of A. tomentosa L. essential oil.

A

B

C

D
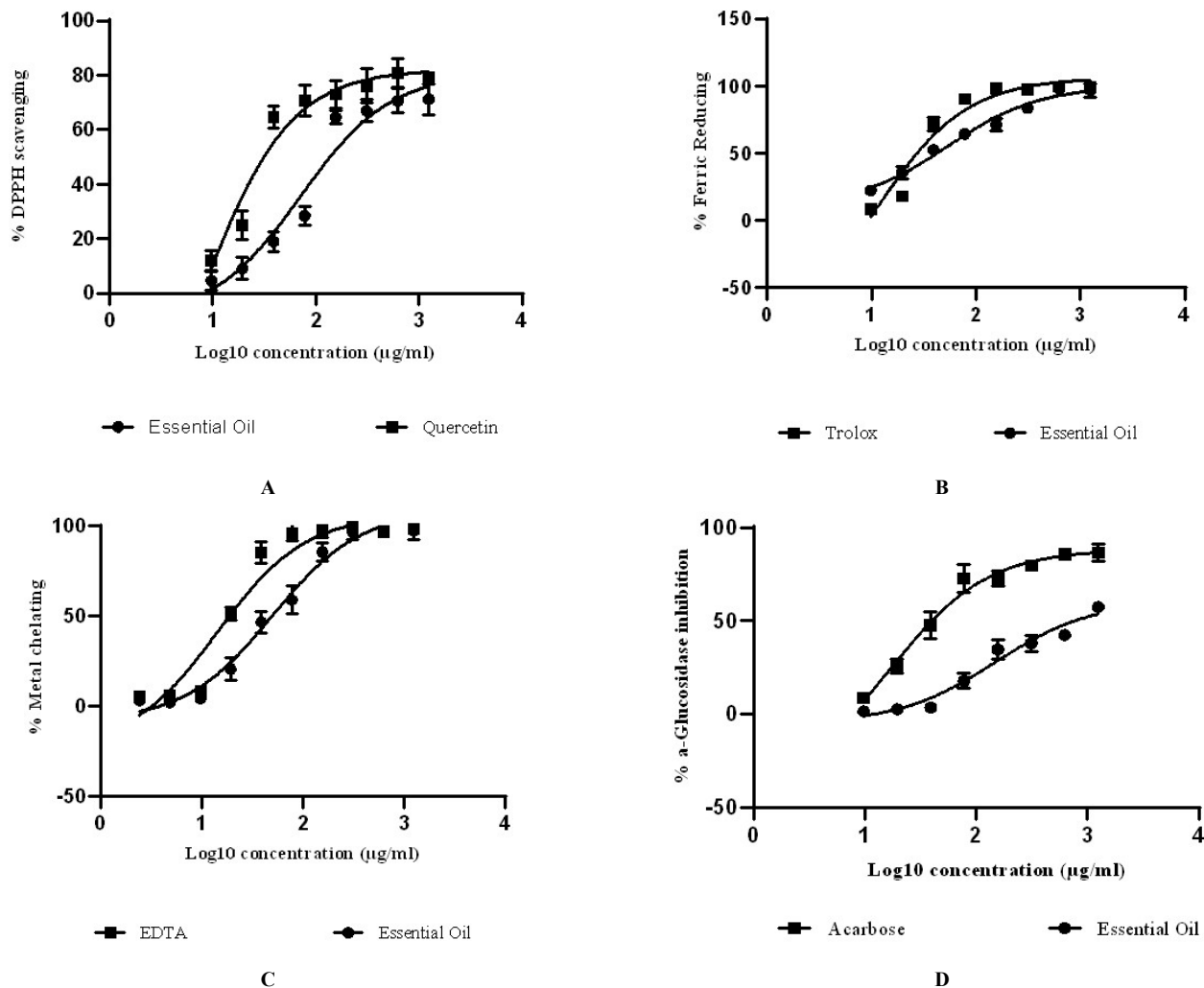
Figure 2. (A) DPPH scavenging, (B) ferric reducing, (C) metal chelating and (D) - % α-Glucosidase inhibition activities *A. tomentosa* essential oil and the positive controls (Trolox, EDTA, Quercetin and Acarabose). Values represented mean ± SD of triplicate analyses.

Other compound such m-Cymene (6.65 %), Di-n-octyl phthalate (4.83 %), α-Pinene (4.56 %) and 1,8-Cineol (3.55 %) present in moderate quantities, which also varies from the study of Chizzola[30] in which Linalool (4.7 %), α-terpineol (3.3 %), β-pinene (2.4 %) and terpinen-4-ol (2.3 %) were present in moderate quantities. As mentioned by previous studies different factors such as animal activity, harvesting time, temperature, developmental stage, geography, and growth conditions can cause quantitative and qualitative variations in the chemical constituents of essential oil[30-34.]

Comparing to closely related species *Achillea millefolium* several studies were reported that some chemotypes have sabinene, camphor, linalool, 1,8-cineole, β-pinene, or ascaridol as main essential oil components, chamazulene have been reported as major constituent in the essential oil of other chemotypse with high proportions of β-caryophyllene or nerolidol in their oils[36-38].

The structure-types and individual compounds of the essential oils appeared to be valuable markers for chemotaxonomic investigations in the view of evolutionary relationships while the level of oxygenation is mainly determined by environmental factors that affect the plant growth[39]. Furthermore, essential oil phytochemicals belong to majority of the terpene family, thousands of phytochmical consituentes of essential oils belonging this family have so far been identified[40].

To the best of our knowledge, this is the first study that reports the antibacterial, antioxidant and enzyme inhibition activity of *A. tomentosa* essential oil. Many investigations were conducted to evaluate the biological activity of *Achillea* spp. other than *A. tomentosa*. Al-jaber et al. [35] reported that the essential oil of *Achillea fragrantissima* inhibit the activity of cholinesterase with IC50 0.05 ± 0.01 µg/µL. The essential oil of *Achillea millefolium* L. was reported to possess strong antioxidant activities and showed bacterial inhibitory effect [38]. On other hand, **Kazemi** [25] reported that the essential oil of *Achillea wilhelmsii* showed appreciable antioxidant activity using DPPH assay.

Table 2. Phytochmical composition of the essential oil of *A. tomentosa* L.

Compound	RI	%
α-Thujene	933	0.46
α-Pinene	941	4.56
Camphene	953	0.72
β-Thujene	964	1.24
Thuja-2,4(10)-diene	968	2.98
3-Carene	1012	27.93
m-Cymene	1019	6.65
1,8-Cineol	1029	3.55
γ-Terpinene	1052	2.52
2-Nonanone	1074	2.25
Linalool	1102	0.59
n-Amyl isovalerate	1107	0.17
Cosmene	1113	0.28
β-Thujone	1118	1
Amylisovalerate	1120	0.24
cis-β-terpineol	1125	0.71
Camphor	1137	2.38
Lavandulol	1157	0.39
2,3-Dehydro-1,8-cineole	1161	0.16
Terpinen-4-ol	1165	1.45
cis-Piperitol	1185	0.62
α-Terpineol	1194	0.67
Myrtenol	1201	0.13
Limonene	1214	11.79
(Z)-Chrysanthenyl acetate	1255	1.95
Lavendulyl acetate	1273	0.65
L-Bornyl acetate	1283	1.24
α-Terpinyl acetate	1351	9.78
Cyclosativene	1373	0.28
α-Copaene	1389	0.97
β-Caryophyllene	1425	0.35
α-Humulene	1457	0.18
4,11-Selinadiene	1472	0.18
a-Selinene	1487	2.51
Bicyclogermacrene	1498	0.22
β-Sesquiphellandrene	1520	0.07
(E)-Nerolidol	1534	0.73
Germacrene B	1559	0.19
Spathulenol	1583	0.25
Caryophyllene oxide	1595	0.13
c-Eudesmol, 10-epi-	1617	0.21
β-Eudesmol	1637	0.15
Isophytol	1949	0.1
Phytol	2115	0.34
Di-n-octyl phthalate	2681	4.83
Total		98.75

4. CONCLUSION

The findings of this research show that the essential oil of *A. tomnentosa* showed considerable antioxidant, antibacterial and enzyme inhibition activities. These findings may have important medical benefits and could direct attention for *A. tomentosa* plant as source for cancer and diabetes prevention and treatment compounds.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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