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Antimicrobial, antioxidant and cytotoxic activity on human breast cancer cells of essential oil from *Pinus sylvestris*. var *mongolica* needle

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ABSTRACT

Pinus sylvestris. var *mongolica* is a major source of timber in Mongolia. The logging process makes many kinds of valuable biomass including bark, cones, and needles, which can be used for obtaining essential oil. The essential oil from the needles of wild growing *Pinus sylvestris*. var *mongolica* growing in Mongolia was chemically analyzed and its antibacterial, antifungal and cytotoxic activities were evaluated. The chemical analyses identified 101 compounds in the essential oil with the major compounds of α-pinene (29.87 %), limonene + β-phellandrene (16.15 %), camphene (4.95 %), bornylacetate (4.34 %), and β-pinene (3.88 %). This oil possessed the inhibitory activity against *B. subtilis, S. cerevisiae, S. aureus* and *E. coli*, successively with minimum inhibition concentration of 0.125, 0.1, 3.0, and 10.0 µg/mL. Importantly, the oil at 50 µg/mL and 100 µg/mL inhibited the growth of MCF-7 cells by 45.3 % and 99.7 %. The half of inhibition concentration of DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging was 14.36 ± 0.28 mg/mL. The results, therefore, suggested that the essential oil of a Mongolian Scotch pine could potentially be used as a preservative material in cosmetic and food products, as a bioactive agent in anti-inflammatory and wound healing products in view of its antibacterial activity. Given our findings that this essential oil has such profound activity against MCF-7 cancer cells, a further investigation concerning the full extent of this essential oil's anticancer activities seems warranted. Furthermore, given the promising antimicrobial effects of this essential oil against various bacterial species, an investigation concerning its effect against drug-resistant bacteria would be of immense interest.

Keywords: Pinaceae, *B. subtilis*, volatile component, α-pinene

INTRODUCTION

There is an enormous potential to extend the utilization of biomass accessible in vast volumes of unused deposits, including wood, bark, and leaves. In the way from logging and pruning processes, wasted materials are found in huge quantities and can be used as valuable raw material because of their bioactive chemicals [1]. Recently, there has been an expanded interest in the chemical composition of essential oil from various Pinaceae species due to their enormous biomass squandered by the logging industry [2, 3]. In Mongolia, a total of nine species of the genus Pinaceae are distributed, occupying 71 % of the forest area and *Pinus sylvestris* var. *mongolica* Litv is one of them [4].

Pinus sylvestris L (Scotch pine), evergreen coniferous trees, broadly dispersed for the most part in the Northern Hemisphere. *Pinus sylvestris*. var *mongolica*, named as Mongolian Scots pine, is native to Russian Siberia, the northeast area of China and Mongolia. Needles, buds, essential oil and resin of Mongolian Scotch pine are used in traditional medicine for the treatment of liver, rheumatic, and skin diseases and many respiratory infections accompanied by emphysema, common colds, cough, bronchitis, bronchial asthma, laryngitis and influenza [5, 6] due to their various bioactive secondary metabolites [7]. Essential oil (EO), a mixture of volatile components in the plants' secondary metabolic processes, is the most significant group of

compounds with various pharmacological properties of the Scotch pine [1]. The EO of Scotch pine needles is a colorless and pale yellow liquid that smells like dry-balsamic, turpentine and it possesses wide therapeutic effects, including antibacterial [8, 9], antifungal [10], antiseptic [11], and anticancer [12]. The EO has been used in the diseases of the respiratory system like cough or catarrh and applied commonly in massages, clinical showers, and packs [1, 5]

Many studies have currently focused on investigating the new molecules with therapeutic properties in Scotch pine based on its traditional usage. However, there is still limited information on the biological activities of the essential oils from the needles of Mongolian Scotch pine. This study aimed to approve the potential of pharmacological utilization of the needle essential oils from *Pinus sylvestris*. var *mongolica* growing in Mongolia. This is the first report on the antimicrobial and anti-oxidant activities and the cytotoxic effect on breast cancer cells of the essential oil.

EXPERIMENTAL

Plant material: Pine needles were collected in September 2018 from the Bayanzurkh Mountain located in the eastern side of Ulaanbaatar, Mongolia. The voucher specimen (No.56485) is garnered in the Herbariums of the Institute of Botany, Mongolian Academy of Sciences.

Isolation of essential oil: The pine needles were dried and crushed to 4 - 6 cm. The air-dried aerial pine needles (70 - 80 g) were further hydro-distilled in the Clevenger type apparatus for 3 h. The essential oil yield was 0.4 %. The essential oil was dried over anhydrous calcium chloride, then put away in fixed vials at 4 °C until examination.

Gas chromatography – mass spectrometry analysis (GC-MS): GC analysis of EO has performed on Hewlett Packard HP 5890 II Gas Chromatography with fused silica DB-Wax column (30 m x 0.25 mm; film thickness: 0.25 µm). Nitrogen was carrier gas with a linear velocity of 38 mL/min, the split ratio in 30 : 1. The temperature of the detector and injector were 250 °C, column temperature was programmed from 80 °C to 200 °C at a rate of 2 °C/min. The EO sample (1 %) in dichloromethane was injected with an amount of 0.5 µL. Quantitative data is determined by an electronic integration of the flame ionization detector (FID) peak area. GC/MS analysis was conducted on an instrument HP 5971A with MS detector 5890 II, was operated in EI mode (70 eV). It is using a Supelcowax 10 column (60 m x 0.25 mm; film thickness: 0.25 µm); He was as a carrier gas with linear velocity 10 mL/min, split ratio 30 : 1. The column temperature was programmed from 80 °C to 120 °C at a 3 °C/min rate and the injector and detector temperatures were 250 °C, 280 °C respectively.

The identification of the separated components is obtained by matching with the library data of mass - spectra and comparing Kovat's indices with those genuine components with published data.

Culture medium and inoculum preparation: *Bacillus subtilis* MNL-0919, *Bacillus cereus* MNL-0920, *Saccharomyces cerevisiae* MNL-0921, *Paeonia anomala* MNL-09196, *Geotrichum candidum* MNL-09102, *Staphyllacocus aureus, Aspergillus niger*, *Penicillium* sp., and *Fusarium* sp. were used as test organisms in the study from the microbial collection of the Institute of Biology, Mongolian Academy of Sciences. Yeast extracts were sub-cultured into peptone dextrose (YEPD) agar for 5–7 days at 37 °C, bacteria and fungi were sub-cultured at Potato dextrose agar for 24 h, respectively. Mueller–Hinton broth was used as a suspension for colonies from bacteria plates. Fungi were suspended in Sabouraud dextrose agar, and turbidity was coordinating to 0.5 McFarland standard (10^8 CFU/mL). Then the organisms were incubated properly as specified for each species for a period of 18–24 h [13].

Determination antibacterial activity of the essential oil (Agar well diffusion assay): The antimicrobial activity of EO was tested against Gram-positive bacteria *B. cereus, B. subtilis, S. aureus, yeast P. anomala, S. cerevisiae*, and *G. candidum* using the disc-diffusion method [14]. Standardized inoculum of each bacteria and yeasts in the amount of 100 µL were spread onto sterile Muller–Hinton Agar and Sabouraud Dextrose Agar, respectively. A well with 8 mm diameter was cut from the agar; subsequently, 100 µL of the pure and diluted essential oils filled each well, then the plates were incubated as appropriately, as specified for each organism at 28 - 40 °C for 2 or 3 days, each sample was tested in triplicates. The antimicrobial activity of essential oils was formed clear zones by inhibiting microbial growth. The clear zone was measured in millimeters at 24 and 48 hours after the incubation. 50 % dimethyl sulfoxide (DMSO) solution was used as a solvent and a control.

Determination of the essential oil on growth parameters of micro-organisms: Suitable volumes (40, 20, 10, 5, and 2.5 µL) of the EO were straightforwardly added to 40 mL of ME stock bringing in concentration from 1 to 0.0625 µL/mL. Then, the broths, including the EOs with approximately 10^5 CFU/mL culture suspensions incubated under smooth shaking for 48 h at 28 - 37 °C, appropriately to each organism in Erlenmeyer flasks with cotton wool vent-peg and, further sealed with parafilm. Samples were collected every 1 or 2 h. The absorbance of samples was measured at 580 nm; absorbance change revealed the growth rate per time unit (1/h). The growth rate was evaluated by determining the slope of the straight line matched on the growth curves in the exponential phase. The length of the lag phase was calculated by the X value of the straight line at the initial absorbance. All of the calculations were performed by MS Excel. The calibration curve of a cell count versus absorbance at 580 nm gave the maximum total cell count formed in

the stationary phase. Measurements were done three times for each sample.

Determination of minimal inhibitory concentrations (MICs) of the essential oil: Macro dilution assay was used to determine the MICs of the EO, [15]. For the assay, 1 mL of EO in concentrations from 0.0625 µL/mL to 1 µL/mL were added to 30 mL of ME medium in Erlenmeyer flasks, followed by incubation with approximately 10^5 CFU/mL bacteria and yeast for 24 h at 28 °C or 37 °C respectively. Then, the absorbance of the suspensions was measured at 580 nm. MICs were taken the most reduced concentration at which no noticeable growth happened where any colonies were framed in plate count assay. Negative controls contained EO components in ME medium. The absorbance increments measuring over 5 % at time 0 were served as growth positive samples.

Cell culture: Human ER-positive breast cancer MCF - 7 cell was bought from Korean Cell Line Research Foundation and was cultured in Dulbecco's Modified Eagle Medium compounded with 10 % fetal bovine serum and 1 % penicillin. The cells were incubated in a humidified atmosphere, including 5 % carbon dioxide at 37 °C.

In vitro cytotoxic assay: The effects of EO on the viability of malignant cells were determined by ez-cytox cytotoxic assay [16]. Briefly, human breast cancer cells (MCF - 7) were grown in 96 - well microtiter plates, with each well containing 10^4 CFU/mL. After 24 h, 10 µL of test samples with concentrations 50 and 100 µg/mL dissolved in DMSO were added to each well. One plate without a sample is considered as a day 0 control. After cell culturing during 48 h at 37 °C, ez-cytox were fixed, followed by determination of optical densities at 450 nm using a Microplate Reader (Tecan, Switzerland). The percentage of growth inhibition was calculated using the following equation:

$$\% \text{ Growth} = \frac{[\text{OD (reagent)} - \text{OD (day 0)}] \times 100}{[\text{OD (negative control DMSO 10 \%)} - \text{OD (day 0)}]}$$

Where, OD is absorbance values or optical density. Etoposide, a potential anticancer agent, was used as a positive control.

Determination of antioxidant activity (2,2-Diphenyl-1-picrylhydrazyl free radical-scavenging capacity) of the essential oil: Measurement of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich) radical scavenging capacity was performed according to Karamać *et al* [17]. Briefly, 2 mL methanol solution of 0.5 mmol/L DPPH was mixed with 5, 10, 20, 40 mL of different concentrations of EO of Mongolian Scotch pine. After 20 min incubation, the absorbance was measured at 517 nm with a spectrophotometer. Methanol was served as the positive control. The percentage of free radical-scavenging capacity was calculated by the following equation:

$$\text{Radical scavenging capacity (\%)} = (A_{blank} - A_{sample})/A_{blank} \times 100$$

Where, A_{sample} is the absorbance of DPPH with essential oil, A_{blank} is the absorbance of DPPH with methanol. All measurements were carried out in triplicate and reported as the average value. The results were expressed as half of minimum inhibition concentration-IC_{50} (mg/mL) and trolox equivalent per mL of EO or fraction (µg TE/mg EO).

Statistical analysis: Antioxidant data were calculated and expressed as concentrations, at which 50 % of free radical was scavenged (IC_{50} values ± standard deviation). All experiments were performed in triplicate and the Excel software was used for the calculation of IC_{50} values. $P < 0.05$ was determined to be significant.

RESULTS AND DISCUSSION

Chemical compositions: The essential oil (EO) of needles from Mongolian Scotch pine obtained by hydrodistillation showed amber color with a mild aromatic odor. The average yield was 0.4 % on a dried weight basis. The chemical composition of the oil is presented in Table 1.

A total of 101 constituents, representing 98.36 % of the total EO, were identified by GC/MS. The results showed that monoterpene hydrocarbons (59.20 %) were dominated group and sesquiterpenes (19.29 %) resented the second largest group. The major compounds were the monoterpene α-pinene, followed by limonene + β-phellandrene, camphene, bornyl acetate, ∆-cadinene and β-pinene. In general, the monoterpenes significantly release into the air because of their high enough vapor pressures at normal atmospheric conditions [18]. The published report has previously shown that the chemical compositions of the EO of Scotch pine consist mainly of 50 - 90 % monoterpene hydrocarbons, and the other components are sesquiterpene hydrocarbons as well as the oxygenated mono- and sesquiterpenes. The EO is mainly dominated by α-pinene, camphene, β-pinene, 3-carene, β-myrcene, limonene, β-phellandrene, p-cymene, terpinolene, bornyl acetate, and β-caryophyllene, and diterpenes (isopimaral and isoabienol) occurred as mains in few samples [19, 20]. Within the cases, the principal components including α-pinene, 3-carene, and β-phellandrene or β-pinene clarified 37.6 %, 23.6 %, and 10.1 % of the whole change, individually, permitting the visualization of more than 70 % of the EO of Scotch pine. For that reason, chemically, Scotch pine can be divided into three groups: α-pinene, 3-carene, and β-phellandrene or β-pinene based on the amount of those compounds accumulated in needle EO [20]. The needles EO of Mongolian Scotch pine belongs to α-pinene chemotype similar to Scotch pine native to Kosovo, Slovakia, Greece, and Russia (Siberia) [1].

Table 1. The chemical compositions of essential oil from Mongolian Scotch pine needles.

No	Composition	RT	RI	%
1.	tricyclene	7.29	921	1.18
2.	3-thujene	7.46	926	0.13
3.	**α-pinene**	**7.68**	**932**	**29.87**
4.	**camphene**	**8.16**	**947**	**4.95**
5.	verbenene	8.32	952	0.04
6.	sabinene	8.95	973	0.18
7.	**β-pinene**	**9.04**	**975**	**3.88**
8.	6-methyl-hept-5-en-2-one	9.42	987	0.05
9.	β-myrcene	9.55	991	1.34
10.	α-phellandrene	9.98	1004	0.11
11.	α-terpinene	10.41	1017	0.07
12.	meta-cymene	10.69	1022	0.27
13.	**limonene+ β-phellandrene**	**10.87**	**1028**	**16.15**
14.	1,8-cineol	10.93	1031	0.03
15.	cis-β-ocimene	11.18	1038	0.05
16.	trans-β-ocimene	11.54	1048	0.80
17.	Ɣ-terpinene	11.89	1058	0.13
18.	terpinolene	12.94	1088	0.38
19.	trans-2,3-epoxy-pinane	13.29	1097	0.06
20.	para-cis-menth-2-en-1-ol	14.11	1121	0.11
21.	α-campholenal	14.28	1126	0.05
22.	trans-pinocarveol	14.73	1138	0.25
23.	camphor	14.93	1144	0.16
24.	p-mentha-1,5-dien-8-ol	15.42	1168	0.05
25.	pinocamphone	15.51		0.04
26.	pinocarvone	15.52	1162	0.03
27.	borneol	15.69	1166	0.24
28.	terpinen-4-ol	16.1	1177	0.26
29.	cryptone	16.41	1187	0.42
30.	α-terpineol	16.58	1191	0.19
31.	myrtenol	16.77	1197	0.22
32.	trans-3-methyl-6-(1-methylethyl)-2-cyclohexen-1-ol	17.15	-	0.07
33.	verbenone	17.23	1210	0.12
34.	trans-carveol	17.56	1219	0.16
35.	cumine aldehyde	17.66	1241	0.03
36.	cis-carveol	17.95	1233	0.07
37.	thymol methyl ether	18.12	1236	0.62
38.	cumine aldehyde	18.28	1241	0.08
39.	carvone	18.42	1245	0.09
40.	geraniol	18.77	1255	0.26
41.	2-undecanone	18.88	-	0.20
42.	5-pentyl-3H-furan-2-one	19.15	1266	0.04
43.	phellandral	19.41	1276	0.33
44.	**bornyl acetate**	**19.88**	**1287**	**4.34**
45.	cumin alcohol	19.99	1293	0.11
46.	undecan-2-one	20.11	1294	0.27
47.	carvacrol	20.29	1302	0.05
48.	∆-elemene	21.97	1338	1.00
49.	α-ylangene	22.7	1372	0.03
50.	α-copaene	22.78	1378	0.38
51.	β-bourbobene	23.07	1387	0.44
52.	β-cubebene	23.31	1392	0.14
53.	β-elemene	23.3	1392	0.15
54.	β-longipinene	23.54	1401	0.04
55.	methyl eugenol	23.73	1406	0.06
56.	caryophyllene	24.25	1422	1.06
57.	β-copaene	24.48	1432	0.22
58.	aromadendrene	24.77	1440	0.28
59.	guaia-6,9-diene	24.91	1445	0.30
60.	cis-muurola-3,5-diene	25.14	1448	0.19
61.	humulene	25.3	1456	0.24
62.	cis-muurola-4(14),5-diene	25.53	1465	0.25
63.	trans-cadina-1(6),4-diene	25.85	1476	0.20
64.	Ɣ-muurolene	26.01	1480	1.05
65.	D-germacrene	26.09	1484	0.85
66.	β-selinene	26.24	1488	0.74
67.	bicylosesquiphllandrene	26.42	1494	0.33
68.	valencene	26.57	1494	1.25
69.	α-muurolene	26.66	1502	0.92
70.	bornyl 2-methylbutanoate	26.86	1510	0.13
71.	(E,E)-α-farnesene	26.94	1510	0.06
72.	Ɣ-cadinene	27.15	1517	2.74
73.	**∆-cadinene**	**27.42**	**1527**	**4.95**
74.	zonarene	27.42	1528	0.10
75.	trans-cadina-1,4-diene	27.62	1536	0.33
76.	α-cadinene	27.77	1541	0.21
77.	α-calacorene	27.93	1546	0.46
78.	salviadienol	28.24	1555	0.23
79.	β-calacorene	28.52	1565	0.19
80.	citronellyl 3-methylbutanoate	28.85	1577	0.15
81.	**spathulenol**	**29.01**	**1580**	**2.78**
82.	caryophyllene oxide	29.11	1586	0.79
83.	valencene	29.34	1494	0.14
84.	salvial-4(14)-en-1-one	29.5	1598	0.16
85.	β-oplopenone	29.94	1610	0.28
86.	1,10-di-epi-cubenol	30.09	1618	0.23
87.	α-corocalene	30.22	1624	0.12
88.	1-epi-cubenol	30.39	1632	0.68
89.	isospathulenol	30.73	1640	0.07
90.	**T-Muurolol**	**30.76**	**1644**	**2.47**
91.	∆-cadinol	30.95	1649	0.37
92.	α-cadinol	31.18	1658	1.33
93.	10-hydroxy-cis-calamenene,	31.31	1662	0.04
94.	cadina-3,10(15)-dien-5beta-ol	31.62	1684	0.05
95.	cadalene	31.68	1677	0.07
96.	germacra-4(15),5,10(14)-trien-1-ol	31.9	1688	0.07
97.	eudesma-4(15),7-dien-1beta-ol	31.99	1688	0.15
98.	3beta-hydroxy-muurola-4,9-diene	32.53	1701	0.07
99.	pentadecanal	32.76	1712	0.16
100.	hexahydrofarnesyl acetone	36.17	1846	0.06
101.	nor- (isomer 2) - dehydroabietan	40.33	2020	0.07
	Monoterpene hydrocarbones			59.20
	Oxygenated monoterpenes			9.33
	Sesquiterpene hydrocarbones			19.29
	Oxygenated sesquiterepes			10.54
	Unidentified			1.64
	Total			**100**

They are also rich in α-pinene, camphene, β-pinene, limonene or β-phellandrene which possess high biological activities including antimicrobial, anti - inflammatory antimetastatic, and apoptotic effects [21 - 25]. Moreover, the compositions of EO of Scotch pine were studied in different geographical areas since the contents of the main compounds and the biological activities of EO are suggested to be varied considerably depending on the geographical areas [3]. For example, Murbach and Andrade *et al*. reported that the EO of cultivated Scotch pine in Australia, which contains bornylacetate (32.7 %), camphene (21.6 %), and α-pinene (10.9 %), inhibited the growth of *S. aureus* and *E. coli* with MIC values of 2.58 mg/mL and 26.22 mg/mL, respectively. The authors also reported that the EO of Scotch pine of Central Balkans, containing α-pinene (41.9 %), camphene (4.7 %), and δ-3-carene (3.6 %), showed the inhibitory effects against these two bacteria in the range of 5 – 20.00 mg/mL (MICs) [26]. After analyzed the chemical composition, we conducted the antimicrobial activity in the following research.

Antimicrobial activity: The antimicrobial activity of EO of Mongolian Scotch pine needle is determined the inhibition zone against the growth of Gram - positive bacteria, *B. subtilis*, *B. cereus*, and yeast strains, *S. cerevisiae*, *P. anomala*, *G. candidum*, using two different concentrations, 100% (pure) and 50 % of the essential oil (in DMSO), after incubated for 24 and 48 hours. The results of antimicrobial activity are shown in Table 2. Interestingly, the pure EO exhibited different degrees of toxic activity against most of the tested bacterial and yeast strains except the *G. candidum*.

We observed no difference in the sensitiveness of the bacteria growth depending on the investigation times 24 h or 48 h. The sensitivity of test organisms to the pure EO decreased in the following sequence: *S. cerevisiae* > *P. anomala* > *B. subtilis* > *B. cereus* > *S. aureus.*

Table 2. The antimicrobial activity of the essential oil in clear zone of inhibition (mm).

Micro-organisms	Time (h)	The inhibition zone (mm)	
		EO 100%	EO 50%
Gram positive bacteria			
B. subtilis	24	5	4
	48	5 ± 0.5	4
S. aureus	24	4	-
	48	4	-
B. cereus	24	5	-
	48	5	-
Yeast			
S. cerevisiae	24	10	-
	48	10	-
P. anomala	24	6	-
	48	6	-
G. candidum	24	-	-
	48	-	-

Notice: 100 % - pure essential oil, 50 % - diluted in DMSO; (clear zone > 6 mm) – moderate; (0<clear zone<6 mm)-optimal; (clear zone = 0 mm) – non active

The diluted (50 %) EO showed the activity against *B. subtilis* only with a range of 4 mm clear zone as similar to the pure EO. To obtain more detailed results, the tests of effects on bacteria growth parameters are performed with three species those showing the highest, medium, and the lowest sensitivity to the EO. The effects on growth parameters were determined by total cell number versus absorbance calibration curve using spectrophotometer measurement for Gram-positive bacteria (*S. aureus* and *B. subtilis*) and yeast *S. cerevisiae* in agar media. The lag phases and the maximum growth rates of the test organisms are shown in Table 3.

Table 3. Effects of the essential oil on the duration of lag phases of bacteria and yeast

Essential oil concentration (µl/mL)	*S. aureus*		*B. subtilis*		*S. cerevisiae*	
	Lag phase	Growth rate	Lag phase	Growth rate	Lag phase	Growth rate
Control	4.518 ± 0.568	0.289 ± 0.371	7.375 ± 0.019	0.586 ± 0.013	3.733 ± 0.258	0.242 ± 0.005
0.625	3.778 ± 2.389	0.092 ± 0.025	36.6 ± 1.714	0.443 ± 0.414	5.569 ± 0.367	0.284 ± 0.043
0.125	5.261 ± 3.069	0.083 ± 0.036	> 48.00	0	12.229 ± 0.401	0.368 ± 0.037
0.25	11.16 ± 0.781	0.065 ± 1.278	> 48.00	0	20.776 ± 0.875	0.537 ± 0.275
0.5	13.65 ± 0.015	0.157 ± 0.783	> 48.00	0	24.399 ± 0.057	0.277 ± 0.241
1.0	17.372 ± 0.964	0.206 ± 0.197	> 48.00	0	>48.00	0

Notice: Lag phase: h, mean ± SD, Growth rate : 1/h, mean ± SD, SD: Standard deviation*, * Various letters point to significant differences (p<0.05).

The lag phases of the tested bacteria and yeast strain were considerably prolonged by increasing the EO concentrations in all of the cases. The most sensitive species was approved as *B. subtilis*, the lag phases of this bacterium could lengthen over 48 h at low concentrations of the EO, and, there was no growth at 0.125 µL/mL of it. For *S. cerevisiae*, the decrease of the lag phase of this strain was observed at 1.0 µL/mL concentration during 48 h investigation time. The maximum total cell counts were calculated from the growth curves, where indicated the different variance from the control. A moderate change on maximum total cell counts of *B. subtilis* was shown at a low concentration of EO, whereas 0.5 µL/mL EO decreased

to no growth of the maximum total cell counts in this bacterium. In the case of *S. aureus*, the maximum total cell counts fluctuated at all the concentration of EO. Besides, we also examined the effect of the EO on growth parameters of gram - negative bacteria *E. coli* and compared the minimum inhibitory concentrations (MIC) against three tested species.

As described in the experimental part, the MIC was determined by measuring absorbance accordingly with the growth rate of *S. aureus, B. subtilis* and *S. cerevisiae* in ME medium, and the results are shown in Table 4.

Table 4. MIC (μL/mL) of the essential oil in growth medium.

Essential oil	*B. subtilis*	*S. aureus*	*S. cerevisiae*	*E. coli*
Concentration	0.125	3.0	1.0	10.0

The EO had interesting antimicrobial potential as a result of low MIC values. MIC values oscillated between 0.125 (*B. subtilis*) to 10.0 μL/mL (*E. coli*). The antimicrobial activity of the essential oil of Mongolian Scotch pine might be resulted due to its major chemical compounds. α-Pinene compound is well known to have good antimicrobial activity [27] and it has been shown to exhibit the antimicrobial activity against *E. coli, C. freundii, C. sporogenes, E. faecalis, E. carotovora, F. suaveolens, K. pneumoniae, M. luteus, B. subtilis, S. aureus, S. epidermidis, P. aeruginosa* [24, 28]. β-Pinene and limonene could, therefore, explain the antimicrobial property [27 - 29]. Mechanistically, the action of terpenes has been suggested to cause the membrane disturbance through lipophilic compounds and interaction with the intracellular sites. [30, 31]. In addition, it is also possible that the components in lower percentage might be involved in some types of synergism with the other active major compounds [32]. For example, Jiali *et al.* reported that the α - pinene shows inhibition to *B. subtilis* and *E. coli* at the concentrations of 65 and 125 mg/mL [29], while the EO of Mongolian Scotch pine has the MIC values of 0.125 and 10.0 μg/mL against the bacteria.

As the most interesting observation from the current results, the MIC values were comparatively higher than the obtained results from the agar diffusion method and the previous researches. The main components of the EO might have limited diffusion through the agar medium because of their high volatility during its incubation [18]. In the MIC assay system, the essential oil was tested in an emulsion form, and the less dense oil was partitioned well to the liquid culture system. This may enhance the antibacterial effects in the MIC assay.

Antioxidant activity: The antioxidant activity of EO of Mongolian Scotch pine was assessed in a series of in vitro assays. The DPPH assay is a measured form of DPP-H produced by donating hydrogen atoms or electrons of the essential oil for in transformation of DPPH· using a spectrophotometrical method. In Table 5, the results were expressed as IC_{50} and trolox equivalent per mg of EO or fraction (μg TE/mg EO).

Table 5. DPPH value of the EO from Mongolian Scotch pine needle.

DPPH value	Concentration
Essential oil IC50 (mg/mL)	14.36 ± 0.28
Trolox IC50 (μg/mL)	9.54 ± 0.14
Essential oil TEAC (μg TE/mg)	0.66 ± 0.02

Notice: ± mean - Standard deviation

The half maximal inhibitory concentration (IC_{50}) scavenging DPPH of the EO was 14.36 mg/mL, equal to 0.66 μg TE/mg. The result can be inferred that the EO of Mongolian Scotch pine has low antioxidant activity [33, 34], as similar to other species that rich in monoterpenes accumulation, including *P. sylvestris, Cupressus sempervirens, Eucalyptus globulus* and *Rosmarinuse officinalis* [35, 36]. Concerning literature data, very little is known about the antioxidant activity of the essential oil of needles of Scotch pine. Kurti and Giorgi have determined the poor antioxidant activity (0.224 μg TE/mL) for EO of Scotch pine needles in Kosovo. This pine contains an abundant monoterpene hydrocarbons consisted of α-pinene (32.45 %) and β-pinene (13.65 %), limonene (9.35 %), δ-cadinene (7.05 %), 3-carene (6.45 %) and β-caryophyllene (4.50 %) [37]. Notably, the main constituents were very similar to the results of this study, whereas our DPPH value was higher than the previous determination, as about three times higher. This can also suggest that the antioxidant activity of EO might not be depending on the main components, but also the minor compounds that adding potential synergistic effects to the main components.

Cytotoxic activity toward breast cancer cells: In this work, we also focused for the first time that needle EO of Mongolian Scotch pine has the potential to suppress the viability of human breast cancer MCF-7 cells. The inhibition activity of the EO against the breast cancer cell growth was determined by MTT assay. A dose-dependent decrease in viability of human breast cancer cells was observed after 48 h of treatments with 50 and 100 μg/mL of the EO. As the result, the essential oil inhibited the growth of MCF-7 cell line by 43.7 % at 50 μg/mL, while the same concentration of the etoposide (control) inhibited the cell growth by 32.3 %. A high cytotoxic effect of the EO on the human breast cancer cell line (MCF-7) was observed with 100 μg/mL concentration of EO by inhibiting the cell growth to 0.8 %. Earlier studies noted that the growth of this cancer cell line is directly stimulated by the estragon [38]. For that point, the EO of Mongolian Scotch pine could be a potential candidate for endocrine

treatment. Previously, the extracts prepared from the needles of various pine species have been shown to exert some anticancer effects [12, 39, 40]. Hoai *et al*. reported that the EO of Estonian Scotch pine needle possessed a strong cytotoxic activity on both MCF-7 and MDA-MB-231 cell lines, and those IC_{50} values were 28.67 µg/mL and 29.23 µg/mL, respectively [12]. The authors stated that the oil could not only responsible for chemoprevention but also for chemotherapeutic to endocrine insensitive breast tumors. Therefore, EO of Mongolian Scotch pine needle may seem to contain some compounds or fraction with a high potential to be developed as candidates for prevention or therapeutic adjuvants to breast cancer.

CONCLUSIONS

For the first time, the present work provides the research results on the chemical compositions and some biological activities of the essential oil of *Pinus sylvestris*. var *mongolica*. As compared with the previous studies [26, 29, 37], the large differences in antimicrobial and antioxidant activities seem not related to the major chemical components in the essential oil, rather with observed possibility of the synergistic effects of the minor components to the major components. Besides, our results showed the essential oil could be an easily accessible source of the potential candidate for developing novel therapeutic anticancer agents.

Given our findings that this essential oil has such profound activity against MCF-7 cancer cells, a further investigation concerning the full extent of the anticancer activities of this essential oil seems warranted. Furthermore, given the promising antimicrobial effects of this essential oil against various bacterial species, an investigation concerning its effect against drug - resistant bacteria would be of immense interest. A detailed study on its anticancer and antibiotic activity against drug resistant bacteria is required based on the results in this work.

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