

**ANTIBACTERIAL, ANTIOXIDANT AND CYTOTOXIC STUDIES OF THE
ESSENTIAL OIL AND ETHANOL EXTRACT OF AERIAL PARTS OF *Artemisia rutifolia*
*Steph.ex. Spreng***

ABSTRACT: Mongolia is rich in medicinal plants. In recent years, interest in plant-derived food additives has grown. This study was aimed to evaluate antioxidant, cytotoxic activities of aerial parts ethanol extracts from *Artemisia rutifolia Steph.ex Spreng* grown in Mongolia.

The essential oil composition variability of *A.rutifolia Steph.ex Spreng* aerial parts collected from three different slopes of Kharkhira mountain was assessed by gas chromatography mass spectrometry (GC/MS).

Chromatographic analysis of *A.rutifolia Steph.ex Spreng* essential oils showed the predominance of monoterpene hydrocarbons represented mainly by santolina triene, β -myrcene.

The antioxidant and cytotoxic activities of the essential oil and ethanol crude extracts were determined by using DPPH and MTT assays. The antioxidant activity of *A.rutifolia Steph.ex Spreng* ethanol extract is 2.12 times higher than the antioxidant activity of the essential oil.

The essential oil of *A.rutifolia Steph.ex Spreng* with a concentration of 150 mg/ml or 3 μ g/disk inhibits the growth of *S.enterica* 9.3 ± 0.76 mm, *B.subtillus* 10.3 ± 0.58 mm, *S.aureus* 9.6 ± 1.5 mm and has a moderate bacterial activity.

The results clearly showed that the essential oil presented satisfactory cytotoxic activity against two human tumor cell lines *HepG2* (human liver cancer cell line); *AGS* (human stomach cancer cell line). Our work revealed that the ethanol extracts and essential oil of *Artemisia ritifolia Steph.ex Spreng* grown in Mongolia has potential as sources of new antioxidant, and cytotoxic compounds, respectively.

Essential oil of *A.rutifolia Steph.ex Spreng* contains caryophylline-7.19% and caryophylline oxide-5.82%, so the essential oil at a dose of 25 mg/ ml inhibits the growth of human stomach cancer cell (*AGS*).

The results of the study were certified by the utility model certificate of "Soap Fragrance Elixir" with the registration number 20-003068.

Keywords: *Artemisia rutifolia Steph.ex Spreng*, essential oil; antimicrobial; antioxidant, and cytotoxic activities

Introduction

The genus *Artemisia* (*Asteraceae*) consists of about 500 species, distributing throughout the world, one of the most numerous plant groupings, which comprises about 1000 genera and over 20000 species [25,26].

The genus consists of a small herbs and shrubs, found in northern temperate regions and comprises of about 500 species from South Asia, North America, and European countries^[11]. In Mongolia, 103 *Artemisia* species have been found^[4].

The *Artemisia* species have wide and various applications to the plant and human disease control, as well as in the pharmaceutical industry^[3].

Artemisia has indiscriminately served as a treatment for maladies such as viruses, malaria, bacteria, hepatitis, fungi, cancer, and inflammation^[28].

The most important derivatives of *Artemisia annua* L. are arteether, artemether, artemiside, artemisinin, artemisone, artesunate, and dihydroartemisinin.

Artemisinin also use against some cancers such as liver cancer, brain glioma, leukemia, nasopharyngeal cancer, gallbladder cancer, gastric cancer, cervical cancer, lung cancer, breast cancer and colon cancer^[29].

Artemisia rutifolia Steph.ex Spreng grows in Afghanistan, China, India, Kazakhstan, Kyrgyzstan, Mongolia, Nepal, Pakistan, Russia and Tajikistan^[7].

Stems and leaves of *A.rutifolia Steph.ex Spreng* contain sugar, carbohydrates, nutrients, alkaloids, vitamin C, chlorophyll, sesquiterpene lactone-canine (Berekovskaya 1991), cirsilineol, tricinin, chrosoeriol^[6].

The aerial part of the *A. rutifolia Steph.ex Spreng* contains high levels of phenolic compounds such as bile acids, caffeic acid, chlorogenic acid, cyanuric acid, p-coumaric acid, m-coumaric acid, ferulic acid, vanilla acid, myricetin and quercetin^[17].

The essential oil of the *A. rutifolia Steph.ex Spreng* has a laxative effect on bacteria, fungi and helminths. Pharmacological studies have shown that canine is tolerant to cold and Chrysofenetin has anti-cancer effects^[2].

A.rutifolia Steph.ex Spreng has been used in traditional Pakistan medicine to treat fever, asthma, abdominal pain, inflammation, cancer and other diseases^[8].

The herbal teas have anti-asthma and anti-inflammatory, as well as diuretic properties. Fresh herbs are used to relieve toothache, sore throats, stomach and heart diseases^[7].

The *A. rutifolia Steph.ex Spreng* essential oil with camphor and borneol has anti-inflammatory, anthelmintic and heart-stimulating properties^[9].

In Russian traditional medicine, *A.rutifolia Steph.ex Spreng* is used as a medicinal herb, while

in veterinary practice it is used to bandage the wounds of pets^[9].

Artemisia rutifolia from the Chitral region of Pakistan has been reported with its traditional uses against gastrointestinal problems. This plant is also used against fever and stomach worms^[20].

In Mongolian traditional medicine, the leaves of *A.palustris* show the beneficial influence for treatment of skin diseases, while the seeds are useful for treatment of bronchitis and tuberculosis^[18].

Essential oil of *Artemisia macrocephala Jacquem. ex Besser*, especially hamazulene shows anti-inflammatory and anesthetic activities^[19].

Essential oil of *A.rutifolia Steph.ex Spreng* kills bacteria, fungi and parasites^[21].

The herb in Mongolian-Tibetan medicine is used for yellowing of the eyes, violations of the regulatory system mkkhris (the system responsible for food rhenium, absorption, energy processes, assis-milation) and Botkin's disease^[27].

The aim of this study was to evaluate the antioxidant and cytotoxic effects of essential oil and ethanol extract from *A.rutifolia Steph.ex Spreng* grown in Mongolia.

The antioxidant activities of the essential oil and the ethanol extracts were tested by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay.

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) colorimetric method was used for determining cytotoxic activity of samples.

To the best of our knowledge, there are no published reports on the biological activities of the essential oil and ethanol extracts of *A.rutifolia Steph.ex Spreng* grown in Mongolia. Therefore, it is important to develop a better understanding of their mode of biological action for new application in human health.

Methods

Chemicals

Dimethyl sulfoxide (DMSO), and DPPH were purchased from Millipore-Sigma (Germany) and WST was purchased from DoGen (Korea). RPMI 1640 medium and fetal bovine serum were purchased from GIBCO (USA). Penicillin and streptomycin were purchased from Himedia (India).

The human hepatocellular cell line (*HepG2*) and human gastric cancer cell lines (*AGS*) were purchased from ATCC (USA). All other chemicals were of analytical grade and purchased from Millipore-Sigma (Germany) and DUKSAN Co. (Korea).

Plant material

Samples were collected from Kharkhiraa Mountain in September 2019 in Uvs aimag, Mongolia. Voucher specimens have been deposited in the herbarium of the Khovd State University, Mongolia.

Isolation and Analysis of the Essential oil

The aerial parts (1.1 kg) of the freshly collected plants were finely chopped and hydro-

distilled for 3 h using a Clevenger-Adams type apparatus^[12]. The yield of the essential oil produced during the steam distillation was 0.96% (v/w). The oil was then stored at 4°C prior to analysis.

The GC-MS analysis of the essential oil sample was carried out using a Agilent 6890 gas chromatograph equipped with mass selective detector MSD 5973 (Agilent) on capillary column HP5 (5% diphenyl and 95% dimethylsiloxane, 30 m x 0.25 mm x 0.25 µm (film thickness).

The temperature of injector was 280°C. The column temperature was programmed as follows: 2 min at 50°C, temperature increase at a rate of 4 deg/min to 240°C and then at a rate of 20 deg/min to 280°C, isothermal period of 5 min. Helium was used as a carrier gas (1.0 ml/min).

MS conditions were as follows: ionization voltage of 70 eV, acquisition mass range 30–650, data acquisition rate of 1.2 scan/s. 1.0 µl of sample (solution of the essential oil in hexane, 8.0 µl per 0.5 ml) was injected in a split mode with split ratio 100:1. A mixture of normal hydrocarbons C₈–C₂₄ was added to the sample as a standard for determining linear retention indices.

Essential oil components were identified by comparison of their full mass spectra and linear retention indices (RI) with these parameters for authentic samples listed in the handbook^[13]

Essential oil components were identified by comparison of their mass spectra and linear retention indices (relative to C₈–C₂₄ alkanes) with those reported in database developed in our laboratory^[11,12].

Extraction and fractionation

The air-dried and powdered whole plant (150 g) was extracted with 99% ethanol (2 L x 3) using sonicator under room temperature. The resultant extracts were combined and evaporated in a rotary vacuum evaporator (Buchi R-205, Switzerland) at 40°C to afford crude extracts. The obtained ethanol crude extract was weighed (30g) and stored in the refrigerator for the later analysis.

Determination of the Antioxidant activity

The assay was carried out according to the method of Brand-William et al.^[13] to investigate the free radical scavenging activity of samples. Briefly, the samples were dissolved in ethanol at the concentration of 100 mg/ml and then serially diluted by ethanol.

On each well of a 96-well plate, 100 µl of samples of different concentration were mixed together with 100 µl of 60 µM DPPH prepared in ethanol. After incubation of 20–30 minutes for reaction, the absorbance of supernatants was measured at 517 nm by using Multi-detection Reader (Bio Tek Co.). Ethanol was used as negative control and -tocopherol as positive control. The scavenging capacity (SC) of the sample was calculated using the following formula:

$$SC (\%) = [1 - A_s/A_c] 100$$

Where, A_s=is the net absorbance of the sample, A_c=is the net absorbance of negative control. The IC₅₀ value of a sample is the concentration of sample at which 50% activity of DPPH (absorbance) is inhibited. It was calculated by linear regression.

Determination of antimicrobial activity

To investigate the antimicrobial activity of essential oil and ethanol extract from *A.rutifolia Steph.ex Spreng*, we evaluated its effect on four different bacteria, such as *S.enterica*, *B.subtillus*, *S.aureus*, *E.coli* by Agar diffusion method.

Determination cytotoxic activity

HepG2 cell was cultured in RPMI-1640 medium supplemented with 0.2% sodium bicarbonate, 1% penicillin-streptomycin and 10% fetal bovine serum at 37°C in 5% CO₂ incubator. The four samples were prepared as 30 mg/ml stock solutions in DMSO. The *HepG2* cell was treated by samples with final concentration of 300 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml and 10 µg/ml, and incubated for 24 hours.

RPMI-1640 medium with 10% WST was added to the treated cells. After 1-hour incubation, the cultured cells were quantified by spectrophotometer, measuring the absorbance of the dye solution at 450 nm. Results of each extract were compared to that of DMSO only treated control cells, 1% v/v DMSO. The IC₅₀ was calculated for each sample by IC50 Calculator by AAT Bioquest. Avoiding the possibility of metabolic activity alteration thus tetrazolium dye reduction without affecting cell viability, the results were then checked under microscope by examination of live condition^[14].

Result and Discussion

Composition of Essential Oil

The composition of essential oil from *A.rutifolia Steph.ex Spreng* is presented in the Table 1.

We were able to identify only 28 components representing more than 88% of the total oil. The main constituents were found to be santolina triene (more than 22%) and β-myrcene (more than 21%).

A comparison of the chemical composition of essential oil of *Artemisia rutifolia Steph.ex Spreng* is shown. /Table-2 /.

Essential oils of Mongolian *Artemisia rutifolia Steph.ex Spreng* include α-pinene, β-pinene, camphene, sabinene, β-myrcene, 1,8 cineole, camphor, terpine-4-ol, β-elemene, caryophyllene and spatulenol. Containing compounds such as α-pinene, camphene, sabinene, 1,8-cineol, camphor this species is characterized by the hemotype characteristics of *A. rutifolia Steph.ex Spreng* essential oil.

For the first time, 17 terpenoid compounds were determined in essential oil from *A.rutifolia Steph.ex Spreng*, including santolina triene, pseudo-limonene, α-cedrene, caryophyllene, humulene, dehydro-

sesquicinel, selina-4,11-diene, germacrene-D, valencene, α -selinene, α -bulnesene, γ -cadinene, d-cadinene, cedrol, eremoligenol, α -bisabolol and aciphilyc acid.

Antioxidant activity

DPPH is free radical compound that has been widely used to determine free radical scavenging activity^[13].

The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability or radical scavenging activity. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form DPPH (non radical) with the loss of this violet color^[15].

The DPPH assay is used to analyze antioxidant activities by mechanism in which antioxidants act to inhibit lipid oxidation, so scavenging of DPPH radical and therefore determinate free radical scavenging capacity. The method was applied according to Brand-Williams et al^[13].

Ethanol extract was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution (30 mg/mL) for antioxidant assays. The extract was prepared by two times dilution method in 96-well microtiter plate. Also, Gallic acid standard solutions were prepared in 96 well micro liter plate for building of standard curve, which is used for calculation of antioxidant activity of samples.

The final results were expressed as ug/ml of Gallic acid equivalent (table-2).

The antioxidant activity of *A.rutifolia Steph.ex Spreng* ethanol extract is 2.12 times higher than the antioxidant activity of the essential oil.

The process of preperation of the soap fragrance using the essential oil of *Artemisia rutifolia Steph.ex Spreng*.

The acidity, pH, and antibacterial activity of soap fragrances were determined by the National Food Safety Reference Laboratory of the General Agency for Specialized Inspection of Mongolia.

The number of acids in soap fragrances was determined by titration method, the solution environment by standard method /MNS1474: 2000/, the acid number by standard method /MNS1131: 2018/, bactericidal ability by standard method /MNS6236: 2011/, alcohol content by standard method /MNF-2011/, the amount of *Eschirichia coli* bacteria by the standard method /MNS1595: 2017/, the amount of *Staphylococcus aureus* bacteria by the standard method /MNS1636: 2017/ and the amount of *Pseudomonas aruginosa* bacteria was determined by the standard method /MNS2173: 2017/^[31-37].

The environment and acid number of "Soap fragrance" elixir which was made using essential oil *A. rutifolia Steph.ex Spreng* were determined /Table-4/^[30].

Cytotoxic activity

To investigate the cytotoxic activity of ethanol extracts and essential oil from *A.rutifolia Steph.ex Spreng*, we evaluated its effect on a selection of liver cancer cell line *HepG2* and human stomach cancer cell line *AGS* by Rapid colorimetric assay.

These cell lines were submitted to growing concentrations of essential oil and ethanol extract *A.rutifolia. Steph.ex Spreng* for 24 hours. As shown in Figure 2-5, the essential oil of plant significantly active against chosen human cancer cell lines tested the ethanol extract (See Figure 2-5).

Antimicrobial activity

To investigate the antimicrobial activity of essential oil and ethanol extract from *A.rutifolia Steph.ex Spreng*, we evaluated its effect on four different bacteria, such as *S.enterica*, *B.subtillus*, *S.Aureus*, *E.coli* (See Table 3).

Conclusion

This study on essential oil chemical composition and biological activities of *A..rutifolia Steph.ex Spreng* grown in Mongolia were not well performed before.

Essential oils hydrodistilled from *A.rutifolia Steph.ex Spreng* were found to be rich in santolina triene, β -myrcene 1,8 cineol, camphor, caryophyllene, caryophyllene oxide.

The essential oil of *A.rutifolia Steph.ex Spreng* with a concentration of 150 mg/ml or 3 μ g/disk inhibits the growth of *S.enterica* 9.3 \pm 0.76 mm, *B.subtillus* 10.3 \pm 0.58 mm, *S.aureus* 9.6 \pm 1.5 mm and has a moderate bacterial activity.

The antioxidant activity of the ethanol extracts were moderate than essential oil. The results clearly showed that the ethanol extracts presented satisfactory cytotoxic activity against 2 human cancer cell lines tested. The results of this work also demonstrate the potential of *A.rutifolia Steph.ex Spreng* ethanol extracts as a new antioxidant and cytotoxic agents for human health.

Essential oil of *A.rutifolia Steph.ex Spreng* contains caryophyllene-7.19% and caryophyllene oxide-5.82%, so the essential oil at a dose of 25 mg/ml inhibits the growth of human stomach cancer cell (*AGS*).

The antibacterial activity of "Soap fragrance" preperation against *Eschirichia coli*, *Staphylococcus aureus* and *Pseudomonas aruginosa* bacteria meets the requirements of /MNS1595: 2017/, /MNS1636: 2017/, /MNS2173: 2017/ standards.

The results of the study were certified by the Utility model certificate of "Soap Fragrance preperation" with the registration number 20-003068.

Acknowledgments

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31. MNS1474: 2000- The number of acids in soap fragrances was determined by titration method, the solution environment by standard method

32. MNS1131:2018-The acid number by standard method

33. MNS6236: 2011- Bactericidal ability by standard method

34. MNF-2011-Alcohol content by standard method

35. MNS1595:2017-The amount of *Escherichia coli* bacteria by the standard method

36. MNS1636:2017- The amount of *Staphylococcus aureus* bacteria by the standard method

37. MNS2173:2017-The amount of *Pseudomonas aruginosa* bacteria was determined by the standard method

Supplementary Material

Table 1. Composition of the essential oil from *Artemisia .rutifolia Steph.ex Spreng* grown in Mongolia

Chemical	Retention time, min	Retention indices (RI)	Content, %
santolina triene	6.583	908	22.38
α -pinene	7.356	932	0.30
camphene	7.810	947	0.21
sabinene	8.648	973	0.82
β -myrcene	9.247	991	21.84
pseudo-limonene	9.420	1003	5.27
1,8-cineol	10.554	1031	4.63
camphor	14.575	1144	2.13
terpinen-4-ol	15.839	1177	0.68
β -elemene	23.029	1392	0.84
α -cedrene	23.672	1412	0.22
caryophyllene	23.888	1422	7.19
humulene	24.957	1456	0.59
dehydro-sesquicineol	25.412	1471	0.90
selina-4,11-diene	25.628	1477	0.97
germacrene D	25.816	1484	0.99
valencene	26.184	1494	1.20
α -selinene	26.256	1496	2.17
aciphyllene	26.350	1500	1.34
α -bulnesene	26.567	1508	0.84
γ -cadinene	26.819	1517	0.48
δ -cadinene	27.087	1527	0.42
spaphulenol	28.725	1580	1.97
caryophyllene oxide	28.863	1586	5.82
cedrol	29.787	1604	2.27
eremoligenol	30.205	1631	0.69
α -bisabolol	31.750	1688	0.42
aciphilyc acid	36.761	1879	0.91

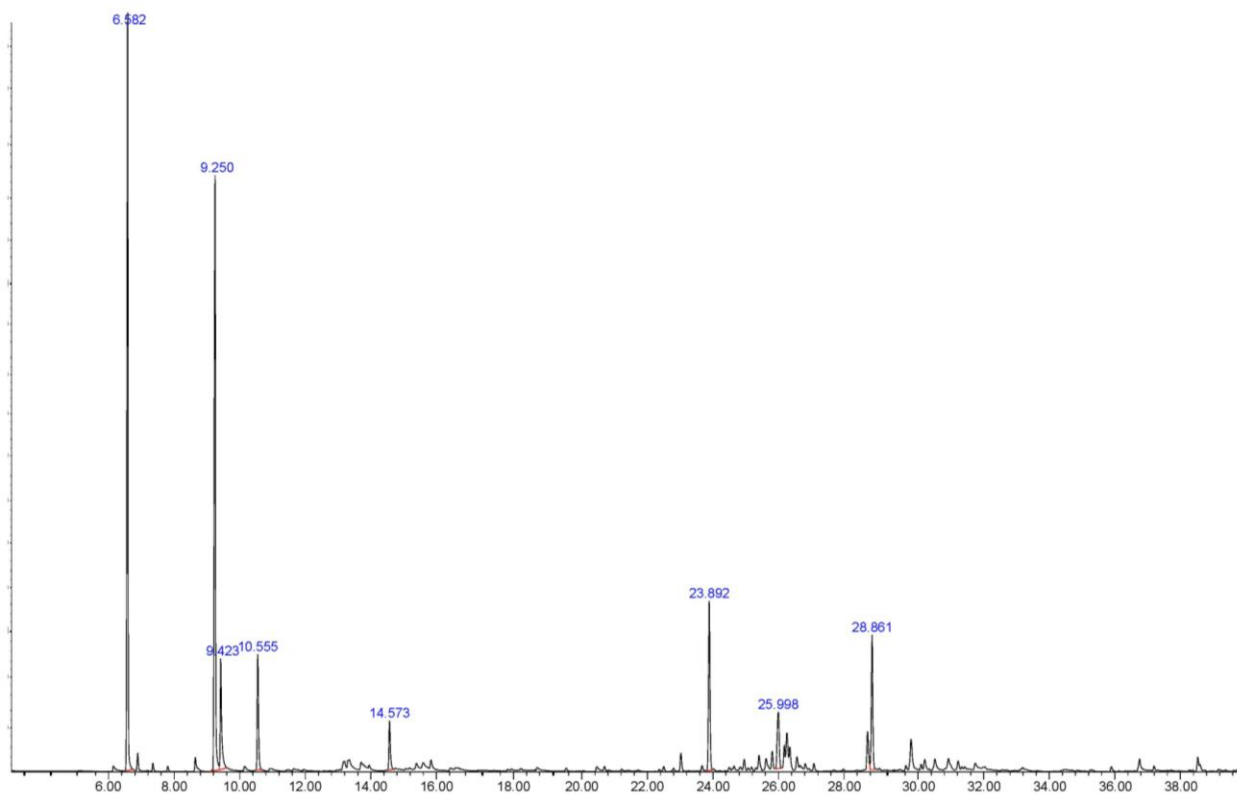


Figure 1. GC/MS chromatogram of essential oil from *Artemisia rutifolia* Steph.ex Spreng grown in Mongolia

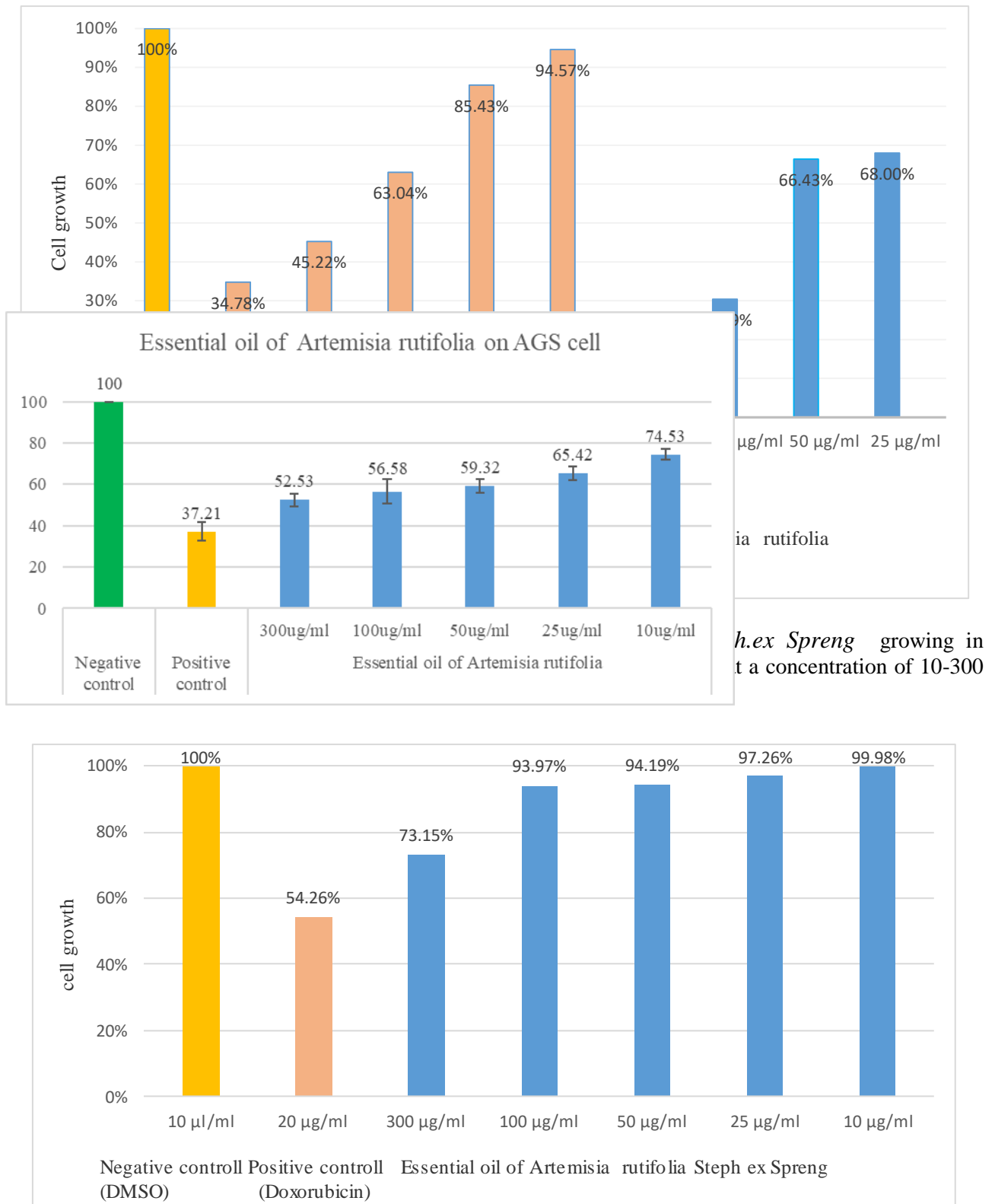


Figure 3. Cytotoxicity (%) of essential oil from *Artemisia rutifolia Steph.ex Spreng* growing in Mongolia against *HepG2* cell line. Cell was treated with the fractions for 24 h at a concentration of 10-300 µl/ml.

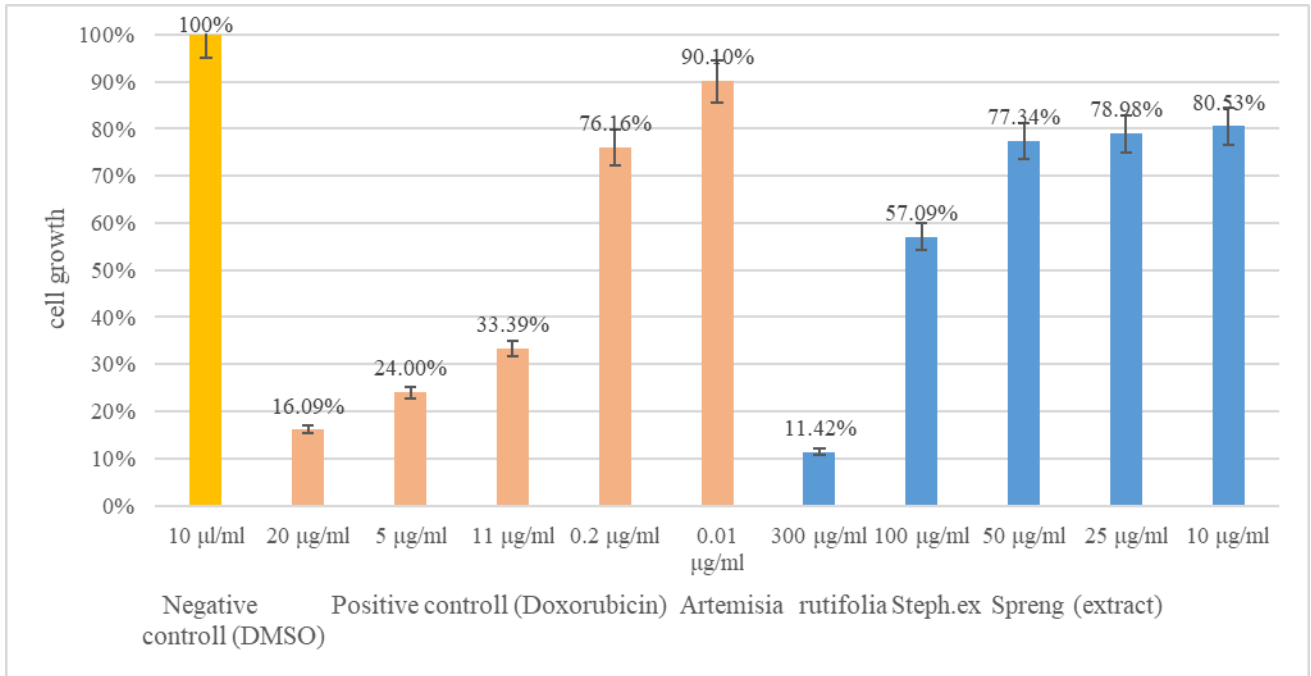


Figure 4. Growth inhibitory effect of ethanol extract *Artemisia rutifolia* Steph.ex Spreng on AGS cells after 24 hours treatment. The results are expressed as percentage of untreated control

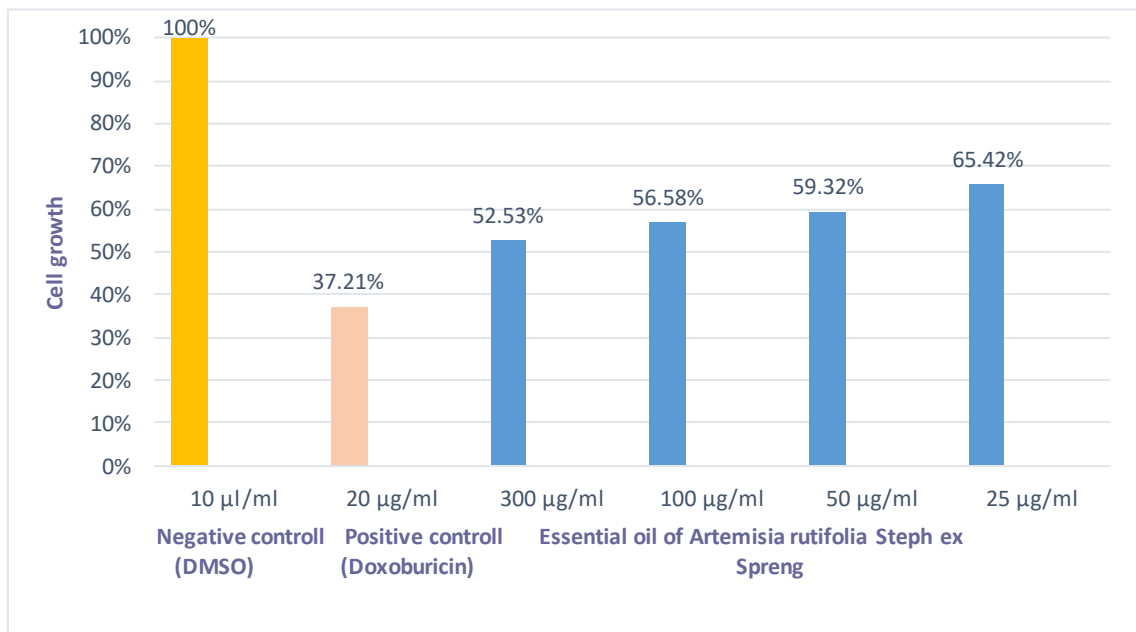


Figure 5. Growth inhibitory effect of essential oil *Artemisia rutifolia* Steph.ex Spreng on AGS cells after 24 hours treatment. The results are expressed as percentage of untreated control

Table 2. Comparison of chemical composition of *Artemisia rutifolia* Steph.ex Spreng essential oil (%)

№	Turpentine compounds	Content%/	Essential oil of <i>Artemisia rutifolia</i> Steph.ex Spreng [22]	Essential oil of <i>Artemisia rutifolia</i> Steph.ex Spreng [24]	Essential oil of <i>Artemisia rutifolia</i> Steph.ex Spreng [23]	Essential oil of <i>Artemisia rutifolia</i> Steph.ex Spreng [7]
1	santolina triene	22.38				
2	α - pinene	0.30	1.246	+	+	1.25
3	camphene	0.21	1.336	+		0.34
4	sabinene	0.82	0.267	+		0.27
5	β -myrcene	21.84	0.153		+	0.15
6	pseudo-limonene	5.27	-	-		
7	1,8-cineol	4.63	25.134	25.14		25.13
8	camphor	2.13	21.735	21.71	8.9	21.74
9	terpinen-4-ol	0.68	3.539	3.54	1.6	
10	β - elemene	0.84	0.712			0.71
11	α - cedrene	0.22				
12	caryophyllene	7.19			+	
13	humulene	0.59				
14	dehydro-sesquiceneol	0.90				
15	selina-4,11-diene	0.97				
16	germacrene D	0.99				
17	valencene	1.20				
18	α - selinene	2.17				
19	aciphyllene	1.34				
20	α - bulnesene	0.84				
21	γ - cadinene	0.48				
22	d- cadinene	0.42				
23	spathulenol	1.97	0.166	+		0.17
24	caryophyllene oxide	5.82				
25	cedrol	2.27				
26	eremoligenol	0.69				
27	α -bisabolol	0.42				
28	aciphilyc acid	0.91				

Table 3. Growth inhibition effect of essential oil and ethanol extract from *Artemisia rutifolia Steph.ex Spreng* on gram positive and negative bacteria

Sample	Zones of growth inhibition in mm							
	<i>S.enterica</i>				<i>B.subtilis</i>			
	Product 3µg/disc		0.6 µg/disc		Product 3µg/disc		0.6 µg/disc	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
<i>Artemisia rutifolia Steph.ex Spreng (essential oil)</i>	9.3	±0.7	6.6	±0.2	10.3	±0.5	6.5	±0.00
<i>Artemisia rutifolia Steph.ex Spreng (ethanol extract)</i>	7.8	±0.7	7.2	±0.5	7.0	±0.5	6.5	±0.5
<i>Positive control (ethanol extract)</i>	21	±1.3	21	±1.3	21	±1.3	21	±1.3
<i>Positive control -kanamycin (essential oil)</i>	20	±0.5	20	±0.5	20	±0.5	20	±0.5
<i>Negative control- (ethanol extract)</i>	6.0	±0.00	6.0	±0.00	6.0	±0.00	6.0	±0.00
<i>Negative control (essential oil)</i>	6.0	±0.00	6.0	±0.00	6.0	±0.00	6.0	±0.00

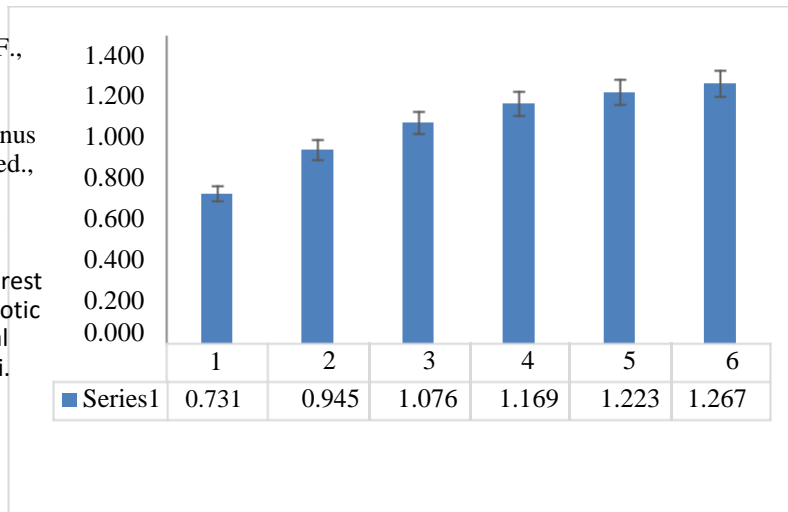
continued

Sample	Zones of growth inhibition in mm							
	<i>S.aureus</i>				<i>E.coli</i>			
	Product 3µg/disc		0.6 µg/disc		Product 3µg/disc		0.6 µg/disc	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
<i>Artemisia rutifolia Steph.ex Spreng (essential oil)</i>	9.6	±0.5	6.0	±0.00	6.5	±0.0	6.5	±0.0
<i>Artemisia rutifolia Steph.ex Spreng (ethanol extract)</i>	6.8	±0.2	6.2	±0.2	6.0	±0.00	6.0	±0.00
<i>Positive control (ethanol extract)</i>	21	±1.3	21	±1.3	21	±1.3	21	±1.3
<i>Positive control (essential oil)</i>	20	±0.5	20	±0.5	20	±0.5	20	±0.5
<i>Negative control (ethanol extract)</i>	6.0	±0.00	6.0	±0.00	6.0	±0.00	6.0	±0.00
<i>Negative control (essential oil)</i>	6.0	±0.00	6.0	±0.00	6.0	±0.00	6.0	±0.00

Table 4. Specifications of soap fragrance elixir

No	Specifications	amount
1	pH	4.48
2	number of acids /mg/g/	1.80
3	amount of alcohol /%/	54.83

Сүүлд ном зүй
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**Anti-oxidative activity
 (Gallic acid equivalent, µg/mL)**

Figure 6. Radical scavenging activity of ethanol extract from *Nepeta sibirica* L. grown in Mongolia

