To evaluate antioxidant, cytotoxic, antibacterial activities of aerial parts ethanol extracts from *Nepeta sibirica L*. grown in Mongolia

Irekhbayar Jambal[a], Amarjargal Ayurzana[b], , Altantsetseg Shatar[c], Batbayar Bayarhuu[b] Oyuntsetseg Chultem.[b]

[a,]IREKHBAYAR Jambal:
Department of Chemistry, School of Arts and Sciences, National University of Mongolia (NUM), Baga toiruu, Sukhbaatar District, Ulaanbaatar 14201, Mongolia. Phone: 976-99163406, Fax: +976-11-322278. E-mail: irekhbayar@num.edu.mn
[b] AMARJARGAL Ayurzana:
Department of Chemistry and biology, School of Natural Science and Technology, Khovd State University, Jargalant soum, Khovd aimag, 84140, *Mongolia. Phone: 976-99439775, Fax: +976- 7595-2500*
E-mail: amar731012@yahoo.com
[c]ALTANTSETSEG Shatar:
Institute of Chemistry and Chemical Technology, Mongolian Academy of Sciences, 4th Building Enkhtaivan Avenue, Ulaanbaatar 210351, Ulaanbaatar, Mongolia. Phone: 976-99131592, E-mail: altaa12000@yahoo.com
[b] BAYARHUU Batbayar
Department of geography and geology School of Natural Science and Technology, *Khovd State University,* Jargalant soum, Khovd aimag 84140, *Mongolia. Phone: 976-99438200, Fax: +976- 7595-2500*
E-mail: bayarhuub@yahoo.com
[b] OYUNTSETSEG Chultem
Department of Chemistry and biology, School of Natural Science and Technology, *Khovd State University,* Jargalant soum, Khovd aimag 84140, *Mongolia. Phone: 976-99664716, Fax: +976- 7595-2500*
E-mail: oyunaa0507@yahoo.com
[a,]IREKHBAYAR Jambal:
Correspondence author: Department of Chemistry, School of Arts and Sciences, National University of
Mongolia (NUM), Baga toiruu, Sukhbaatar District, Ulaanbaatar 14201, Mongolia. Phone: 976-99163406,
Fax: +976-11-322278.
E-mail: irekhbayar@num.edu.mn

ABSTRACT: *Mongolia is rich in medicinal plants. In recent years, interest in plant-derived food additives has grown. Many Lamiaceae species have a characteristic scent or taste, which in many cases are the reason for their application in Mongolian traditional medicine. This study was aimed to evaluate antioxidant, cytotoxic activities of aerial parts ethanol extracts from Nepeta sibirica L. grown in Mongolia. The antioxidant and cytotoxic activities of the essential oil and ethanol crude extracts were determined by using DPPH and MTT assays. The ethanol extracts showed higher antioxidant activity than essential oil. 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 Nepeta sibirica L. grown in Mongolia has potential as sources of new antioxidant, and cytotoxic compounds, respectively.*

Keywords: *Nepeta sibirica* L.; essential oil; antimicrobial; antioxidant, and cytotoxic activities

Introduction

A number of aromatic medicinal plants used for treating infectious diseases have been mentioned in different phytotherapy manuals due to their availability, fewer side effects, and reduced toxicity. The essential oils of these aromatic plants[1] are responsible for their fragrance as well as biological properties.[2] Essential oils are complex mixtures of volatile secondary metabolites that are responsible for both the fragrant and biological effects of aromatic medicinal plants.[3-5] An important characteristic of essential oils and their constituents is their hydrophobicity, which enables them to partition in the lipids of bacterial cell membranes and mitochondria, thus disturbing the structures and rendering them more permeable.[6]

The genus *Nepeta* (Lamiaceae), widely distributed in the Central and South Asia, Europe, the Middle East and in some regions of tropical Africa, comprises about 300 perennial species (Wink, 2003).[21,23]

Nepeta sibirica L. belongs to the family Lamiaceae.[7]

Mongolia is rich in essential oil medicinal plants. Mongolian traditional medicine has long history of more than 2500 years.[8,20] There are about 60 clans, about 200 species[20,22], 300 kinds of essential oil plants and 600 kinds of herbal plants have been registered, among of them, 150-200 kinds are commonly used.[9] Many essential oil plants have not been studied yet. It is important to investigate their chemical compositions and biological activities by using traditional medicine.[10,11,23]

The genus *Nepeta* from the Lamiacea comprises about 280 species and is distributed across most of central and southern Europe, the near East, and central and southern Asia. Plants of this genus have been used in folk medicine as antimicrobial agents, and their chemical composition has been researched widely. *Nepeta sibirica* is a representative plant, which is a source of traditional medicines in Mongolia used for digestive ailments, nervous system diseases and as antiseptics.[19]

Nepeta sibirica L. has been used for the treatment of various diseases and is one of the traditional medicines of Mongolia.[19]

The aim of this study was to evaluate the antioxidant and cytotoxic effects of essential oil and ethanol extract from *Nepeta sibirica* L. 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-diphenyltetrazoliumbromide (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 *Nepeta sibirica* L. 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 mountain of the Uvs aimag (Mongolia) on the July 2019. Voucher specimens have been deposited in the herbarium of the *Khovd State University*, Mongolia.

Isolation 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 is 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 mass spectra and linear retention indices (relative to C8–C24 alkanes) with those reported in database developed in our laboratory. [13,14]

Extraction and fractionation

The air-dried and powdered whole plant (170 g) was extracted with 99% ethanol (2 L × 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 (28 g) 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.[15] 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:

$$SC\ (\%) = [1\text{-}AS/AC]\ 100$$

Where, AS=is the net absorbance of the sample, AC=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 *N. sibiriva* L., 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. [16]

Result and Discussion

Analysis of the Essential oil

The percentage contents of the essential oil component are summarized in Table 1. A total of 23 components were identified, representing 96.00% of the total oil. The terpenoides made up the largest component of the oil and had many representative volatiles. The oxygenated monoterpenes (3.74%), and oxygenated sesquiterpenes (24.82%), sesquiterpenes (64.29%) diterpenoid (were weakly represented (3.15%). The main constituents were found to be -bourborene (14.19%), caryophyllene (9.51%), ***(E)***- -farnesene (7.28%), -bisabolene (8.07%), caryophyllene oxide (12.17%).

These differences might have been derived from local, climatic and seasonal factors (Table 1).

Antioxidant activity

DPPH is free radical compound that has been widely used to determine free radical scavenging activity.[15]

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. [17]

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.[15]

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 (See Figure 6).

The Essential oil of *Nepeta sibirica* L. did not show any antioxidant properties in DPPH assay.

Cytotoxic activity

To investigate the cytotoxic activity of ethanol extracts and essential oil from *N. sibirica* L., 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 *N. sibirica L.* for 24 and 48 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 *N. sibirica* L., we evaluated its effect on four different bacteria, such as *S.enterica, B.subtillus, S.Aureus, E.coli* (See Table 2).

Conclusion

In recent years, interest in plant-derived food additives has grown. Plant extracts might substitute synthetic food antioxidants, which may influence human health when consumed chronically. [18]

This study on essential oil chemical composition and biological activities of *Nepeta sibirica* L. grown in Mongolia were not well performed before.

Essential oils hydrodistilled from *Nepeta sibirica* L. were found to be rich in β-bourborene, caryophyllene, (E)-β-farnesene, β-bisabolene and d-cadinene.

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 *N.sibirica* L. ethanol extracts as a new antioxidant and cytotoxic agents for human health.

Acknowledgments

This research was supported by grant #PIU24/19 “Chemical Research and Cultivation Technology of (*Nepeta sibirica* L)” for the Asian Development Bank supported Implementation and Financing of Local

Development In Higher education innovation project. We are also grateful to the Novosibirsk Institute of Organic Chemistry, Russia and special thanks to Dr Romanenko Elena Petrovna and prof Tkachev Alexey Vasilyevich.

References

1. H. Vandendool, P. D. Kratz. A generalization of the retention index system including linear temperature programmed gasliquid partition chromatography. *J. Chromatogr.* 1963, *11,* 463-471.
2. D. Kalemba, A. Kunicka. Antibacterial and antifungal properties of essential oils. *Curr. Med. Chem*. 2003, *10,* 813-829.
3. U. J. Salzer. The analysis of essential oils and extracts (oleoresins) from seasonings-a critical review. *CRC Crit. Rev. Food Sci. Nutr.* 1977, *9*, 345-373.
4. A. Angioni, A. Barra, M. Arlorio, J. D. Coisson, M. T. Russo, F. F. Pirisi, M. Satta & P. Cabras. Chemical composition, plant genetic differences, and antifungal activity of the essential oil of *Helichrysum italicum*G. Don ssp. microphyllum (Willd) Nym. *J. Agric Food Chem*. 2003, *51,* 1030-1034.
5. F. Senatore, N. A. Arnold & F. Piozzi. Chemical composition of the essential oil of *Salvia multicaulis* Vahl. var. simplicifolia Boiss. growing wild in Lebanon. *J. Chromatogr. A.* 2004, *1052,* 237-240.
6. J. Sikkema, J. A. de Bont & B. Poolman. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* 1995, *59,* 201-222.
7. Daniela Rigano, Nelly Apostolides Arnold etc. Characterisation of the essential oil of *Nepeta glomerata* Montbret et Aucher ex Bentham from Lebanon and its biological activities. *Natural Product Research*. Vol. 25, No. 6, March 2011, 614–626.
8. V. I. Grubov. *Key to the Vascular Plants of Mongolia*. Nauka: Leningrad, Russia. 1982, 245-253.
9. S.Shatar, Sh. Altantsetseg. Essential oil composition of some plants cultivated in Mongolian climate. *J. Essen. Oil Res.* 2000, *12,* 745-750.
10. S. Shatar. *Mongolian fragrant herbs.* Soyombo Press, UB, Mongolia. 1989, 14-16.
11. U. Ligaa, B. Davaasuren, N. Ninjil. Using the Mongolian medicinal herbs in eastern and western medicine. KSA Press, UB, Mongolia, 2005, 114.
12. R. P. Adams. Cedar Wood Oil - Analysis and properties. In: Modern Methods of Plant Analysis: Oils and Waxes. Springer-Verlag Berlin, Germany, 1991, 159-173.
13. Y. Massada. Analysis of Essential Oil by Gas Chromatography and Spectrometry. Wiley, New York, USA, 1976.
14. R. P. Adams. Identification of essential oils components by gas chromatography/mass spectroscopy. Carol Stream, Il, USA: Allured Publication Corporation, 2001.
15. W. Brand-Williams, M. E. Cuvelier, C. Berset. Use of a free radical method to evaluate antioxidant activity. LWT-Food Science and Technology, 1995, 28, 25-30.
16. T. Mosman. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J.Immunol. Methods. 1983, 65, 55-63.
17. P. Molyneux. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol. 2004, 26, 211-219.
18. M. Martinez-Tome, A. Jimenez, S. Ruggieri, N. Frega, R. Strabbioli, & M. Murcia. Antioxidant properties of Mediterranean spices compared with common food additives. Journal of Food Protection, 2001, *64,* 1412-1419.
19. Ts.Tsuruoka, S.Shatar, Sh.Altantsetseg, A.Amarjargal, The essential oil Mongolian *Nepeta sibirica*: a single component and its biological activities. *Journal. Essential Oil. Res*., 2012, Vol 24, No 6, .555-559.
20. European observatory on Health Systems and Policies. HiT profile in brief: Mongolia 2008. http://www.euro.who.int/__data/assets/pdf_file/0 004/98860/E90671sum.pdf?ua=1.
21. Lijana Dienaitėa , Milda Pukalskienėa , Ana A. Matias etc. Valorization of six Nepeta species by assessing the antioxidant potential, phytochemical composition and bioactivity of their extracts in cell cultures. Journal of Functional Foods (2018), https://doi.org/10.1016/j.jff.2018.04.004

22. Abbas Ali, Nurhayat Tabanca, Betul Demirci etc. Chemical Composition and Biological Activity of Essential Oils from Four *Nepeta Species* and Hybrids against Aedes aegypti (L.) (Diptera: Culicidae)., *Rec. Nat. Prod*. 10:2 (2016) 137-147.

23. Lijana Dienaitė, Milda Pukalskienė, Ana A.Matias, etc. Valorization of six Nepeta species by assessing the antioxidant potential, phytochemical composition and bioactivity of their extracts in cell cultures. Journal of Functional Foods (2018), https://doi.org/10.1016/j.jff.2018.04.004

Supplementary Material

Table 1. Main components (%) of the essential oil from *Nepeta sibirica* L. grown in Mongolia

#	Peak Retention time, min	Peak area	%	
1	19.888	1720851	3,74	dihydroedulan
2	22.755	485986	1,06	-copaene
3	23.029	6530313	14,19	**-bourborene**
4	24.134	4375671	9,51	**caryophyllene**
5	24.466	552640	1,20	-copaene
6	24.928	334003	0,73	isogermacrene D
7	25.217	915891	1,99	Humulene
8	25.339	3351565	7,28	***(E)*- -farnesene**
9	25.917	1125476	2,45	g-muurolene
10	26.177	2138309	4,65	ar-curcumene
11	26.545	521448	1,13	-zingiborene
12	26.653	382428	0,83	-muurolene
13	26.928	3713218	8,07	**-bisabolene**
14	27.065	1555738	3,38	g-cadinene
15	27.325	2013387	4,38	**d-cadinene**
16	27.418	1581248	3,44	-sesquiphellandrene
17	29.115	5600713	12,17	**caryophyllene oxide**
18	29.483	224934	0,49	salvial-4(14)-en-1-one
19	29.902	1855150	4,03	humulene-6,7-epoxide
20	30.855	946397	2,06	T-cadinol + T-muurolol (~1:1)
21	31.238	1728117	3,76	-cadinol
22	36.270	1063020	2,31	hexahydrofarnesyl acetone
23	42.941	1447315	3,15	13-b-methyl-13-vinyl-podocarpan-8b-ol (C20)

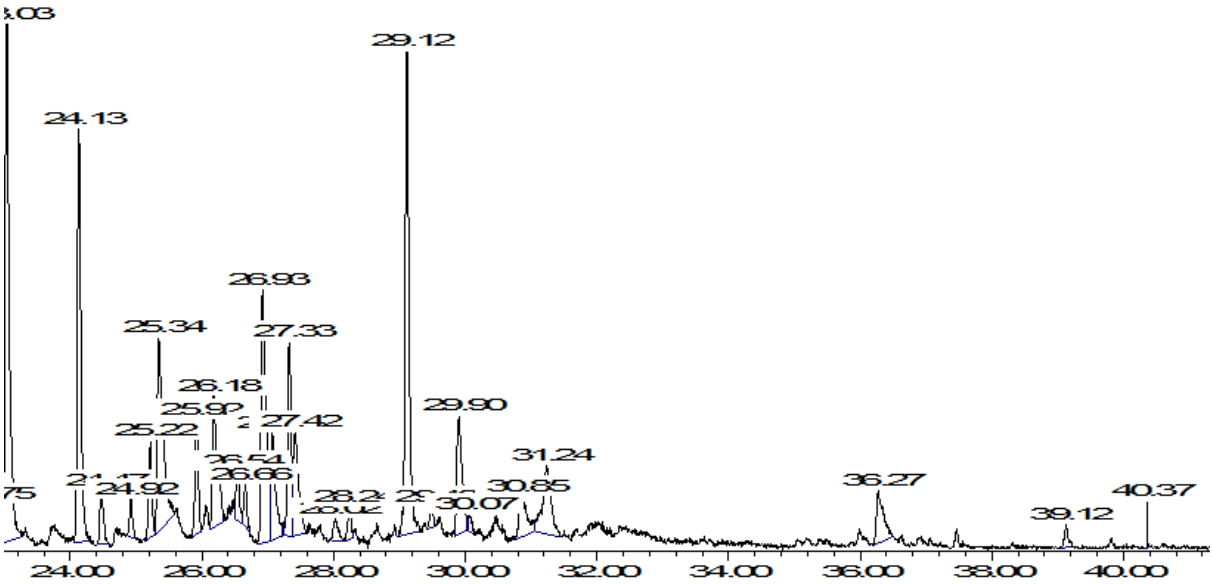


Figure 1. GC/MS analysis of essential oil from *Nepeta sibirica* L. grown in Mongolia

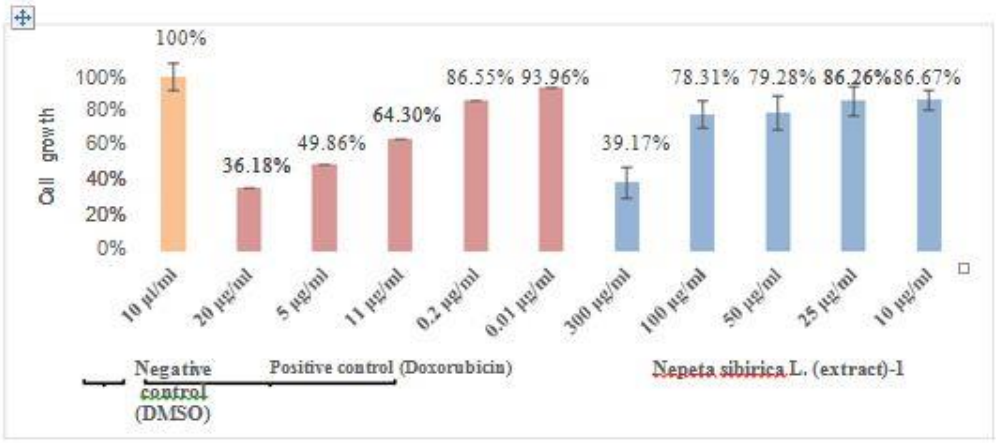


Figure 2. Cytotoxicity (%) of ethanol extract from *N.sibirica* L.growing in Mongolia against HepG2 cell line. Cell was treated with the fractions for 24 h at a concentration of 10-300 µl/ml.

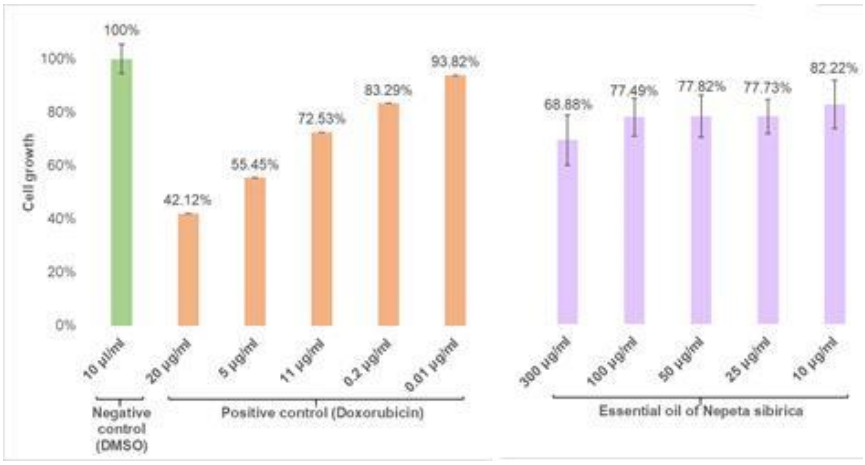


Figure 3. Cytotoxicity (%) of essential oil from *N.sibirica* L.growing in Mongolia against HepG2 cell line. Cell was treated with the fractions for 24 h at a concentration of 10-300 µl/ml.

Figure 4. Growth inhibitory effect of ethanol extract *N. sibirica* L. on AGS cells after 24 hours treatment. The results are expressed as percentage of untreated control

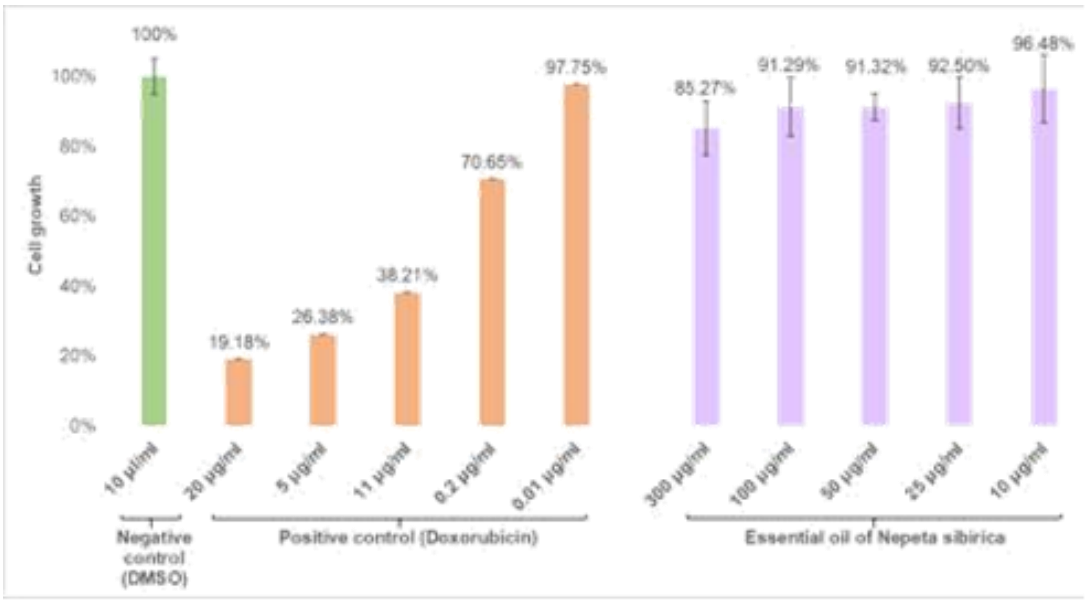


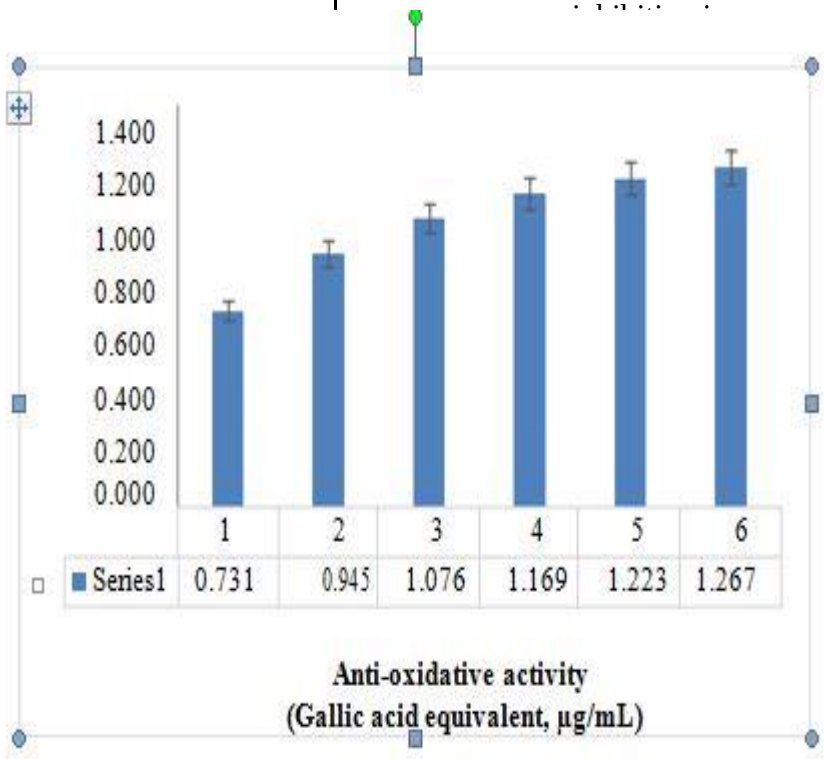
Figure 5. Growth inhibitory effect of essential oil *N. sibirica* L. on AGS cells after 24 hours treatment. The results are expressed as percentage of untreated control

Table 2. Growth inhibition effect of essential oil and ethanol extract from *N. sibirica* L. on gram positive and negative bacteria

Sample	Zones of growth inhibition in mm							
	S.enterica				***B.subtilus***			
	Product 3µg/disc		0.6 µg/disc		Product 3µg/disc		0.6 µg/disc	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
N.sibirica* L. *(essential oil)	12.3	±0.5	6.7	±0.5	8.3	±0.5	7.2	±0.2
N.sibirica L.(ethanol extract)	6.5	±0.00	6.5	±0.00	7.8	±0.58	6.5	±0.00

continued

Sample	Zones of growth inhibition in mm	
	E.coli	
	0.6 µg/disc	
	Mean	SEM
N.sibirica* L. *(essential oil)	7.2	±0.2
N.sibirica L.(ethanol extract)	6.5	±0.00



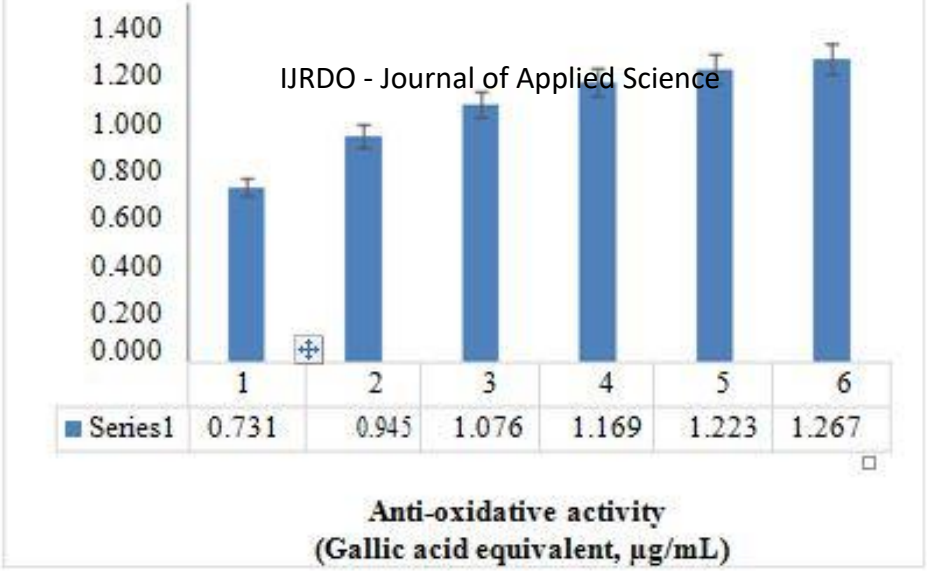


Figure 6. Radical scavenging activity of ethanol extract from *Nepeta sibirica* L. grown in Mongolia