http://dergipark.org.tr/trkjnat
Trakya University Journal of Natural Sciences, 23(2): 125-134, 2022
ISSN 2528-9691
DOI: 10.23902/trkjnat.1043241

Research Article

BIOLOGICAL ACTIVITY EVALUATION OF THREE *Hypericum* L. SPECIES

Nuraniye ERUYGUR[1*], Necati OZPINAR[2], Hülya OZPINAR[3], Mehmet ATAŞ[4], Mehmet TEKIN[5], Ozge ÇEVIK[6]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Selçuk University, Konya, TÜRKİYE
[2]Department of Emergency Aid and Disaster Management, Faculty of Health Sciences, Mustafa Kemal University, Hatay, TÜRKİYE
[3]Department of Pharmaceutical Botany, Faculty of Pharmacy, Sivas Cumhuriyet University, Sivas, TÜRKİYE
[4]Department of Pharmaceutical microbiology, Faculty of Pharmacy, Sivas Cumhuriyet University, Sivas, TÜRKİYE
[5]Department of Pharmaceutical Botany, Faculty of Pharmacy, Trakya University, Edirne, TÜRKİYE
[6]Department of Biochemistry, Faculty of Medicine, Adnan Menders University, Aydın, TÜRKİYE

Cite this article as:

Eruygur, N., Ozpinar, N., Ozpinar, H., Ataş, M., Tekin M. & Çevik O. 2022. Biological activity evaluation of three *Hypericum* L. species. *Trakya Univ J Nat Sci*, 23(2): 125-134, DOI: 10.23902/trkjnat.1043241

Received: 28 December 2021, Accepted: 25 April 2022, Online First: 07 June 2022, Published: 15 October 2022

Edited by:
Yeşim Sağ Açıkel

****Corresponding Author:***
Nuraniye Eruygur
neruygur@gmail.com

ORCID iDs of the authors:
NE. orcid.org/0000-0002-4674-7009
NO. orcid.org/0000-0002-7317-885X
HO. orcid.org/0000-0001-8154-0874
MA. orcid.org/0000-0002-9425-0080
MT. orcid.org/0000-0003-1837-7917
OC. orcid.org/0000-0002-9325-3757

Key words:
Hypericum perforatum
Hypericum thymbrifolium
Hypericum thymopsis
Antioxidant
Anti-Trichomonas
Antimicrobial
Cytotoxicity

Abstract: This study was performed in order to evaluate *in vitro* antioxidant, anti-*Trichomonas vaginalis*, antimicrobial and cytotoxic activities of methanol extracts of aerial parts of *Hypericum perforatum* L., *H. thymbrifolium* Boiss. & Noë and *H. thymopsis* Boiss. The antioxidant activity of methanol extracts was tested with 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging and iron chelating assays. The total phenol and flavonoid contents were also determined spectrophotometrically. Cytotoxicity and apoptosis were evaluated on MCF-7 breast cancer and PC3 prostate cancer cell line. Antimicrobial activities were evaluated by the microdilution method on the bacterial strains *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa (*ATCC 27853) *Escherichia coli* (ATCC 25922) and the fungal strain *Candida albicans* (ATCC 10231). All extracts demonstrated cytotoxic activity on PC3 prostat cancer cell line with a concentration dependent manner, while *H. thymopsis* extract was the most active against cancer cell growth. The obtained results of the present study revealed that the methanol extract of *H. perforatum*, *H. thymbrifolium* and *H. thymopsis* have significant antioxidant and cytotoxic activities. The results provided the basic research data for further phytochemical and biological activity guided investigations on these species for identication and isolation of potential drug active compounds.

Özet: Bu çalışma, *Hypericum perforatum* L., *H. thymbrifolium* Boiss. & Noë ve *H. thymopsis* Boiss'ın toprak üstü kısımlarının metanol ekstrelerinin *in vitro* antioksidan, anti-*Trichomonas vaginalis*, antimikrobiyal ve sitotoksik aktivitelerini değerlendirmek amacıyla yapılmıştır. Metanol ekstrelerinin antioksidan aktivitesi, 2,2-difenil-1-pikrilhidrazil (DPPH), (2,2'-azino-bis (3-etilbenzotiazolin-6-sülfonik asit) (ABTS), ve demir şelatlama metodu ile test edildi. Toplam fenol ve flavonoid içerikleri de spektrofotometrik olarak analiz edildi. Sitotoksisite ve apoptoz, MCF-7 meme kanseri ve PC3 prostat kanseri hücre hattı üzerinde değerlendirildi. Antimikrobiyal aktivite, *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa (*ATCC 27853) *Escherichia coli* (ATCC 25922) bakteri suşları ve *Candida albicans* (ATCC 10231) mantar suşu üzerinde mikrodilüsyon yöntemiyle değerlendirildi. Ekstrelerin tümü, konsantrasyona bağlı bir şekilde PC3 prostat kanseri hücre hattı üzerinde sitotoksik aktivite gösterirken, *H. thymopsis* ekstresi kanser hücresi büyümesine karşı en fazla etkiliydi. Bu çalışmanın elde edilen sonuçları, *H. perforatum*, *H. thymbrifolium* ve *H. thymopsis*'in metanol ekstresinin önemli antioksidan, sitotoksik aktiviteye sahip olduğunu ortaya koydu. Sonuçlar, potansiyel ilaç aktif bileşiklerinin tanımlanması ve izolasyonu için bu türler üzerinde daha fazla fitokimyasal ve biyolojik aktivite rehberli araştırma için temel araştırma verilerini sağlar.

Introduction

The genus *Hypericum* L. is a member of *Hypericaceae* and includes more than 469 species classified into 36 taxonomic sections. Some species of the genus are used in folk medicine and among which *Hypericum perforatum* L. (St. John's wort) is commonly known as a traditional remedy used for mild to moderate depression, melancholia,

abdominal and urogenital pains and ulcerated burns (Mansour *et al.* 2014). Oils prepared from *H. perforatum* flowers and herbs are largely used to treat wounds and ulcers (Baytop 1999). In Turkey, the genus is comprised of about 100 taxa grouped in 19 sections, of which 45 are endemic to the country (Boga *et al.* 2016). The genus has been used remedy for burns, wounds, hemorrhoids and ulcer (Baytop 1984, Özen & Başhan 2003).

Phytochemical studies on the genus *Hypericum* demonstrated the presence of tannins, flavonoids, saponins and antraquinones in the taxa investigated (Silva *et al.* 2005, Toker *et al.* 2006, Yang *et al.* 2015). Pharmacological studies showed that some extracts prepared from the aerial parts of various *Hypericum* members exhibited antioxidant, antidepressant, antinociceptive and antimicrobial activities (Hunt *et al.* 2001, Ozturk *et al.* 2007, Radulović *et al.* 2007, Süntar *et al.* 2010, Barış *et al.* 2011, Mansour *et al.* 2014, Tala *et al.* 2015, Eruygur *et al.* 2019).

Antioxidants have recently been a popular topic, with particular emphasis given on those which have the ability to avoid the alleged harmful effects of free radicals on biological systems and the deterioration of lipids and other food ingredients (Bounatirou *et al.* 2007). Therefore, the search for plants with antioxidant properties from natural sources is getting more and more attention. Due to the potential antimicrobial properties of medicinal plants revealed in studies carried over the last years, it is important to treat from infectious diseases to some chronic diseases. Therefore, plants are considered to be potential sources for the development of new compounds that are even effective against antibiotic-resistant bacteria (Hyun *et al.* 2014). In parallel to this, studies of antimicrobial activity of plants are of great interest.

Trichomonas vaginalis (Donné) is an anaerobic protozoan that is sexually transmitted through female urogenital tract infections and is the agent for trichomoniasis (Taran *et al.* 2006). Sexual contact is the most common way for trichomoniasis to spread from person to person. This is why the rate of illness is high in women who have reached sexual maturity. Trichomonal infection is found in all racial groups and socioeconomic levels and has a cosmopolitan spread. Approximately 333 million new Sexually Transmitted Diseases (STDs) occur every year in the world, of which 170 million are *T. vaginalis* infections. Although metronidazole is frequently used in the treatment of trichomoniasis, metronidazole-resistant *T. vaginalis* has been reported in recent cases (Snipes *et al.* 2000, Schwebke & Barrientes 2006, Kirkcaldy *et al.* 2012, Ertabaklar *et al.* 2016), suggesting the need to find anti-trichomonase effective compounds from natural sources.

Although there are several investigations performed on biological activity characteristics of some *Hypericum* species, there are no reports on the biological activities of extracts of these species. The aim of the present work was to investigate *in vitro* biological acitivies of methanol extracts of *H. perforatum*, *Hypericum thymbrifolium* Boiss. & Noë, and *H. thymopsis* Boiss, which are endemic in Turkey. We comperatively investigated the *in vitro* biological activities of methanol extracts, namely antioxidant, cytotoxicity and antimicrobial activities by DPPH, ABTS, iron chelation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and the microdilution methods, respectively.

Materials and Methods

Plant material and preparation of the extracts

Specimens of *Hypericum thymbrifolium, H. perforatum* and *H. thymopsis* were collected from Sivas province of Turkey during their flowering periods. *Hypericum thymbrifolium* (HTB) was collected from B6 Sivas: Kangal-Gürün road 16. km, 39°07’52.3”N, 37°14’33.8”E, 1541 m, 22 June 2016, M. Tekin 1727 (CUFH), *H. perforatum* (HP) was collected from B6 Sivas: Kangal-Gürün road 16. km, 39°07’52.2”N, 37°14’33.5”E, 1522 m, 05 July 2014, M. Tekin 1622 (CUFH) and *H. thymopsis* (HTS) was collected from B6 Sivas: Kangal-Gürün road 16. km, 39°07’51.9”N, 37°14’33.1”E, 1522 m, 05 July 2014, M. Tekin 1625 (CUFH). The dried plant materials were powdered using a grinder. The extraction was done at room temperature for three times. 100 g of dried and powdered whole aerial parts were extracted seperately with methanol for 48 h accompanied by periodic shaking. Then the filtrates were combined together and concentrated under vaccum on a rotary evaporator (Buchi R-100). The methanol extracts of *H. perforatum* (HP), *H. thymopsis* (HTS) and *H. thymbrifolium* (HTB) were obtained as 9.84%, 12.98% and 10.34%, respectively.

GC-MS characterization of the extracts

Gas Chromatography–Mass Spectrometry (GC-MS) analysis was performed for characterization of the extracts. Chemical constituents of the extracts as revealed by Gas Chromatography (GC) chromatogram are given in Table 1.

Antioxidant Activities

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging effects of the extracts were determined by the method of Blois (1958). The iron chelating activity of extract was performed using the method of Dinis *et al.* (1994). The scavenging activites of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals were measured as described by Re *et al.* (1999).

Determination of total phenol and flavonoid content

The Foline-Ciocalteu spectrometric technique was used to determine the total phenolic content (TPC) (Ainsworth 2007). The TPC was expressed as milligrams of gallic acid (GAE) equivalents per gram of drye matter (mg GAE/g DM). The TFC was determined by the aluminium trichloride colorimetric assay using the method described by Chang *et al.* (2002). The TFC in extracts were expressed in terms of quercetin equivalent (mg of QE/g of dry extract).

Antimicrobial Activities (Micro-well dilution assay)

Minimum Inhibitory Consantration (MIC) values of the extracts against the bacterial strains *Staphylococcus*

aureus (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa (*ATCC 27853) and *Escherichia coli* (ATCC 25922) and the fungal strain *Candida albicans* (ATCC 10231) were evaluated by the microdilution broth method (Eloff 1989). The extracts were dissolved in 40% Dimethyl sulfoxide (DMSO) at 100 mg/mL concentration. In the first line of a microtiter plate, a 10 µL sample was added, which was then diluted with 90 µL of sterile distilled water. Then, 50 µL of sample was placed on the second line of the microtiter plate, which had been serially diluted twice with sterile distilled water. Every well had a final inoculum size of 5×10^5 CFU/mL for bacteria and $0.5\text{-}2.5\times10^3$ CFU/mL for candida (CLSI 2002, 2012). For diluting the bacteria and candida cultures, Mueller Hinton Broth (Accumix® AM1072) and Saboraud Dekstroz Broth (Himedia ME033) were used, respectively. On prepared samples, 50 µL of bacteria and fungi suspension were introduced and incubated for 16-24 hours. The extract concentrations in the wells ranged from 5.000 to 0.009 µg/mL. Positive controls included gentamicin and fluconazole and the negative controls included DMSO. To detect microbial growth, 40 µL of 2 mg/mL 2,3,5-Triphenyltetrazolium chloride (TTC) (Merck, Germany) was added to each well. The microtiter plates were then incubated for another 2 hours at 37°C. The experiment was repeated twice, with a standard deviation of zero.

Cytotoxicity assay

The *in vitro* cytotoxic activities of the extracts were evaluated by the MTT colorimetric assay according to Mosmann (1983) and Eruygur *et al*. (2017). Prostate cancer (PC3) cell lines with exponential growth were planted into 96-well microplates at a density of 5×10^3 cells per well in 100 µL of culture medium and allowed to adhere for 16 hours before being cultured at 37°C in a 5% CO_2 humidified environment. The cells were treated with different concentrations of methanol extracts of the *Hypericum* species. Then plates were incubated for 24 hours, the medium was discharged from the 96-well plate, 10 µL of MTT was added per well, and the plate was kept for 2 h in 5% CO_2 humidified incubator at 37°C to allow reaction of yellow colored MTT reduced by mitochondrial dehydrogenases in viable cells to form pink to purple coloured formazan. The excess MTT was sucked off and the resulting formazan crystals formed were dissolved in 100 µL DMSO. Using a microplate reader (Epoch, USA), the absorbance of purple formazan which is proportional to the number of live cells, was measured at 560 nm. The tests were carried out in triplicate. The following calculations were used to compute the percentage of cell viability:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of the untreated cells} \times 100}$$

Determination of Apoptosis

MCF-7 and PC3 cells were treated with 10 µg/mL plant extracts for 12 hours in 12-well plates at a density of 2×10^4 cells/well in triplicate. A Zeiss Axio inverted microscope (10X) imaging system was used to capture images of cell growth. Quantification of cell growth was done using methylene blue staining. Acridine orange (AO) and ethidium bromide (EB) were used to evaluate apoptosis in the MCF7 and PC3 cancer cell lines. After a 24-hour incubation period with 10 µg/mL plant extract in 12-well plates at density of 2×10^5 cells per well in triplicate. Cells were then stained with 1 µg/mL AO/EB solutions and the fluorescent intensities unique to cell growth were measured using a microscope (Zeiss). Apoptotic cells are grouped with red intensity, while living cells are grouped with green intensity.

Anti-Trichomonase vaginalis activity

The metronidazole-resistant and sensitive *T. vaginalis* strains were employed (ATCC 50143 and ATCC50148, both obtained by American Type Culture Collection).

Culture of *T. vaginalis*

Commercially available Trichomonas Broth (TB, liofilchem, 610061) medium was used and prepared according to the manufacturer's instructions. After the TB was prepared, it was divided among the experimental tubes and autoclaved at 121°C for 15 minutes. The medium was then cooled to 37°C, and 10% inactive horse serum (Sigma, 1234598765) was added to the medium. The strains were introduced to the TB medium and cultured under anaerobic conditions for 3 days at 37°C (Taran *et al*. 2006).

In vitro metronidazole and plant extract susceptibility assay

The plant extract was used to compare the Minimum Lethal Dose (MLD) of the metronidazole-sensitive and resistant *T. vaginalis* strains. 96-well plates were used for this experiment. The strains were grown in metronidazole (Sigma, 1711544348111) concentrations of 400 µM, 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM, 0.6 µM and 0.3 µM after seeding in TB medium at 37°C. 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.6 mg/mL, 0.3 mg/mL, 0.15 mg/mL of plant exract concentration were used. The incubated live protozoa were tested for flagellated and undulating membrane movement on a Thoma slide after 2-4-24 hours and counted in a 1% eosin solution.

Statistical analyses

All the analyses were carried out in triplicate. The mean and standard deviations (Mean ± SD) were used to represent the parameter values. Graphpad 8.0 program was used to find significant differences between the groups using one-way ANOVA and Student's t-test. If the *P* value was less than 0.05, the results were considered significant.

Results

GC-MS analysis of the Hypericum extracts

The chemical compositions of methanol extracts prepared from three *Hypericum* species are given in Table 1. The results of the GC-MS analysis showed that spathulenol, linoleic acid and palmitic acid were the main

compounds, as formerly reported for *H. capitaum* (Boga *et al.* 2016). According to GC-MS analyzing results in a previous study, fatty acids, sterioids and terpenic compounds are dominant in the extracts of all *Hypericum* species. Among the detected constituents, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyranone (DDMP) has been reported to have antiproliferative and proapoptotic effects through inactivation of NF-$_K$B in human colon cancer cells (Ban *et al*. 2007). In another study, this compound was also investigated for DPPH radical scavenging activity and mushroom tyrosinase inhibitory activity (Takara *et al.* 2007). Another most abundant compound found in three *Hypericum* species in this study is palmitic acid. This constituent was also reported for different physiological activities (Carta *et al.* 2017).

Table 1. Chemical characterization of HP, HTB, HTS methanol extracts with GC/MS analysis.

No	Compound name	RT	HP	HTB	HTS
1	2-Furancarboxaldehyde	6.274	-	0.95	-
2	Urea-N15	4.775	-	-	0.78
3	2-Furancarboxaldehyde	6.263	-	-	0.98
4	2-Furanmethanol	6.858	-	0.74	0.73
5	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	11.132	-	0.82	0.56
6	3-fluoro-2,5-dimethyl-2,4-hexadien	13.404	-	-	0.88
7	Benzoic acid	15.206	0.55	-	2.60
8	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	17.077	0.21	0.79	7.08
9	Benzofuran	19.011	-	-	3.89
10	2-Furancarboxaldehyde	20.361	-	1.63	4.07
11	DL-Proline	25.677	-	-	3.50
12	Naphthalene	28.424	-	-	1.52
13	Germacrene-D	29.459	-	-	0.47
14	2(4H)-Benzofuranone	29.940	-	-	0.74
15	Dodecanoic acid	30.735	-	-	0.53
16	(+) spathulenol	31.164	-	0.57	1.59
17	Cyclododecane	31.731	-	1.98	-
18	(-)-Loliolide	35.227	-	-	1.31
19	Neophytadiene	36.263	-	1.46	2.60
20	2-Hexadecen-1-ol	36.263	0.38	-	-
21	Onychine	36.841	-	-	1.77
22	Acetic acid	37.024	-	1.75	-
23	Palmitic acid	37.808	3.07	3.65	16.02
24	Hexadecanoic acid	38.695	6.73	0.62	-
25	9,12-Octadecadienoic acid	40.514	1.54	0.71	2.61
26	9,12,15-Octadecatrienoic acid	40.634	2.50	1.08	7.34
27	Neophytadiene	40.760	-	-	0.81
28	Phytol	40.812	-	1.13	-
29	2,3,10-Trimethylphenanthrene	40.880	-	-	0.10
30	Methyl stearate	40.926	-	-	0.88
31	Heptadecanoic acid	40.955	-	0.41	-
32	Octadecanoic acid	40.966	0.74	-	0.88
33	9,12-Octadecadienoic acid	41.424	0.64	-	-
34	6-Tetradecyne	41.504	0.08	-	-
35	Linolenic acid	41.516	-	6.05	7.45
36	Linoleic acid ethyl ester	41.544	0.95	-	2.29
37	1-Propene-1,2,3-tricarboxylic acid	41.619	1.11	-	-
38	Ethyl 9,12,15-octadecatrienoate	41.636	-	0.74	-

Table 1. Continued.

No	Compound name	RT	HP	HTB	HTS
39	Decanedioic acid	41.699	1.10	-	3.12
40	Octadecanoic acid	41.968	0.10	-	4.35
41	Butyl citrate	42.048	1.79	-	1.69
42	Farnesol	42.334	-	-	0.99
43	(1RS,2SR)-2-methyl-2-(4'-methylpent-3'-enyl)cyclopropanecarbaldehyde	42.574	0.80	-	1.47
44	Butyl citrate	43.238	43.68	1.90	2.36
45	Eicosane	43.450	1.12	-	0.24
46	6-Methoxy-1,2,3,4-tetrahydro-9-phenanthrenol	44.251	-	-	0.53
47	4-(2,2-Dimethyl-6-methylenecyclohexyl)butanol	-	-	-	1.20
48	Hexanedioic acid	44.863	1.80	2.21	0.57
49	3-Cyclohexene-1-methanol	44.983	0.64	-	0.82
50	2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-phenol	45.275	-	0.10	0.31
51	N-O-nitrobenzeylidene-P-bromoanilin	45.332	-	0.64	-
52	2,6,10,14-Hexadecatetraen-1-ol	45.532	0.47	-	-
53	4-(2,2-Dimethyl-6-methylenecyclohexyl)butanal	45.996	0.71	-	-
54	4-(Trimethylsilyl)thianthrene 5-oxide	46.024	-	6.89	-
55	Tricosane	46.087	1.45	-	-
56	2R-Acetoxymethyl-1,3,3-trimethyl-4t-(3-methyl-2-buten-1-yl)-1c-cyclo hexanol	46.293	-	-	0.47
57	trans-Geranylgeraniol	46.391	-	0.29	0.38
58	Lupulon	46.539	-	0.49	-
59	trans-3,5,4'-trimethylstilbene	46.700	-	-	0.71
60	Bicyclo[3.2.1]octan-4-one-1-carboxylic acid	47.472	0.70	-	-
61	8(14),22-Ergostadienol	47.821	-	0.51	-
62	2-octadecyloxy-1,1,2,2-tetradeuteroethanol	49.160	0.60	-	-
63	1-Phenyl-7-methoxy-[1]benzopyrano[4,3-c]pyrazol-4(1H)-one	50.104	-	-	1.18
64	3-Menthene	51.237	-	1.17	0.21
65	Geranyl linalool	52.090	-	1.59	4.95
66	Dulciol C	52.124	1.05	-	-
67	Decane	52.273	-	0.32	0.98
68	1,2-Benzenedicarboxylic acid	52.290	2.76	2.40	0.88
69	Farnesol	52.502	-	-	1.0
70	Lanost-8-en-3-ol	54.304	0.36	0.42	-
71	2,6,10,14-Hexadecatetraen-1-ol	54.848	-	0.97	-
72	Isocymopolone	54.871	0.51	-	-
73	(+)-trans-.alpha.-himachalene	54.968	-	-	0.38
74	Vitamin E	55.735	0.49	2.08	0.73
75	Rocellaric acid	55.918	0.40	-	-
76	Carotene	56.129	-	0.22	-
77	3-[(4'RS,5'RS)-4',5'-diphenyl-1',3'-dioxolan-2'-yl]-2-phenylpropanal	57.308	1.18	-	-
78	Stigmasterol	57.497	0.30	0.50	0.57

Antioxidant activity

The antioxidant activity assay was performed in order to examine the action of extracts on metal chelating, free radical scavenging and antioxidant capacity. The HTB and HTS have been investigated for the first time for their antioxidant activities. According to the results of the DPPH free radical scavenging assay, the HTB extract exhibited a strong antioxidant capacity with an IC_{50} value

of 36.60 ± 1.08 µg/mL, which is higher than the antioxidant capacity of HP (40.97 ± 0.89 µg/mL) and HTS extracts (95.09 ± 0.79 µg/mL), but lower than the standard compound gallic acid (21.30 ± 0.49 µg/mL). In ABTS assay, the HP extract exhibited a strong radical scavenging effect with an IC_{50} value of 0.37 ± 0.21 µg/mL, which is higher than the antioxidant capacity of HTS (3.22 ± 0.31 µg/mL) and HTB extracts (6.81 ± 0.08 µg/mL), and the standard compound gallic acid (0.62 ± 0.19 µg/mL). In iron chelating assay, the HTS extract demonstrated highest iron chelating capacity with an IC_{50} value of 418.4 ± 1.04 µg/mL, followed by the HTB (562.9 ± 0.45 µg/mL) and HP extracts (710.5 ± 0.76 µg/mL), but lower than the standard compound EDTA (37.04 ± 0.41 µg/mL).

Total phenolic (TPC) and flavonoid contents (TFC)

The TPC and TFC of the methanol extracts obtained from the whole plant of HP, HTB and HTS were determined as gallic asic (GAE) and quercetin (QE) equivalents, ranging in 73.58-78.33 mg/g extract and 16.07-31.25 mg/g extract, respectively. The phenolic contents of the all extracts were higher than the flavonoid contents (Fig 1). The HP extract was found to be higher in phenolic content and the HTS extract showed higher flavonoid content than others.

Antimicrobial activity

The antimicrobial activities of methanol extracts are given in Table 2. The difference between the antimicrobial activities of the extracts is not statistically significant (Student's t-test, $P>0.05$). The HP extract exhibited less antimicrobial activity than the other two species. Among the tested microorganisms, *Staphylococcus aureus* (Rosenbach) was more susceptable to all *Hypericum* extracts, with MIC values ranging between 1.25 and 2.5 mg/mL.

Anti-Trichomonas vaginalis activity

The effects of methanol extracts of three *Hypericum* species were tested on metronidazole resistant and susceptible strains of *T. vaginalis* and the results were evaluated at the end of 2, 4 and 24 h. The MLD values of HP extract on metronidazole resistant *T. vaginalis* was 5 mg/mL, 1.25 mg/mL at the end of 4 hours, and 0.6 mg/mL at the end of the 24 hour period (Table 3).

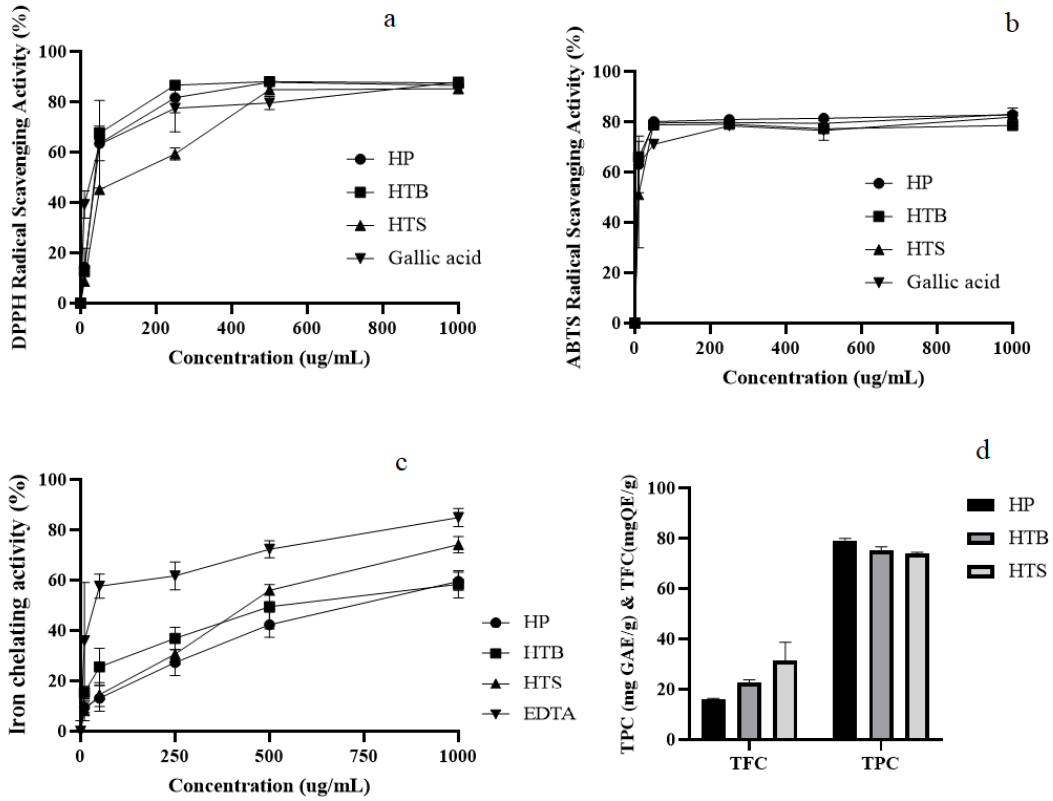

Fig. 1. a. DPPH radical scavenging activity, **b.** ABTS radical scavenging activity, **c.** total phenolic and flavonoid contents, **d.** iron chelating activity of methanol extracts of three *Hypericum* species.

Table 2. Antimicrobial activities of methanol extracts of aerial parts of three *Hypericum* species included in the study (MIC values, mg/mL)

	E. coli	*S. aureus*	*P. aeruginosa*	*E. faecalis*	*C. albicans*
H. perforatum	> 5	2.50	> 5	> 5	> 5
H. thymopsis	> 5	1.25	> 5	> 5	> 5
H. thymbrifolium	> 5	1.25	> 5	> 5	> 5

Table 3. The viable counts of *T. vaginalis* isolates exposed to different concentrations of metronidazole at the end of 2, 4 and 24 hours.

TV strains		Metronidazol Concentration (µM)								
		400	**200**	**100**	**50**	**25**	**12.5**	**0.6**	**0.3**	**Control**
N1	2 hours	14.10^3	14.10^3	14.10^3	15.10^3	17.10^3	18.10^3	18.10^3	18.10^3	18.10^3
N2		0	0	0	0	0	6.10^3	8.10^3	12.10^3	17.10^3
N1	4 hours	14.10^3	14.10^3	14.10^3	15.10^3	17.10^3	18.10^3	18.10^3	18.10^3	18.10^3
N2		0	0	0	0	0	0	6.10^3	10.10^3	18.10^3
N1	24 hours	14.10^3	16.10^3	16.10^3	18.10^3	18.10^3	20.10^3	20.10^3	20.10^3	20.10^3
N2		0	0	0	0	0	0	0	12.10^3	22.10^3
TV strains		***H. perforatum* (mg/mL)**								
		5	**2.5**	**1.25**	**0.6**	**0.3**	**0.15**	**Control**		
N1	2 hours	0	1.10^3	2.10^3	4.10^3	11.10^3	12.10^3	18.10^3		
N2		0	0	2.10^3	5.10^3	6.10^3	11.10^3	17.10^3		
N1	4 hours	0	0	0	2.10^3	3.10^3	7.10^3	18.10^3		
N2		0	0	0	0	0	0	18.10^3		
N1	24 hours	0	0	0	0	2.10^3	8.10^3	20.10^3		
N2		0	0	0	0	0	0	22.10^3		
TV strains		***H. thymbrifolium* (mg/mL)**								
		5	**2.5**	**1.25**	**0.6**	**0.3**	**0.15**	**Control**		
N1	2 hours	0	1.10^3	4.10^3	11.10^3	10.10^3	12.10^3	18.10^3		
N2		0	0	4.10^3	10.10^3	11.10^3	11.10^3	17.10^3		
N1	4 hours	0	1.10^3	2.10^3	4.10^3	9.10^3	10.10^3	18.10^3		
N2		0	0	0	0	2.10^3	3.10^3	18.10^3		
N1	24 hours	0	0	0	3.10^3	10.10^3	11.10^3	20.10^3		
N2		0	0	0	0	0	0	22.10^3		
TV strains		***H. thymopsis* (mg/mL)**								
		5	**2.5**	**1.25**	**0.6**	**0.3**	**0.15**	**Control**		
N1	2 hours	0	5.10^3	5.10^3	8.10^3	10.10^3	15.10^3	18.10^3		
N2		0	0	5.10^3	5.10^3	8.10^3	8.10^3	17.10^3		
N1	4 hours	0	5.10^3	5.10^3	5.10^3	6.10^3	10.10^3	18.10^3		
N2		0	0	0	0	0	4.10^3	18.10^3		
N1	24 hours	0	0	0	5.10^3	6.10^3	10.10^3	20.10^3		
N2		0	0	0	0	0	2.10^3	22.10^3		

TV; *Trichomonas vaginalis*, N1; Metronidazole resistant *T. vaginalis* ATCC50143, N2; Metronidazole sensitive *T. vaginalis* ATCC50148.

The MLD values of HTB extract was 2.5 mg/mL at the end of the 2 and 4 hour periods, 1.25 mg/mL at the end of the 24 hour period, while MLD value of *H. thymopsis* was 5 mg/mL at the end of the 2 and 4 hour periods and 1.25 mg/mL at the end of the 24 hour period.

It has been observed that metronidazole has no effect on the highest concentration-resistant strains. When the effects on metronidazole susceptible *T. vaginalis* strains were examined, it was concluded that HP had a MLD value of 2.5 mg/mL at the end of the 2 hour period, <0.15 mg/mL at the end of the 4 and 24 hour period, and *H. thymbrifolium* had a MLD value of 2.5 mg/mL at the end of the 2 hour period, 0.6 mg/mL at the end of the 4 hour period and <0.15 mg/mL at the end of the 24 hour period. The MLD value of HTS extract was 2.5 mg/mL at the end of the 2 hour period and 0.3 mg/mL at the end of the 4 and 24 hour periods. When the MLD values of metronidazole were examined, it was determined that MLD value was 0.6 µM after 24 hours. In addition to this data, it was also observed that the breeding was significantly suppressed after 24 hours in resistant and susceptible strains even at the lowest concentration (0.15 mg/mL) of HP. The HTS extract at concentrations of 0.15 mg/mL (Table 3) demonstrated the same effect of HP at 0.3 mg/mL.

Cytotoxicity

Cytotoxicity screening in extracts plays an important role in the identification of possible antineoplastic agents of plant origine (Rahman *et al.* 2012). The cytotoxic activity of methanol extracts of three *Hypericum* species was investigated *in vitro* on PC3 prostat cancer cell line and MCF-7 breast cancer cell line by the MTT method. The results are given in Figs 2-3. All extracts demonstrated cytotoxic activity on PC3 prostat cancer cell line with a concentration dependent manner. To further estimate the anticancer effects of the extracts and assess the rates of cell proliferation and apoptosis, cells were fluorescently stained with acridine orange/ethidium bromide (AO/EB) (Figs 4-5). The HTS extract, which showed highest inhibition on cancer cell growth, is the most potential extract than others to be further studied on phytochemical indentification of lead compounds, therefore, GC-MS analysis was performed on methanol extracts in this work.

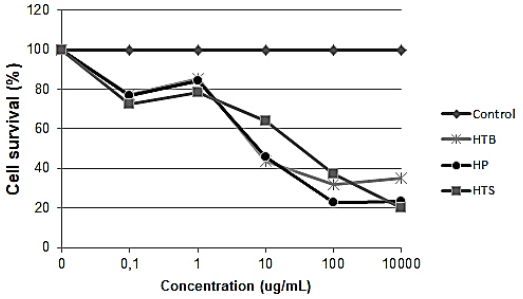

Fig. 2. Cell survival of PC3 cell lines with incubated different concentrations of methanol extracts of HTB, HTS and HP for 24 h.

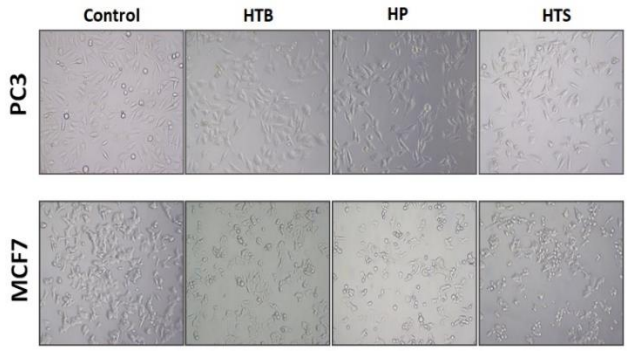

Fig. 3. Cell growing imaging of MCF7 breast cancer and PC3 prostate cancer cells incubated with 10 µg/mL concentration of plant extracts for 24 h.

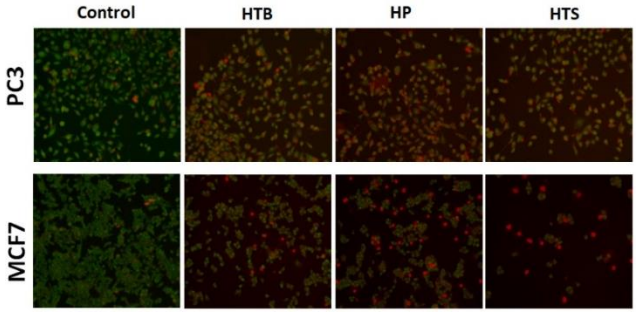

Fig. 4. AO/EB staining image in MCF7 breast cancer and PC3 prostate cancer cells incubated with 10 µg/mL concentration of *Hypericum* methanol extracts for 24 h.

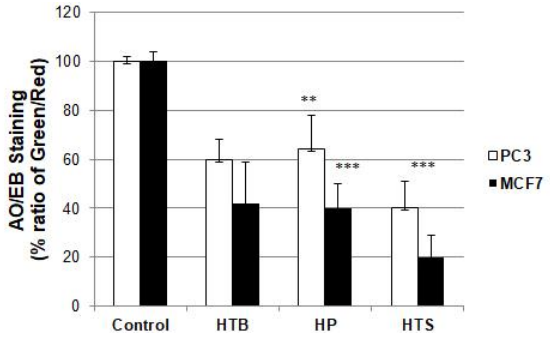

Fig. 5. Ratio of AO/EB staining in MCF7 and PC3 cancer cells incubated with 10 µg/mL concentration of *Hypericum* methanol extracts for 24 h (*** $P<0.001$ and ** $P<0.01$ compared to the control groups).

Discussion

As awareness of the risks posed by the use of synthetic drugs increases, attention is focused on the potential of medicinal plants to provide both effective and safe treatments. It is important to underline the fact that this is the first report about the antioxidant, antimicrobial, cytotoxic, and *anti-Trichomonas vaginalis* activity of three Turkish *Hypericum* species methanol extracts. The phytochemical constituents were determined by GC-MS. Fatty acids, sterioids and terpenic compounds are the most abundant constituents in the methanol extracts of all *Hypericum* species. 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyranone (DDMP) and palmitic acid were found to be rich, and they were shown to have different physiological activities. When compared with previous results on *Hypericum* species, total phenolic content of *Hypericum triquetrifolium* and *Hypericum scabroides* was found as equivalent to 267 and 333 µg gallic acid (Kizil *et al.* 2008), which was more higher than the amount in our study. According to Barıs *et al.* (2011), high TPC and antioxidant activity were associated with strong antibacterial efficacy against microbes. Three *Hypericum* extracts revealed a broad range of significant antibacterial activity against all tested bacteria and fungal species. The effect of methanol extracts against *Staphylococcus auresus* was higher than the other microorganism, which may be promising in the prevention and treatment of *S. aureus*-induced diseases. Boga *et al.* (2016) investigated different extracts of *H. capitatum* for antimicrobial activit by disc diffusion method and found all extracts have weak to moderate antimicrobial activity, methanol extract showed the higher antimicrobial activity. Radulovic *et al.* (2007) also reported antimicrobial activity of nine *Hypericum* species in different regions of the Balkans (Radulović *et al.* 2007). In another study, *H. empetrifolium* root and aerial part extracts were screened for antimicrobial activity, the best antimicrobial activity was obtained for aerial part extract than roots against *Candida parapsilosis* (Langeron & Talice) with a MIC value of 4.88 µg/mL, against *C. tropicalis* (Castellani) Berkhout with 19.53 µg/mL and against *C. albicans* (C.P. Robin) Berkhout with 78.12 µg/mL (Boga *et al.* 2021). The n-hexane subextract from *Hypericum scabrum* showed the best activity against *Bacillus subtilis* (Ehrenberg) Conn with 39.06 µg/mL MIC value (Ergin *et al.* 2022).

In a previous report, the supercritical fluid extract and isolated compounds from *Hypericum polyanthemum* were investigated for anti-*Trichomonas vaginalis* activity and benzopyran HP1 demonstrated best selectivity against metronidazole-resistant and susceptible isolated protozoan with no cytotoxicity on mammalian cells (Cargnin *et al.* 2013).

According to the results of several cytotoxicity studies on palmitic acid showed that it has cytotoxic activity (Harada *et al.* 2002, Oh *et al.* 2012, Moravcova *et al.* 2015), which is also the highest constituent in HTS, suggesting that this compound found in this species may responsible for the cytotoxic effect.

Conclusion

To date, the current research can be considered as the basic investigation focused on the biological activity and chemical compound characterization of three Hypericums species. In this study, three hypericum species were compared both in terms of chemical content, antioxidant activities and inhibition activity of different enzymes, which are the target mechanisms in the pathology and treatment of different diseases. Our findings showed that *Hypericum* species were particularly effective on metronidazole resistant *T. vagialis* strains. We think that this study will guide the development of new chemotherapeutics that can be used in the treatment of

metronidazole-resistant tricomoniasis which has been increasing in recent years. In addition, no report about the cytotoxicity and antimicrobial activity of the extracts of HTS and HTB was given so far. For this reason, this study will be the basis and pioneering for further studies on these species.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Author Contributions: Concept: N.E., M.T., O.C., Desing: N.E., N.O., Execution: N.O., M.A., Material supplying: M.T., Data acquisition: N.E., N.O., O.C., Data analysis/interpretation: N.E., N.O., Writing: N.E., N.O., Critical review: H.O., M.T., O.C.

Conflict of Interest: The authors have no conflicts of interest to declare.

Funding: The authors express their sincere gratitude to Scientific Research Foundation of Sivas Cumhuriyet University for their financial support of the study with the project number ECZ-020.

References

1. Ainsworth, E.A. & Gillespie, K.M. 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. *Nature Protocols*, 2(4): 875-876.
2. Ban, J.O., Hwang, I.G., Kim, T.M., Hwang, B.Y., Lee, U.S., Jeong, H.S., Yoon, Y.W., Kim, D.J. & Hong, J.T. 2007. Anti-Proliferate and pro-Apoptotic Effects of 2,3-Dihydro-3,5-Dihydroxy-6-Methyl-4H-Pyranone through Inactivation of NF-KB in Human Colon Cancer Cells. *Archives of Pharmacal Research*, 30(11): 1455-1463.
3. Barış, D., Kızıl, M., Aytekin, Ç., Kızıl, G., Yavuz, M., Çeken, B. & Ertekin, A.S. 2011. *In Vitro* Antimicrobial and Antioxidant Activity of Ethanol Extract of Three *Hypericum* and Three *Achillea* Species From Turkey. *International Journal of Food Properties*, 14(2): 339-355.
4. Baytop, T. 1984. *Therapy With Medicinal Plants in Turkey (past and Present).* Istanbul University Press, Istanbul, 520 pp.
5. Baytop, T. 1999. *Therapy with Medicinal Plants in Turkey Past and Present*. 2nd Edition, Nobel Tıp Kitabevi, Istanbul, 480 pp.
6. Blois, M.S. 1958. Antioxidant Determination by the Use of a Stable Free Radical. *Nature*, 181: 1199-1200.
7. Boga, M., Ertas, A., Eroglu-Ozkan, E., Kizil, M., Ceken, B. & Topcu, G. 2016. Phytochemical analysis, antioxidant, antimicrobial, anticholinesterase and dna protective effects of *Hypericum capitatum* car. *capitatum* extracts. *South African Journal of Botany*, 104: 249-257.
8. Boga, M., Ersoy, E., Ozkan, E.E., Cinar, E., Kara, E.M., Canturk, Y.Y. & Zengin, G. 2021. Volatile and phenolic profiling of a traditional medicinal plant, *Hypericum empetrifolium* with *in vitro* biological activities. *Journal of Ethnopharmacology*, 272: 113933
9. Bounatirou, S., Smiti, S., Miguel, M.G., Faleiro, L., Rejeb, M.N., Neffati, M., Costa, M.M., Figueiredo, A.C., Barroso, J.G. & Pedro, L.G. 2007. Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus* Hoff. et Link. *Food Chemistry*, 105(1): 146-155.
10. Cargnin, S.T., de Brum Vieira, P., Cibulski, S., Cassel, E., Vargas, R.M.F., Montanha, J., Roehe, P., Tasca, T. & von Poser, G.L. 2013. Anti-*Trichomonas vaginalis* activity of *Hypericum polyanthemum* extract obtained by supercritical fluid extraction and isolated compounds. *Parasitology International*, 62(2): 112-117.
11. Carta, G., Elisabetta, M., Sebastiano, B. & Claudia, M. 2017. Palmitic Acid: Physiological Role, Metabolism and Nutritional Implications. *Frontiers in Physiology*, 8(11): 1-14.
12. Chang, C., Yang, M., Wen, H. & Chem, J. 2002. Estimation of Total Flavonoid Content in Propolis by Two Commplementary Colorimetric Methods. *Journal of Food and Drug Analysis***,** 10(3): 178-182.
13. Dinis, T.C.P., Madeira, V.M.C. & Almeida, M.L.M. 1994. Action of phenolic derivates (acetoaminophen, salycilate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Archives of Biochemistry and Biophysics*, 315(1): 161-169.
14. Eloff, J.N. 1989. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for Bacteria. *Planta Medica*, 64: 711-713.
15. Ergin, K.N., Karakaya, S., Göger, G., Sytar, O., Demirci, B. & Duman, H. 2022. Anatomical and Phytochemical Characteristics of Different Parts of *Hypericum scabrum* L. Extracts, Essential Oils, and Their Antimicrobial Potential. *Molecules*, 27: 1228.
16. Ertabaklar, H., Yaman Karadam, S., Malatyalı, E. & Ertuğ, S. 2016. Investigation of *in vitro* Metronidazole Resistance in the Clinical Isolates of *Trichomonas vaginalis*. *Microbiyoloji Bulteni*, 50(4): 552-558.
17. Eruygur, N., Atas, M, Cevik, Ö. & Tekin, M. 2017. Investigating of Phytochemicals, antioxidant, antimicrobial and proliferative properties of different extracts of *Thymus spathulifolius* Hausskn. and Velen. endemic medicinal plant from Sivas, Turkey. *International Journal of Secondary Metabolite*, 4(3): 155-166.
18. Eruygur, N., Ucar, E., Akpulat, H.A., Shahsavari, K., Safavi, S.M. & Kahrizi, D. 2019. *In vitro* antioxidant assessment, screening of enzyme inhibitory activities of methanol and water extracts and gene expression in *Hypericum lydium*. *Molecular Biology Reports*, 46(2): 2121-2129.
19. Harada, H., Yamashita, U., Kurihara, H., Fukushi, E., Kawabata, J. & Kamei, Y. 2002. Antitumor activity of palmitic acid found as a selective cytotoxic substance in a marine red alga. *Anticancer research*, 22(5): 2587-2590.

20. Hunt, E.J., Lester, C.E., Lester, E.A. & Tackett, R.L. 2001. Effect of St. John's Wort on free radical production. *Life Sciences*, 69(2): 181-190.

21. Hyun, T.K., Kim, H.C. & Kim, J.S. 2014. Antioxidant and Antidiabetic Activity of *Thymus quinquecostatus* Celak. *Industrial Crops and Products*, 52: 611-616.

22. Kirkcaldy, R.D., Augostini, P., Asbel, L.E., Bernstein, K.T., Kerani, R.P., Mettenbrink, C.J., Pathela, P., Schwebke, J.R., Secor W., Workowski, K.A., Davis, D., Braxton, J. & Weinstock, H.S. 2012. *Trichomonas vaginalis* antimicrobial drug resistance in 6 US Cities, STD surveillance network, 2009-2010. *Emerging Infectious Diseases*, 18(6): 939-943.

23. Kizil, G., Kizil, M., Yavuz, M., Emen, S. & Hakimoğlu, F. 2008. Antioxidant activities of ethanol extracts of *Hypericum triquetrifolium* and *Hypericum scabroides*. *Pharmaceutical Biology*, 46(4): 231-242.

24. Mansour, S., Djebli, N., Ozkan, E.E. & Mat, A. 2014. *In vivo* antiinflammatory activity and chemical composition of *Hypericum scabroides*. *Asian Pacific Journal of Tropical Medicine*, 7(1): 514-520.

25. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65(1-2): 55-63.

26. Moravcova, A., Červinková, Z., Kučera, O., Mezera, V., Rychtrmoc, D. & Lotkova, H. 2015. The effect of oleic and palmitic acid on induction of steatosis and cytotoxicity on rat hepatocytes in primary culture. *Physiological research*, 64(5): 627-636.

27. Oh, J.M., Choi, J.M., Lee, J.Y., Oh, S.J., Kim, H.C., Kim, B.H., Ma, J. Y. & Kim, S.K. 2012. Effects of palmitic acid on TNF-α-induced cytotoxicity in SK-Hep-1 cells. *Toxicology in Vitro*, 26(6): 783-790.

28. Oztürk, N., Korkmaz, S. & Oztürk, Y. 2007. Wound-healing activity of St . John's Wort (*Hypericum perforatum* L.) on Chicken Embryonic Fibroblasts. *Journal of Ethnopharmacology*, 111(1): 33-39.

29. Özen, H.C. & Bashan, M. 2003. The composition of fatty acids in *Hypericum scabrum*, *H. scabroides* and *H. amblysepalum*. *Turkish Journal of Chemistry*, 27(6): 723-725.

30. Radulović, N., Stankov-Jovanovic, V., Stojanovic, G., Smelceroovic, A., Spitller, M. & Asakawa Y. 2007. Screening of *in vitro* antimicrobial and antioxidant activity of nine *Hypericum* species from the Balkans. *Food Chemistry*, 10(1): 15-21.

31. Rahman, M.M., Hossain, M.A., Siddique, S.A., Biplab, K.P. & Uddin, M.H. 2012. Antihyperglycemic, antioxidant, and cytotoxic activities of *Alocasia Macrorrhizos* (L.) Rhizome Extract. *Turkish Journal of Biology*, 36: 574-579.

32. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine*, 26(9-10): 1231-1237.

33. Schwebke, J.R. & Barrientes, F.J. 2006. Prevalence of *Trichomonas vaginalis* Isolates with resistance to metronidazole and tinidazole. *Antimicrobial agents and chemotherapy*, 50(12): 4209-4210.

34. Silva, B.A., Ferreres, F., Malva, J.O. & Dias Alberto, C.P. 2005. Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. *Food Chemistry*, 90(1-2): 157-167.

35. Snipes, L.J., Gamard, P.M., Narcisi, E.M., Beard, C.B., Lehmann, T. & Secor, W.E. 2000. Molecular epidemiology of metronidazole resistance in a population of *Trichomonas vaginalis* clinical isolates. *Journal of Clinical Microbiology*, 38: 3004-3009.

36. Süntar, I.P., Akkol, E.K., Yilmazer, D., Baykal, T., Kirmizibekmez, H., Alper, M. & Yeşilada, E. 2010. Investigations on the *in vivo* wound healing potential of *Hypericum perforatum* L. *Journal of Ethnopharmacology*, 127: 468-477.

37. Takara, K., Otsuka, K., Wada, K., Iwasaki, H., & Yamashita, M. 2007. 1, 1-Diphenyl-2-picrylhydrazyl radical scavenging activity and tyrosinase inhibitory effects of constituents of sugarcane molasses. *Bioscience, biotechnology, and biochemistry*, 71(1): 183-191.

38. Tala, M.F., Talontsi, F.M., Zeng, G.Z., Wabo, H.K., Tan, N.H., Spiteller, M. & Tane, P. 2015. Antimicrobial and cytotoxic constituents from native Cameroonian medicinal plant *Hypericum riparium*. *Fitoterapia*, 102: 149-155.

39. Taran, M., Rezaeian, M. & Izaddoost, M. 2006. *In vitro* antitrichomonas activity of *Allium hirtifolium* (Persian shallot) in comparison with metronidazole. *Iranian Journal of Public Health*, 35(1): 92-94.

40. Toker, Z., Kizil, G., Ozen, H.C., Kizil, M. & Ertekin, S. 2006. Compositions and antimicrobial activities of the essential oils of two *Hypericum* Species from Turkey. *Fitoterapia*, 77: 57-60.

41. Yang, D., Li, Z., Yang, Y., Li, X. & Xiao, W. 2015. Chemical constituents from *Hypericum beanii*. *Chinese Herbal Medicines*, 7(4): 375-379.