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Phytochemical analysis, antioxidant, antimicrobial, anticholinesterase and DNA protective effects of *Hypericum capitatum* var. *capitatum* extracts

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ABSTRACT

This is the first study on phytochemical analysis and biological evaluation of *Hypericum capitatum* Choisy var. *capitatum* (HCC) endemic to Turkey with exception of essential oil analysis. Hyperoside and isoquercetin (25.7 ± 0.02 and 17.8 ± 0.02 μg/mg extract, respectively) were determined as the main compounds of the methanol extract. The major compounds of essential oil of HCC were found to be spathulenol and iso-longifolene (12.9% and 11.2%, respectively), and linoleic and palmitic acids (26.8% and 18.3%, respectively) were the main fatty acids. The methanol extract whose antioxidant activities studied for the first time was found to have moderate activity in β-carotene bleaching method (IC_{50}:41.69 ± 1.29 μg/mL) and was found to be the most active extract in scavenging of DPPH free radical (IC_{50}:16.82 ± 0.58 μg/mL) and ABTS cation radical (IC_{50}:9.24 ± 0.28 μg/mL) and CUPRAC assays. Among the prepared four extracts of HCC, the methanol extract showed highest activity against *Escherichia coli* with an MIC value of 10 μg/mL, and the methanol extract was also found to have high DNA damage protective activity. The methanol extract exhibited high butyrylcholinesterase inhibitory activity (70.74%). Thus, *H. capitatum* var. *capitatum* can be regarded as a potential natural antioxidant, antimicrobial, anticholinesterase source and DNA damage inhibitor.

1. Introduction

The genus *Hypericum*, a member of Hypericaceae family, is represented by 100 taxa, 45 being endemic to Turkey (Robson, 1988). In Turkish folk medicine, the genus *Hypericum* is known as "sarı kantaron, kantaron, binbirdelik otu, mayasıl otu" and most of them, especially *H. perforatum* have been used for the treatment of burns, wounds, hemorroids, diarrhea, and ulcers (Baytop, 1984). The traditional name of *H. capitatum* var. *capitatum* is kırmızı kantaron otu, and its aboveground part is used to release of pain for animals (Akan et al., 2013). *Hypericum* species contain naphthodianthrones (especially hypericin and pseudohypericin), acylphloroglucinol derivatives (especially hyperforin and adhyperforin), flavonoids (especially quercetin, quercitrin, hyperoside, and biapigenin), tannins, *n*-alkanes, and xanthones as secondary metabolites (Bombardelli and Morazzoni, 1995; Bruneton, 1995; Fu et al., 2006). In recent years, there is a remarkable interest in the characterization of new constituents of genus *Hypericum* as a source of several promising compounds with various biological activities, including cytotoxicity, anti-tumour, antimicrobial, and anti-inflammatory activities (Hu et al., 1999; Schmidt et al., 2000).

There are many studies in the literature about phenolic compounds obtained from plants and their pharmacological effects in the last decade. Moreover, essential oil composition and their biological activities have also gained importance in this field. A literature survey showed that there have been only one previous phytochemical report (Bağcı and Yüce, 2011) and no bioactivity studies, including antioxidant, anticholinesterase, antimicrobial, and DNA damage protection activity, on HCC. Therefore, we aimed to evaluate these activities on petroleum ether (HCP), acetone (HCA), methanol (HCM), and water (HCW) extracts of HCC. Essential oil of HCC and fatty acid compositions of petroleum ether extract were determined by using GC and GC/MS methods. Qualitative and quantitative flavonoids, flavonoid glycosides, and phenolic acid constituents of HCC were analysed with HPLC-DAD instrument. Total phenolic and flavonoid contents of the crude extracts were determined as pyrocatechol and quercetin equivalents, respectively. β-Carotene-linoleic acid bleaching assay, DPPH free radical scavenging assay, ABTS cation radical scavenging assay, and cupric reducing

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antioxidant capacity of the crude extracts were carried out to indicate the antioxidant activity. The anticholinesterase and antimicrobial potential of the extracts were determined by Ellman and disc diffusion and minimal inhibition concentration methods, respectively. The protective effect of the methanol extract of HCC on DNA cleavage induced by UV photolysis of H_2O_2 using pBluescript M13+ plasmid DNA was also investigated.

2. Materials and methods

2.1. Instruments and chemicals

Eleven compounds were determined by using Shimadzu HPLC. A Shimadzu UV spectrophotometer (Kyoto, Japan) and BioTek Power Wave XS microplate reader (Vinooski, VT, USA) were used for the activity assays. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), butylated hydroxytoluene (BHT), HPLC grade acetonitrile, methanol, ethyl acetate, and sodium dihydrogen phosphate dihydrate were purchased from Merck (Darmstadt, Germany); Milli-Q ultrapure water was obtained from Millipore (Darmstadt, Germany), formic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, linoleic acid, Tween 40, potassium persulfate, pyrocathecol, 5,5-dithiobis-(2-nitro benzoic acid) (DTNB), copper (II) chloride dihydrate ($CuCl_2.2H_2O$), neocuproine (2,9-dimethyl-1,10-phenanthroline), EDTA (ethylenediaminetetraacetic acid), acetylcholinesterase (AChE: from electric eel) (Type-VI-S, EC 3.1.1.7, 425.84 U/mg), and butyrylcholinesterase (BChE: from horse serum) (EC 3.1.1.8, 11.4 U/mg) were obtained from Sigma (Steinheim, Germany); α-tocopherol and acetylthiocholine iodide were from Aldrich (Steinheim, Germany); hypericin, chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, kaempferol, quercetin, amentoflavone, and hyperforin galanthamine hydrobromide were from Sigma-Aldrich (Steinheim, Germany); pseudohypericin was purchased from Phytoplan (Heidelberg, Germany); Folin Ciocalteu phenol reagent was from Applichem (Darmstadt, Germany); and butyrylthiocholine iodide and orthophosphoric acid (85%) were from Fluka (Steinheim, Germany).

2.2. Plant material

The whole plant of *H. capitatum* Choisy var. *capitatum* was collected from northwestern Turkey (Çavuşbaşı, Beykoz, Istanbul) in August 2012 by Dr. A. Ertaş, and identified by Dr. Yeter Yeşil. A sample was stored in the Herbarium of Istanbul University, Faculty of Pharmacy (ISTE 98059).

2.3. Preparation of the extracts for determination of biological activities

Powdered form of the whole plant material was weighed (100 g) and sequentially macerated three times with petroleum ether (250 mL each), acetone (250 mL each), methanol (250 mL each) and water (250 mL each) at 25 °C for 24 h. After filtration, the solvent was evaporated to obtain the crude extracts. The petroleum ether extract was analyzed to determine its fatty acid composition by GC/MS.

2.4. Chromatographic HPLC conditions

In this study, for quantitative analysis, a European Pharmacopoeia method was used for hypericin, pseudohypericin, chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, kaempferol, quercetin, amentoflavon, and hyperforin compounds with Shimadzu HPLC-DAD instrument (Table 1, Figs. 1–3).

The methanol extract have been analyzed by HPLC-DAD. The HPLC system consisted of a Shimadzu 10A model (Shimadzu Analytical and Measuring Instruments, Kyoto, Japan), a pump (LC-10 AD), a diode-array detector (DAD) (SPD-M10A), and an autosampler (SIL-10 AD).

Separation was accomplished with an ACE C18 column, 250 × 4.6 mm i.d., 5 μm (Advanced Chromatography Technologies, Alberdeen, Scotland). The elution conditions were as follows: flow rate, 1 ml/min; column temperature, 40 °C; injection volume, 10 μL; detection, 590 nm for hypericin, 275 nm for hyperforin, and 360 nm for phenolic compounds.

The solvent system was used as an isocratic to identify and quantitate pseudohypericin and hypericin. Separation was carried out using solvent A [ethyl acetate/15.6 g $\cdot L^{-1}$ sodium dihydrogen phosphate adjusted to pH 2 with phosphoric acid/methanol (39:41:160, v/v/v)]. The solvent system was used as a gradient to identify and quantitate phenolic compounds and hyperforin. The mobile phase consisted of solvent A (0.3% formic acid in water (v/v) and solvent B (0.3% formic acid in acetonitrile (v/v). The following gradient was applied: 0–8 min, 82% A; 8–18 min, 82–47% A; 18–18.1 min, 47–3% A; 18.1–29 min, 3% A; 29–40 min, 3–82% A (European Pharmacopoeia, 2008). All solvents were filtered through a 0.45 μm filter prior to use and degassed in an ultrasonic bath.

The control of the system and the data analysis procedure were performed with Shimadzu LC Solutions software.

Table 1 shows the main components of the methanol extract of *H. capitatum* var. *capitatum* analyzed by HPLC-DAD and retention times, the equations, and r^2 values obtained from calibration curves.

2.5. Standard preparation

The calibration curves were prepared with external standards solution within the different concentration range in methanol. The experiment was conducted for three times in order to provide the same conditions. The calibration curves were constructed by using average of peak areas.

2.6. Sample preparation

The crude methanol extract was dissolved in mixture of methanol/water (8:2, v/v) (European Pharmacopoeia, 2008). All samples were

Table 1
Chemical compounds of the methanol extract of *H. capitatum* var. *capitatum*.

Compounds	Retention time (min)	Calibration equation values	Linear regression (r^2)	HCC (μg/mg extract)
Pseudohypericin	4.86	$y = 2.582269e + 007x + 1741.874$	0.9998	4.6 ± 0.030
Hypericin	13.93	$y = 6.03411e + 007x + 297.2292$	0.9999	1.3 ± 0.002
Chlorogenic acid	4.33	$y = 5110294x + 1490.398$	0.9999	3.3 ± 0.010
Rutin	8.89	$y = 1.383368e + 007x + 5188.182$	0.9999	Nd
Hyperoside	10.19	$y = 2.849917e + 007x + 526.7023$	0.9999	25.7 ± 0.020
Isoquercitrin	10.75	$y = 1.671137e + 007x - 3712.788$	0.9999	17.8 ± 0.020
Quercitrin	14.41	$y = 1.205178e + 007x - 3518.974$	0.9999	5.4 ± 0.010
Kaempferol	17.09	$y = 5.183916e + 007x + 4373.856$	0.9999	Tr
Quercetin	17.84	$y = 3.688175e + 007x + 18905.43$	0.9999	0.3 ± 0.001
Amentoflavon	20.27	$y = 2.207879e + 007x + 772.0972$	0.9996	Nd
Hyperforin	27.75	$y = 6212343x$	0.9997	0.1 ± 0.001

% Values of the compounds in the extracts were the means of three replicates ± standard deviation. Tr: trace; Nd: not detected.

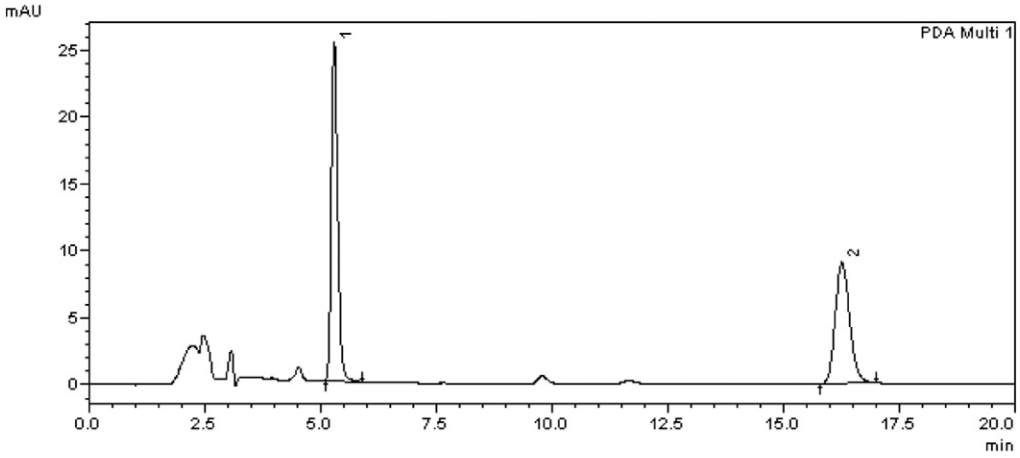

Fig. 1. PDA chromatogram of the methanol extract at 590 nm (1: pseudohypericin; 2: hypericin).

filtered through a 0.45 μm filter into a vial for HPLC analysis. Each sample was prepared and injected for three times.

2.7. Esterification of total fatty acid and GC–MS conditions

The study of Gören et al. (2012) was referred for the esterification of petroleum ether extract of *H. capitatum* var. *capitatum*. A hundred milligram of the petroleum ether extract was refluxed in 0.1 M KOH solution in 2 mL of methanol for 1 h, the solution was cooled, and 5 mL of water was added. The aqueous mixture was neutralized with 0.5 mL of HCl solution, and it was extracted with diethyl ether: hexane (1: 1; 3.5 mL) mixture. The separating organic phase was washed with 10 mL water and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum and then fatty acid methyl esters were obtained. Thermo Scientific Polaris Q GC–MS was used in the current study. The same protocol of Gören et al. (2012) was used for working conditions of GC–MS study and comparison of identification and quantification of the compounds.

2.8. Preparation of essential oil and GC–MS conditions

In order to isolate the essential oil from the aerial parts of the plant (100 g), a Clevenger-type apparatus was used. Initially, dried aerial parts of the plant was crumbled into small pieces and soaked in distilled water (500 mL) for 3 h. Then anhydrous Na_2SO_4 was used for drying step of the obtained essential oil and the samples were stored at +4 °C. For the dilution of the essential oil, CH_2Cl_2 (1:3 volume/volume) was used before the GC-FID (gas chromatography/flame ionization detector) and GC–MS analysis. Thermo electron trace GC-FID detector was used for GC-FID analysis and GC–MS analysis was applied using the same GC and thermo electron DSQ MS.

The following GC conditions were used for both GC/MS and GC/FID analyses: The GC oven temperature was settled at 60 °C for 10 min and the temperature was reached to 280 °C by increasing 4 °C/min and then fixed at 280 °C for 10 min. Helium was chosen as a carrier gas and a nonpolar Phenomenex DB5 fused silica column (30 m × 0.32 mm, 0.25 μm film thickness) was used with helium at 1 mL/min (20 psi). The split ratio was set to 1:50, the injection volume was 0.1 μL, and the EI/MS spectrum was recorded at 70 eV ionization energy. The mass range was m/z 35–500 amu. In the calculation of Kovats Indices (KI), alkane mix (C_8–C_{24}) was used as reference points by the same conditions (Ertas et al., 2014).

GC–MS conditions and comparison of identification and quantification of the compounds were conducted with exact same manner by Ertas et al. (2014). The compounds were identified by comparing their retention times and mass spectra with those obtained from authentic samples and/or the NIST and Wiley spectra as well as data from the published literature.

2.9. Determination of total phenolic and flavonoid contents

The concentrations of phenolic content in the crude extracts were expressed as micrograms of pyrocatechol equivalents (PEs) (Slinkard and Singleton, 1977). The solution (8 μL) of the samples in methanol was added to 176 μL of distilled water and 4 μL of Folin–Ciocalteu's

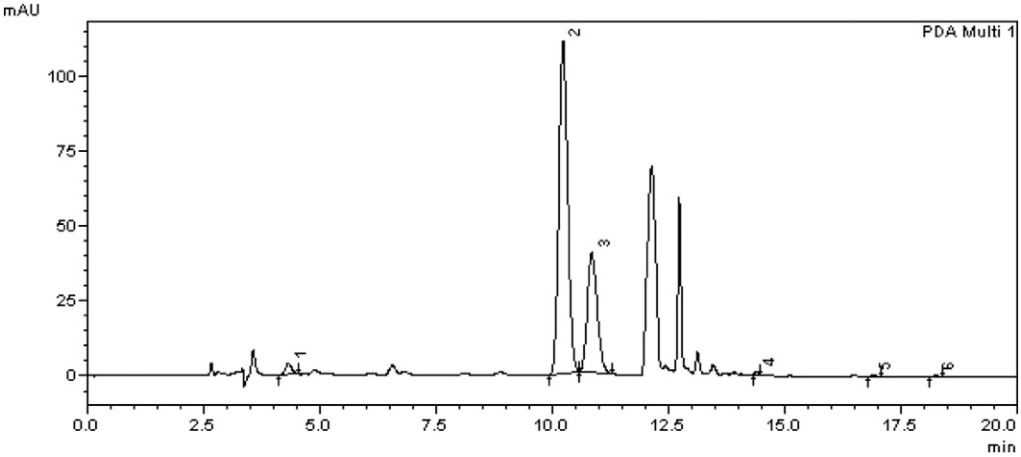

Fig. 2. PDA chromatogram of the methanol extract at 360 nm (1: chlorogenic acid; 2: hyperoside; 3: isoquercitrin; 4: quercitrin; 5: kaempferol; 6: quercetin).

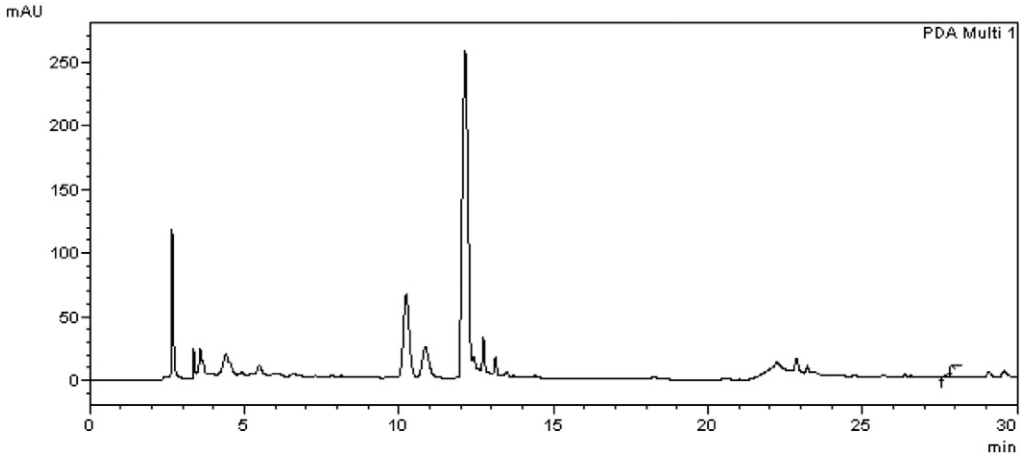

Fig. 3. PDA chromatogram of the methanol extract at 275 nm (1: hyperforin).

Reagent, and mixed thoroughly. After 3 min, 12 μL sodium carbonate (2%) was added to the mixture and shaken intermittently for 2 h at room temperature. The absorbance was read at 760 nm. The concentration of phenolic compounds was calculated according to the following equation that was obtained from standard pyrocatechol graphic:

$$\text{Absorbance} = 0.0128 \text{ pyrocatechol } (\mu g) + 0.0324 \quad \left(R^2 = 0.9984\right)$$

Measurement of flavonoid content of the crude extracts was based on the method described by Moreno et al. with a slight modification and results were expressed as quercetin equivalents (Moreno et al., 2000). An aliquot of 20 μL of the solution (contains 1 mg of crude extract in methanol) was added to test tubes containing 4 μL of 10% aluminium nitrate, 4 μL of 1 M potassium acetate and 172 μL of methanol. After 40 min at room temperature, the absorbance was determined at 415 nm. The concentration of flavonoid compounds was calculated according to the following equation:

$$\text{Absorbance} = 0.1701 \text{ quercetin } (\mu g) - 0.0778 \quad \left(R^2 = 0.9939\right)$$

2.10. Antioxidant activity of the extracts

We used the β-carotene linoleic acid test system (Miller, 1971), DPPH free radical (Blois, 1958), ABTS cation radical scavenging activity (Re et al., 1999), and cupric reducing antioxidant capacity (CUPRAC) methods to determine the antioxidant activity (Apak et al., 2004).

2.10.1. β-Carotene linoleic acid test system

β-Carotene (0.5 mg) in 1 mL of chloroform was added into linoleic acid (25 μL) and Tween 40 emulsifier (200 mg) mixture. After evaporating chloroform, 100 mL of distilled water saturated with oxygen was added followed by shaking, and 160 μL of this mixture was transferred into different test tubes containing 40 μL of the sample solutions at different concentrations. The emulsion was added to each tube, and the zero time absorbances of the values were read at 470 nm. The mixture was incubated for 2 h at 50 °C (Miller, 1971). The bleaching rate (R) of β-carotene was calculated according to the following equation:

$$R = \frac{\ln \frac{a}{b}}{t}$$

where ln = natural log, a = absorbance at time zero, and b = absorbance at time t (120 min). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using following equation:

$$\text{AA (Inhibition \%)} = \frac{R_{\text{Control}} - R_{\text{Sample}}}{R_{\text{Control}}} \times 100$$

2.10.2. DPPH free radical scavenging activity method

DPPH solution (0.1 mM) in methanol 160 μL was added to 40 μL of sample solutions in methanol at different concentrations. After 6 min, the absorbance values were read at 517 nm. The DPPH free radical scavenging potential was calculated using the following equation:

$$\text{DPPH scavenging effect (Inhibition \%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

A_{Control} is the initial concentration of the DPPH.

A_{Sample} is the absorbance of the remaining concentration of DPPH in the presence of the extracts or positive controls (Blois, 1958; Hariprasath et al., 2015).

2.10.3. ABTS cation radical decolorization assay

Seven millimolar ABTS in H_2O was added to 2.45 mM potassium persulfate to produce $ABTS^{\bullet+}$ and solution was stored in the dark at 25 °C for 12–16 h. The prepared solution was diluted with ethanol to get an absorbance of 0.700 ± 0.025 at 734 nm. $ABTS^{\bullet+}$ solution (160 μL) was added to each sample solution (40 μL) at different concentrations. After 6 min, the percentage inhibition at 734 nm was read for each concentration relative to a blank absorbance (methanol). The following equation was used to calculate the scavenging capability of $ABTS^{\bullet+}$ (Re et al., 1999):

$$ABTS^{\bullet+} \text{ scavenging effect (Inhibition \%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

2.10.4. Cupric reducing antioxidant capacity (CUPRAC) method

Aliquots of 61 μL of 1.0×10^{-2} M copper (II) chloride, 61 μL of NH_4OAc buffer (1 M, pH 7.0), and 61 μL of 7.5×10^{-3} M neocuproine solution were stirred; x μL sample solution (2.5, 6.25, 12.5, and 25 μL) and $(67 - x)$ μL distilled water were added to reach the final volume 250 μL. The tubes were left to stand for one hour. Afterward, the absorbance at 450 nm was measured against a reagent blank (Apak et al., 2004).

2.11. Anticholinesterase activity of the extracts

A spectrophotometric method developed by Ellman et al. (1961) was used to test the acetyl- and butyryl-cholinesterase inhibitory

Table 2
Chemical composition of the essential oil from *H. capitatum* var. *capitatum*.

Rt (min)[a]	Constituents[b]	Composition%	RI[c]	Rt (min)[a]	Constituents[b]	Composition%	RI[c]
10.87	Isononane	2.5	865	30.23	Guaiene	5.2	1477
13.60	α-Pinene	5.7	939	30.48	Valencene	5.9	1484
14.17	Camphene	1.1	954	30.67	β-Selinene	3.5	1490
14.83	3-Methyl nonane	0.9	970	30.87	α-Selinene	2.8	1498
15.21	β-Pinene	0.9	979	31.01	β-Himachalene	3.4	1505
17.15	Cineole	1.0	1031	31.26	τ-Cadinene	0.5	1514
18.02	2-Methyl decane	0.8	1063	31.42	β-Cadinene	0.7	1523
19.19	Linalool	0.6	1097	32.06	Elemol	2.1	1550
24.16	Anisaldehyde	0.4	1250	32.93	Spathulenol	12.9	1578
25.80	Dihydrocarvyl acetate	2.7	1344	33.13	Caryophyllene oxide	0.9	1583
26.77	α-Terpinyl acetate	0.9	1349	33.28	Globulol	0.8	1585
27.71	α-Copaene	2.2	1377	33.50	Longiborneol	1.4	1592
28.22	Iso-Longifolene	11.2	1390	34.64	α-Cadinol	1.1	1654
28.49	β-Cubebene	0.5	1393	34.94	Bulnesol	1.9	1672
28.96	Caryophyllene	1.4	1409	35.11	Cadalene	0.5	1677
29.16	β-Gurjunene	5.7	1427	35.52	2-Methyl heptadecane	0.5	1746
29.26	τ-Elemene	1.1	1430	40.61	Arachidic acid	1.1	2366
29.47	β-Farnesene	0.9	1447	43.88	Cholic acid	0.4	2896
29.77	α-Himachalane	0.9	1451		Total	96.2	
29.83	α-Humulene	5.2	1455				
30.00	Alloaromadendrene	4.0	1466				

[a] Retention time (as minute).
[b] Compounds listed in order of elution from a nonpolar Phenomenex DB-5 fused silica column.
[c] RI, retention indice (DB-5 column).

activities. Aliquots of 150 μL of 100 mM sodium phosphate buffer (pH 8.0), 10 μL of sample solution, and 20 μL BChE (or AChE) solution were stirred and incubated for 15 min at 25 °C, then DTNB (10 μL) is added to mixture. In the next step, by the addition of butyrylthiocholine iodide (or acetylthiocholine iodide) (10 μL), the reaction was started. At the end, final concentration of the tested solutions was 200 μg/mL. BioTek Power Wave XS at 412 nm was used to monitor the hydrolysis of these substrates.

$$\text{Inhibition } \% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

2.12. Antimicrobial activity of the extracts

Five different microorganisms including Gram-positive bacteria (*Streptococcus pyogenes* ATCC19615 and *Staphylococcus aureus* ATCC 25923), Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922), and yeast (*Candida albicans* ATCC10231), which were purchased from Refik Saydam Sanitation Center (Turkey) were used for detecting the antimicrobial activity of the samples. The disc diffusion method was employed for this purpose (NCCLS, 1997; Yoon et al., 2015). The minimum inhibitory concentration was determined by the broth macrodilution method according to NCCLS (2009). Ampicillin and fluconazole were used as positive controls for bacteria and yeast, respectively.

2.13. DNA damage protective activity

Measurement of the DNA damage protective activity of the methanol extract was checked on pBluescript M13(+) plasmid DNA. Plasmid DNA was oxidized with OH radicals generated from UV photolysis of H_2O_2 in the presence of the extract and checked on 1% agarose according to Kızıl et al. (2009). The percent inhibition of the DNA cleavage was calculated using the method described by Fukuhara and Miyata (1998).

2.14. Statistical analysis

The results of the antioxidant, anticholinesterase, and antimicrobial activity assays were mean ± SD of three parallel measurements. The statistical significance was estimated using a Student's *t*-test, p values <0.05 were regarded as significant.

3. Results and discussion

3.1. Quantitative analysis of phenolic compounds by HPLC

The methanol extract was found to be have the highest activities such as total pheolic and flavonoid content, antioxidant, and antibutyrylcholinesterase. Therefore, the chemical composition of methanol extract was choosen for further investigation. Methanol extract of HCC was analyzed for quantification of the eleven compounds. All of these compounds were determined from different *Hypericum* species (Çırak et al., 2011; Aybastıer et al., 2013; Eroğlu-Özkan et al., 2013; Camas et al., 2014). Rutin and amentoflavon were not detected by this method. Hyperoside and isoquercitrin, were found to be the most abundant amount in the studied compounds for HCC (25.7 ± 0.02 μg/mg and 17.8 ± 0.02 μg/mg extract, respectively) (Table 1). In the literature, to the

Table 3
Fatty acid analysis of the petroleum ether extract of *H. capitatum* var. *capitatum*.

Rt (min)[a]	Constituents[b]	Composition %
12.00	Lauric acid, methyl ester	1.1
14.39	10-Undecenoic acid, methyl ester	3.7
18.60	Myristic acid, methyl ester	1.4
24.94	Palmitoleic acid, methyl ester	0.3
25.27	Palmitic acid, methyl ester	18.3
29.75	Phytol	1.4
30.64	Linoleic acid, methyl ester	26.8
30.77	Oleic acid, methyl ester	16.6
30.86	Linolenic acid, methyl ester	8.9
31.00	Vaccenic acid, methyl ester	0.1
31.54	Stearic acid, methyl ester	10.1
36.69	11-Eicosenoic acid, methyl ester	1.4
37.38	Arachidic acid, methyl ester	3.0
39.36	Docosane	2.2
43.82	Behenic acid, methyl ester	4.7
	total	100

[a] Retention time (as minute).
[b] Compounds listed in order of elution from a nonpolar Phenomenex DB-5 fused silica column.

Table 4
Total phenolic-flavonoid contents,* Antioxidant activities,* and anticholinesterase activities* of *H. capitatum* var. *capitatum* extracts, BHT, α-TOC, and galantamine.

Samples	Phenolic content (μg PEs/mg extract)[a]	Flavonoid content (μg QEs/mg extract)[b]	Lipid peroxidation	IC_{50} (μg/mL) DPPH free radical	ABTS cation radical	Inhibition % against AChE	Inhibition % against BChE
HCP	112.20 ± 2.87	7.24 ± 0.03	>200	>200	>200	NA	47.98 ± 0.20
HCA	65.45 ± 2.07	9.88 ± 0.95	>200	>200	88.84 ± 1.40	NA	**63.36 ± 0.43**
HCM	**215.85 ± 0.18**	**15.90 ± 0.28**	**41.69 ± 1.29**	**16.82 ± 0.58**	**9.24 ± 0.28**	14.00 ± 1.02	**70.74 ± 0.71**
HCW	189.43 ± 3.62	12.78 ± 0.68	92.85 ± 1.05	>200	**9.76 ± 0.14**	14.63 ± 0.38	45.60 ± 0.40
α-TOC[c]	–	–	15.54 ± 0.12	18.76 ± 0.41	9.88 ± 0.23	–	–
BHT[c]	–	–	10.35 ± 0.67	48.86 ± 0.50	10.67 ± 0.11	–	–
Galantamine[c]	–	–	–	–	–	85.55 ± 0.55	79.22 ± 1.22

Significance of bold data show the highest phenolic and flavonoid contents values, the lowest IC_{50} values for Lipid peroxidation, DPPH free radical scavenging, ABTS cation radical scavenging assays and highest inhibition percentages for butyrylcholinesterase enzyme activity.
NA: not active.
* Values expressed are means ± SEM of three parallel measurements ($p < 0.05$).
[a] PEs, pyrocatechol equivalents ($y = 0.0128x + 0.0324$ $R^2 = 0.9984$).
[b] QEs, quercetin equivalents ($y = 0.1701x - 0.0778$, $R^2 = 0.9939$).
[c] Standard compounds.

best of our knowledge, there is no study on detection of qualitative and quantitative flavonoids, flavonoid glycosides and phenolic acid constituents of HCC with HPLC-DAD instrument.

3.2. Chemical compositions of the essential oil and fatty acid

The essential oils of HCC were determined by GC/MS analysis. The identified compounds are listed in Table 2. According to the results, the major compounds of the essential oil were spathulenol (12.9%) and iso-longifolene (11.2%). As far as our literature survey, one paper is available for the essential oil composition, but no publication shows on fatty acid composition of HCC is available. According to the report of Bağcı and Yüce (2011) the main constituents of the essential oil of HCC were α-pinene (20.3%) and caryophyllene oxide (11.8%). There are a lot of literature reports about different results on essential oil components of the same plants from various locations (Çırak and Bertoli, 2013). The composition of the essential oil of HCC investigated by Bağcı and Yüce (2011) was found to be quite different from our results. Since the same extraction method and similar GC study conditions were used, the differences between essential oil results may be due to from different collected locations and climatic conditions.

The petroleum ether extract of HCC was analysed by GC/MS technique for fatty acid composition. As shown in Table 3, high percentages of linoleic acid (26.8%), palmitic acid (18.3%), and oleic acid (16.6%) were observed in the extract. As far as our literature survey, no report is available for the fatty acid composition of HCC. Previous study on fatty acid composition of *Hypericum* species indicated that the main fatty acid contents were palmitic, oleic and linolenic acids (Ozen et al., 2004a, 2004b; Türkoğlu et al., 2015). There are few studies on fatty acid composition of *Hypericum* species in the literature, such as, palmitic acid (12.16%), oleic acid (3.45%), and linolenic acid (17.15%) were the dominant fatty acids in the extracts of *H. uniglandulosum* (Türkoğlu et al., 2015), another report by Shafaghat (2011), the main components of the flower extract of *Hypericum scabrum* were tetracosane (12.2%) and palmitic acid (9.3%), the leaf extract was palmitic acid (7.4%) as major components. According to a report by Ozen et al. (2004b), the flowers of *H. lysimachioides* var. *lysimachioides* contain unusual 3-hydroxy fatty acids (3-hydroxytetradecanoic acid and 3-hydroxyoctadecanoic acid), along with other normal fatty acids. *Major* components were linolenic and palmitic acids for both leaves and flowers. Our results generally compatible with the previous study.

3.3. Total phenolic and flavonoid contents of the extracts

Total phenolic and flavonoid contents of the petroleum ether, acetone, methanol, and water extracts prepared from the whole plant of HCC were determined as pyrocatechol (PEs) and quercetin (QEs) equivalents, respectively ($y = 0.0128$ pyrocatechol (μg) + 0.0324, $R^2 = 0.9984$ and $y = 0.1701$ quercetin (μg) − 0.0778, $R^2 = 0.9939$). As seen in Table 4, the phenolic contents of the tested four extracts are higher than their flavonoid contents, and the methanol extract was found to have the highest phenolic and flavonoid content.

The phenolic and flavonoid amounts of the methanol extract were identified to be the richest. The amounts of total phenolic and flavonoid from methanol extract were found to be 215.85 ± 0.18 μg/mg extract and 15.90 ± 0.28 μg/mg extract, respectively. The amount of phenolic components was observed to be higher than that of flavonoid components. The phenolic compounds of the HCC are mainly polar; hence, phenolic content of methanol extract was found to have much higher than the flavonoid contents. The results were shown in Table 4. In the literature, total phenolic and flavonoid content in the examined *H. perforatum* extracts were expressed in terms of gallic acid and quercetin equivalents respectively. Moreover, the total phenolic and flavonoid amounts in the examined methanol extract of *H. perforatum* were

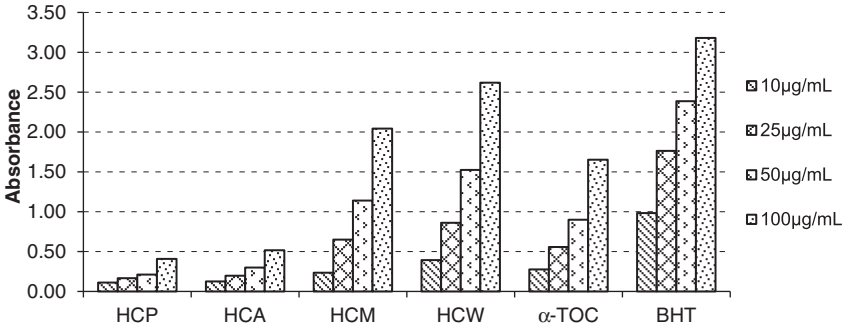

Fig. 4. Cupric reducing antioxidant capacity of the *H. capitatum* var. *capitatum* extracts, α-tocopherol, and BHT. Values are means ± SD, $n = 3$.

Table 5
Zones of growth inhibition (mm) and MIC values showing the antimicrobial activities of the extracts compared to positive controls.

Microorganisms		Gram positive		Gram negative		Yeast
		S. aureus	*S. pyogenes*	*E. coli*	*P. aeruginosa*	*C. albicans*
Petroleum ether extract	[a]DD	–	11 ± 0.1	–	7 ± 0.2	8 ± 0.3
	MIC	–	2000 ± 0.4	–	2200 ± 0.7	2200 ± 0.1
Acetone extract	[a]DD	12 ± 0.2	13 ± 0.2	12 ± 0.5	9 ± 0.5	16 ± 0.3
	MIC	250 ± 0.3	280 ± 0.4	15 ± 0.2	300 ± 0.2	25 ± 0.5
Methanol extract	[a]DD	10 ± 0.5	11 ± 0.3	8 ± 0.2	10 ± 0.4	20 ± 0.1
	MIC	300 ± 0.5	250 ± 0.3	10 ± 0.1	260 ± 0.7	250 ± 0.1
Positive controls	[b]DD	35 ± 0.2	19 ± 0.2	20 ± 0.1	–	30 ± 0.3
	MIC	1.95 ± 0.3	7.815 ± 0.1	7.815 ± 0.4	–	3.125 ± 0.2

–: not active.
[a] DD: Inhibition zone in diameter (mm) around the discs (6 mm) impregnated with 30 mg/mL of plant extracts.
[b] DD: Inhibition zone in diameter (mm) of positive controls that are ampicillin for bacteria and fluconazole for yeast. *Minimum* inhibitory concentration (MIC) values are given as μg/mL.

355.01 mg/g and 167.37 mg/g, respectively; these values were higher than the ethylacetate and water extracts (Altun et al., 2013). Our results were compatible with literature.

3.4. Antioxidant and anticholinesterase activities of the extracts

The petroleum ether, acetone, methanol, and water extracts prepared from the whole plant of HCC were screened for their possible antioxidant activity by using four complementary methods, namely, of β-carotene bleaching, DPPH free radical scavenging, ABTS cation radical decolorisation, and cupric reducing antioxidant capacity assays. The methanol and water extracts exhibited moderate lipid peroxidation inhibitory activity in β-carotene bleaching method with IC_{50}: 41.69 ± 1.29 μg/mL and 92.85 ± 1.05 μg/mL values (Table 4). In the report of Rainha et al., the *Hypericum* species and Azorean *H. foliosum* samples presented a moderate capacity to inhibit linoleic acid oxidation and this report is compatible with our results (Rainha et al., 2011). The methanol extract was found to be more active than BHT and α-Toc, which used as standards in DPPH free radical scavenging assay with IC_{50}:16.82 ± 0.58 μg/mL value (Table 4). Our results were in agreement with Eroğlu-Özkan et al. (2013) who studied similar DPPH radical scavenging activity of other *Hypericum* species. The methanol and water extracts of HCC exhibited strong inhibition in ABTS cation radical scavenging with IC_{50}:9.24 ± 0.28 μg/mL and 9.76 ± 0.14 μg/mL values, respectively, and these extracts showed better activity than α-Toc and BHT standards (Table 4). In the study of Chandrashekhar et al. (2009), methanol extract of *H. hookerianum* has been shown good activity in ABTS cation radical scavenging. The methanol and water extracts of the plant exhibited high absorbance values in CUPRAC assay at all concentrations and better than α-Toc used as a standard compound (Fig. 4).

As shown in Table 4, the acetone and methanol extracts exhibited meaningful anticholinesterase activity against butyrylcholinesterase enzyme at 200 μg/mL as 63.36 ± 0.43% and 70.74 ± 0.71% inhibition, respectively. In the report of Altun et al. (2013), ethylacetate extract of *H. perforatum* showed highest inhibitory activity against butyrylcholinesterase enzyme with 50.79 ± 3.07% inhibition.

3.5. Antimicrobial activity of the extracts

The antimicrobial activities results are presented in Table 5. The petroleum ether extract was active on *S. pyogenes* (Gram positive), *P. aeruginosa* (Gram negative), and *C. albicans* (Yeast). The acetone

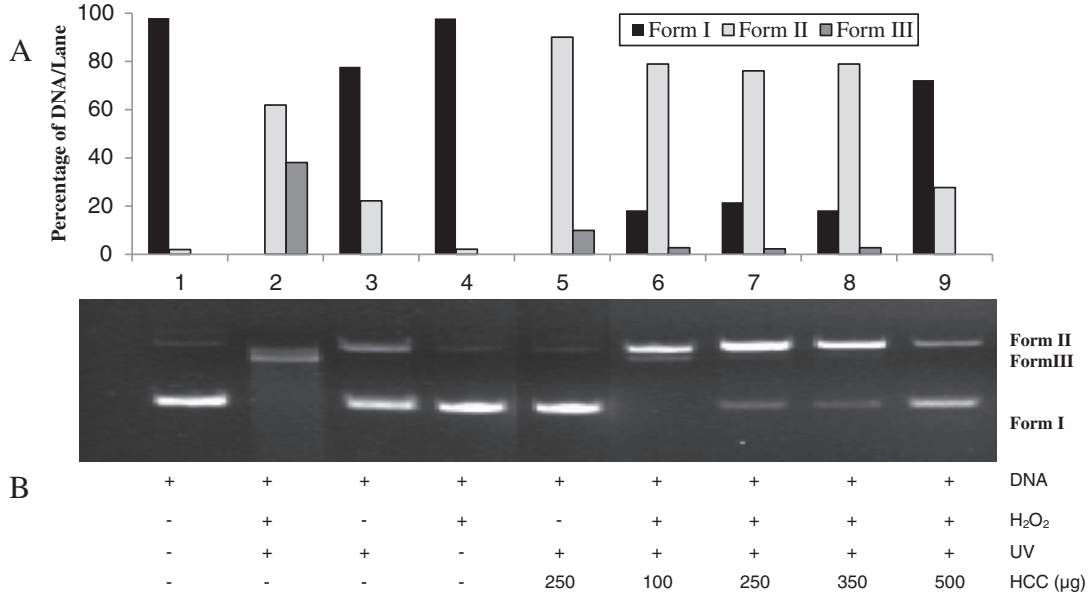

Fig. 5. The quantified band intensity for the scDNA (Form I), ocDNA (Form II), and linDNA (Form III) with Quantity One 4.5.2. version software (A). Electrophoretic pattern of pBluescript M13 + DNA after UV photolysis of H_2O_2 in the presence or absence of methanol extract of *H. capitatum* var. *capitatum*. Reaction vials contained 200 ng of supercoiled DNA (31.53 nM) in distilled water, pH 7). Electrophoresis was performed using 1% agarose at 40 V for 3 h in the presence of ethidium bromide (10 mg/mL) (B). Electrophoresis running buffer: TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.2). Gel was scanned on Gel documentation system (Gel-Doc-XR, BioRad, Hercules, CA, USA). Bands on the gels were quantified using discovery series Quantity One programme (version 4.5.2. BioRad Co.)

and methanol extracts were active on all tested microorganisms with different zone diameters, indicating weak (inhibition zone < 12) and moderate antimicrobial activity (inhibition zone < 20–12). The methanol extract recorded the highest inhibition zone diameter against on *C. albicans* (20 mm inhibition zone diameter) and the lowest MIC value against on *E. coli* (10 ± 0.1 μg/mL).

Various *Hypericum* species were investigated for determining their possible antimicrobial activity by many authors (Sakar et al., 1988; Decosterd et al., 1991; Rath et al., 1996). Unal et al. (2008) reported that various extracts of three *Hypericum* species, including *H. heterophyllum*, *H. hyssopifolium* subsp. *elongatum* var. *elongatum*, and *H. scabrum* L. showed remarkable antibacterial activity against *Bacillus megaterum*, *Bacillus subtilis*, *Proteus vulgaris*, *S. pyogenes*, and *S. aureus*, and all tested *Hypericum* species showed strong antibacterial activity against Gram-positive bacteria. In contrast with antibacterial activity, these *Hypericum* species was found to have no activity against *C. albicans*. In the report of Radulović et al. (2007), the antimicrobial activity of the methanol extracts of nine *Hypericum* species from the Balkans, including *H. barbatum*, *H. hirsutum*, *H. linarioides*, *H. maculatum*, *H. olympicum*, *H. perforatum*, *H. richeri*, *H. rumeliacum*, and *H. tetrapterum*, was studied using disk diffusion method. All mentioned *Hypericum* extracts possess strong antimicrobial activity. It was determined that *H. aviculariifolium* extract was as active as *H. perforatum* against Gram-positive bacteria at a concentration range between 195.31 and 97.65 μg/mL. In our study, the methanol extract exhibited the highest inhibition zone diameter against *C. albicans* (20 mm inhibition zone diameter) and the lowest MIC value against on *E. coli* (10 ± 0.1 μg/mL).

3.6. DNA damage protective activity of the extracts

As DNA damage protective activity, the addition of the methanol extract (100 μg/mL) to the reaction mixture suppressed the formation of linear plasmid DNA but has no significant effect on protection of supercoiled DNA (Fig. 5B, lane 6). The addition of the extract (250, 350 and 500 μg/mL) to the reaction mixture suppressed the formation of open circular and linear plasmid DNA (Fig. 5B, lanes 6–9) and induced a partial recovery of supercoiled DNA. Fig. 5B, lane 2 represents the effects of OH radical generated from UV photolysis of H_2O_2. The methanol extract of HCC (200 μg) alone has no significant effect on DNA cleavage (Fig. 5, lane 5). DNA damage inhibition activities of the methanol extract of HCC at the concentrations of 250, 350, and 500 μg/mL were found to be 18.67%, 22.07%, and 73.75%, respectively. Fig. 5B (lanes 6–9) shows that the methanol extract of HCC has concentration-dependent DNA damage protective activity. In the report Kızıl et al. (2009), ethanol extracts of *Hypericum scabroides* and *Hypericum triquetrifolium* have DNA damage protective activity.

4. Conclusions

The methanol extract of HCC has antioxidant activity since the high phenolic content have potential to function as antioxidant by scavenging free radicals, cation radicals, and cupric reducing power and DNA damage inhibition activity. Our results showed that HCC can be regarded as a potential source of natural antioxidant, antibutyrylcholinesterase, antimicrobial, and DNA damage inhibitor in the pharmaceutical industry. Thus, further phytochemical and biological studies need to be done to characterize the active constituents of *H. capitatum* var. *capitatum*.

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