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Investigation of antioxidant and anticholinesterase activity of *Hypericum Perforatum* L. extracts

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Abstract

Hypericum perforatum L. is widely known for its therapeutic properties, including wound healing, antispasmodic effects, and anxiety treatment. This study was examined the total phenolic and flavonoid content, antioxidant capacity, and anticholinesterase activity of aqueous, ethanolic, and water-ethanol extracts from the plant's aerial parts and also green chemistry and sustainability were also investigated. Extraction was performed under reflux at 60°C. The anticholinesterase effect of *Hyperium perforatum* L., which is known to be used in the treatment of neurological diseases such as coxalgia, paralysis, spastic paralysis, menopausal neurosis, spinal convulsion etc., was investigated *in vitro*. Three different extracts were prepared as 100% ethanol, 50% ethanol-50% water and 100% water. The antioxidant activity of the prepared extracts was examined using Folin-Ciocalteu in terms of total phenolic-flavanoid content and then the anticholinesterase activity of extracts was investigated by Ellman method. LC-HR-MS identified Rhamnocitrin as the most abundant compound in the water-ethanol extract, while Quercitrin and Hederagenin were descent in the ethanol extract. The ethanol extract demonstrated the highest total flavonoid content and enzyme inhibition rates, especially for AChE (65%) and BChE (75%). In contrast, the water extract had the highest phenolic content but lower enzyme inhibition, especially for BChE (16%). Greenness metrics analysis demonstrated superior recyclability for the aqueous extract, earning it the highest score in sustainability. In general, the ethanol extract exhibited the most potent biological activity, while the aqueous extract excelled in green chemistry principles. These findings support the potential of *H. perforatum* extracts for further development in natural product research.

Keywords: Chlorine dioxide, catalysts, oxidation agent, aromatic aldehydes, aromatic acids

1. Introduction

Hypericum perforatum L. (commonly known as St. John's Wort) is a species in the genus *Hypericum* and family Hypericacecae, which includes 400 species worldwide [1]. *H. perforatum* is a plant native to Europe, North Africa, and Western Asia; however, due to its cultivation as a medicinal or ornamental plant, it is now prevalent in temperate regions such as Australia, India, New Zealand, South Africa, and South America [2]. *H. perforatum*, which grows abundantly and wild throughout most of Türkiye, is known by various names in our country, including "Sarı Kantaron, Binbir Delik Otu, Yara Otu, Kanotu, Mayasıl otu, Kuzukıran" [3]. In Türkiye, there are 119 taxa, 49 of which are endemic [4]. Consumption of *H. perforatum* derived products has risen sharply, making it one of the most widely used medicinal plants worldwide [5]. Its medicinal uses include treating skin wounds, eczema, burns, digestive issues, and psychological disorders [6]. The plant's aboveground parts, branches, leaves, flowers, fruits, and seeds are used internally as an appetite, stimulant, sedative, antispasmodic, and deworming agent. It is also used to treat diarrhea, stomach ulcers, abdominal pain, urinary tract infections, prostate issues, colds, and coughs, and as an expectorant. Externally, it is reported to be used for treating wounds, burns, oral infections, and mouth sores [7]. The aboveground parts of *Hypericum perforatum* possess antidepressant properties due to hypericin; sedative, anti-inflammatory, and analgesic effects due to biflavonoids and hyperforin; and diuretic and astringent actions attributed to flavonoids and tannins. Its volatile oil has antiphlogistic properties. Internally, it is used for treating moderate depression,

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anxiety, and nervous disorders, particularly menopause-related anxiety and stress relief. Externally, extracts prepared with herbal oils are applied in the treatment of wounds, burns, and minor cuts [8].

In the aerial parts of the plant, there are 0.05-0.3% naphthodianthrone derivatives (hypericin, pseudohypericin, isohypericin), 2.5% flavonoids (hyperoside, rutin, quercitrin, isoquercitrin, quercetin, kaempferol), about 0.26% biflavonoids (biapigenin), 2-4.5% phloroglucinols (hyperforin, adhyperforin), 0.05-0.3% volatile oils (*n*-alkanes, monoterpenes), 6.5-15% catechin and condensed tannins (catechin, epicatechin, leucocyanidin), phenolic acids (caffeic, chlorogenic, ferulic), β-sitosterol, xanthones (1,3,6,7-tetrahydroxyxanthone), phenylpropanoids, and vitamins A and C [9].

Numerous studies have highlighted the therapeutic potential of *H. perforatum* due to its diverse biological activities. Research has shown that extracts of *H. perforatum* possess significant antioxidant properties, which can decrease oxidative stress and reduce cellular damage [10]. Furthermore, the anticholinesterase activity of *H. perforatum* has been extensively documented, indicating its potential in treating neurodegenerative diseases such as Alzheimer's. A study by [11] revealed that specific extracts inhibited acetylcholinesterase activity, thereby enhancing cholinergic neurotransmission. Additionally, *H. perforatum* exhibits antimicrobial properties, as evidenced by [12], who reported its effectiveness against various pathogens, including bacteria and fungi. Furthermore, this plant is renowned for its wide range of biological activities, including antitumor [13], cytotoxic [14], anticancer [15], antiproliferative [16], anti inflammatory [17], leishmanicidal activity [18], antibacterial [19], neuroprotective activity [20], antifungal [21] features. These findings collectively underscore the significance of *H. perforatum* in traditional and modern medicine, emphasizing its role as a natural source of bioactive compounds with multiple health benefits.

In this study, we introduced a novel extraction method for *H. perforatum*, emphasizing its significance as the first documented approach in the literature. Our method involved water bath extraction at 60°C using three different solvents: ethanol, water, and a (1:1) mixture of ethanol and water. This innovative approach allowed for a comprehensive assessment of the extracts, as we performed LC-HR/MS (Liquid Chromatography-High Resolution Mass Spectrometry) analysis to quantify phenolic content. Additionally, we evaluated the total phenolic and total flavonoid content, along with the anticholinesterase activity in these extracts. While previous studies have typically utilized methanol, ethyl acetate, or water extraction via maceration and focused primarily on antioxidant activities [12] or anticholinesterase effects [11], our research uniquely combines temperature-controlled extraction with varied solvent ratios. This not only enhances the extraction efficiency but also presents a more holistic view of the antioxidant and anticholinesterase activities of *H. perforatum.* The importance of green chemistry is increasing day by day. Based on this, we also worked on green chemistry and sustainability for extraction methods via AGREE: Analytical Greenness Calculator software. There are 12 metrics related to "green chemistry principles". Based on minimize waste, analyst safety, energy consumption, renewebl materal etc., it gives a score between 0 to 1. We used AGREE for calculating different type of solvents in this extraction techniques. It gave a chance to compare these three different solvents in this extraction technique.

2. Experimental

2.1. Chemicals and reagents

The chemicals used in the experiment and their sources of supply are listed below:

Quercetin (Merck, Germany), pirocatechol (Merck, Germany), DTNB (5,5-dithiobis-(2-nitrobenzoic acid)) (Merck, Germany), acetylcholinesterase (Merck, Germany), butyrylcholinesterase (Merck, Germany), chloroform (Merck, Germany), dichlorometane (Merck, Germany), methanol (Merck, Germany), ethanol (Merck, Germany), aluminum nitrate (Merck, Germany), potassium acetate (Merck, Germany), galantamine hydrobromide (Sigma Aldrich, Germany), acetylthiocholine iodide (Sigma Aldrich, Germany), Folin-Ciocalteu reagent (Merck, Applichem, Germany), butyrylthiocholine iodide (Merck, Fluka, Germany), sodium carbonate (Merck, Germany), ammonium acetate (Merck, Germany), sodium hydrogen phosphate (Merck, Germany), sodium dihydrogen phosphate (Merck, Germany)

2.2. Instrumentations and analytical conditions

The Thermo LC system consisted of a Thermo Orbitrap Q-Exactive HRMS equipped with electrospray ion (ESI) source were used for the analysis (Thermo, USA). Data acquisition were performed on TRACE FINDER software. The chromatographic separations was performed on C18 (3µm*150mm*3.00 mm; Fortis) analytical column. The mobile phases were prepared including %0.1 formic acid water (A) and methanol (B). The flow rate for the analysis was determined as 0.35 mL/min and the elution gradient given in the Table 1 was used.

Table 1. Gradient elution

Gradient Time (min)	Flow rate (mL/min)	% B (mobile phase)
0.00	0.35	50
1.00	0.35	50
3.00	0.35	100
6.00	0.35	100
7.00	0.35	50
10.00	0.35	50

The compounds of each extract were ionized with the help of ESI in positive and negative mode. MS parameters were set as; sheath gas flow rate 45 L/min, auxillary gas flow rate 10 L/min, sprey voltage 3.8 kV, capillary temperature 320 °C, and auxillary gas heater temperature 320 °C [22,23].

2.3. Plant material

H. perforatum was collected from Irvindi-Balıkesir in June 2020 and identified by Cagla Kızılarslan Hancer. It was stored in the herbarium of the Faculty of Pharmacy of Istanbul University (ISTE no: 117.295).

2.4. Extraction method

The herba of *H. perfloratum* L. of 1 kg of dried plant was turned into powder in a blender. It was divided into three equal parts, each weighing approximately 250 g. Three separates extracts were prepared: ethanol, water, and water:ethanol (50:50; v/v) using water bath extraction at 60°C.

2.4.1. Preparation of ethanol extracts (HE):

250 g of plant was placed in a volumetric flask. 500 mL of ethanol was added. Extracted under reflux into volumetric flasks for two hours (Fig. 1). After that, it was cooled to room temperature and filtered by filter paper. The remaining ethanol was evaporated by rotary evaporator. Plant extract was stored -80°C until the analysis.

2.4.2. Preparation of water extracts (HS):

Approximately 245 g of plant was placed in volumetric flask and 600 mL of water was added. Extracted under reflux for three hours. It was cooled to room temperature and filtered via filter paper. Then, the extract was divided into four equal parts again and 700 mL of water was added. After waiting in the water bath for two hours, it was removed from the water by aid of rotary evaporator. Plant extracts were stored -80°C until the analysis.

2.4.3. Preparation of water:ethanol extracts (HSE):

250 g of plants weighed and 600 mL of mixture of ethanol:water (50:50; v/v) was added. The extract was kept under reflux for two hours and allowed to cool. When it reached room temperature, it was filtered with the help of filter paper. The remaining solvent was removed by rotary evaporator. It was stored -80°C until the analysis.

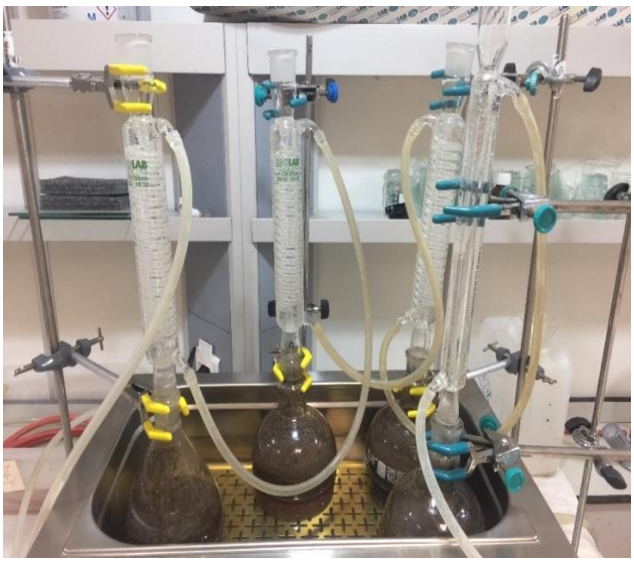


Figure 1. Water bath extraction at 60°C

2.5. Investigation of total phenolic content

The Folin-Ciocalteau method, which is commonly used for the determination of total phenolic compounds, is based on the absorbance measurement according to the color intensity formed by the Folin-Ciocalteau reagent, which gives its name to the method. Pyrocatechol (o-Benzenediol) was used as the standard phenolic compound. As a result of the analysis, a high absorbance value is an indicator of a high amount of phenolic substance [24]. The total phenolic content of the all extracts was determined using the Folin-Ciocalteau reagent as equivalent to pyrocatechol [25]. Pyrocatechol stock solution was prepared at a concentration of 100 ppm. 0,1,2,3,4,5,6,7, and 8 µL of the stock solution were taken, respectively, and their volumes were completed to 184 µL with distilled water. Solutions were prepared from samples taken from aqueous, aqueous:ethanol (50:50; v/v) and ethanolic extracts obtained from the *Hypericum perforatum* L. at a concentration of 1000 ppm. 4 µL of sample solutions including 1 mg of extract were taken and completed to 184 µL with distilled water. 4 µL of FCR reagent was added to the pyrocatechol solutions and samples, and after waiting for 3 min, 12 µL of 2% Na_2CO_3 solution was added. The mixture was left at room temperature for 2 hours and the absorbance values of the samples were measured at 760 nm. Total phenolic contents of the extracts were determined using the following equation obtained from the calibration curve of standard pyrocatechol (Eq 1.). All the samples were measured triplicates.

$$Absorbance = 0.0286\ (\mu g) - 0.0733 \quad (1)$$
$$(R^2 = 0.9953)$$

2.6. Investigation of total flavonoid content

Flavonoids are phenolic compounds with a diphenylpropane structure, which consist of two phenyl

rings combined with a propane chain. Quercetin was used as the standard flavonoid compound.

Total flavonoid contents of the obtained extracts were determined as quercetin equivalents by the aluminum nitrate method [26]. Stock solution of quercetin was prepared at a concentration of 100 ppm. 0,1,2,3,4,5,6,7, and 8 uL were taken from the stock solution and the volumes of each were completed to 192 µL with 80% ethanol by volume. 4 µL of 1 M potassium acetate was added and waiting for 1 min. Then, 4 uL of 10% aluminum nitrate was added. After incubation for 40 minutes, absorbance were measured at 415 nm. Solutions were prepared from samples taken from all extracts of *Hypericum perforatum* L. at a concentration of 1000 ppm. The absorbance of 1000 ppm solutions taken from each extract were measured at 415 nm. Total flavonoid contents of the extracts were determined using the following equation obtained from the calibration curve of standard quercetin (Eq 2.). All the samples were measured triplicates.

$$Absorbance = 0.0394\ (\mu g) - 0.0471 \quad (R^2 = 0.9959) \qquad (2)$$

2.7. Activity of anti-cholinesterase

The Ellman method, also known as a spectrophotometric method, is a method used to measure the inhibitory activities of acetylcholinesterase and butyrylcholinesterase enzymes for the determination of anticholinesterase activity [27]. The principle of this method is based on the *in vitro* hydrolysis of acetylcholine by acetylcholinesterase or butyrylcholinesterase to be followed [28]. This method, a colorimetric method in which acetylcholine is cleaved to thiocholine by AChE and then reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to give the yellow 5-thio-2-nitrobenzoate anion, was performed in 96-well microplates.

Ellman Method

Acetylcholinesterase obtained from electric fish and butyrylcholinesterase obtained from horse serum were used as enzymes, and acetylcholine iodide and butyrylcholine iodide were used as substrates in Ellman method. DTNB, which is yellow in color, was used to measure the inhibitory activity of acetylcholinesterase and butyrylcholinesterase enzymes. 130 µL of phospate buffer with pH:8 was added to each well of 96-well microplates, 10 µL of the solutions of extracts prepared at a concentration of 4000 ppm in ethanol, and 20 µL of BChE solution was added to the AChE and other samples in microplates. It was incubated at 25°C for 10 minutes. Then, 20 µL of DTNB solution and 20 µL of acetylcholine iodide and/or butyrylcholine iodide as substrate were added. The 5-thio-2-nitrobenzoic acid anion, which was formed as a result of the reaction of thiocholine with DTNB, which was released by the enzymatic hydrolysis of acetylcholine iodide and butyrylcholine iodide on the other microplate samples, respectively, was examined spectrophotometrically at 412 nm. Ethanol was used as a control and galantamine was used as standard. AChE and BChE activity (% inhibition) was calculated using the following equation (Eq.3). All the samples were measured triplicates.

$$\%\ Inhibition = \frac{(\text{Control} - \text{Sample})}{\text{Control}} x\ 100 \qquad (3)$$

2.8. Assesment of the greenness

With technological developments, new studies carried out in the chemical and pharmaceutical sectors, reducing the scale, and thus minimizing the damage to the environment has become an important criterion. In recent years, researchers have been working to prevent environmental pollution and develop eco-friendly analytical methods. The newly developed 'eco-friendly' analytical methods aim to keep the amount of solvent and other chemicals consumed at a minimum level to minimize the irritating, toxic, corrosive and pollution effects of them. In addition, reducing the energy consume in analyses is possible by reducing the number of process steps for analysis. One of the points that the 'green chemistry' principle attaches importance to is automation. As the method of working with the automation system increases, the analyst's safety will increase, and his/her effort will decrease. In summary, green chemistry principles prioritize the required automation system, using recyclable materials, minimization of polluting solvents, other chemicals and waste, reduction of energy consumption, and analyst safety.

There are some analytical green calculation (greenness) programs prepared by considering the principles of green chemistry. Wojnowski et al. developed the software AGREE: Analytical Greenness Calculator to assess the environmental and occupational hazards associated with a given analytical procedure based on 12 principles [29]. The score obtained from these metrics is converted into a graph showing extent to which each of the 12 principles is complied with this program. The score between 0 to 1 obtained from each principle visually represents the extent to which the green chemistry principle is complied with in which steps with the colors green, yellow and red [30].

2.9. Statistical calculations

The activity results are demonstrated as the mean ± standard deviation from three independent tests. The

results were found to be within the 95% confidence interval relied on the Student's t-test. Measurement curves were plotted between absorbance and concentration, and the corresponding regression equations were determined. Linear regression analysis, based on the least squares method, was carried out by evaluating the slope, intercept, and correlation coefficients.

3. Results and discussion

3.1. Results of total phenolic and total flavonoid quantifications in extracts

The total phenolic content of the all extracts were determined by taking pyrocatechol as the standard (Fig. 2).

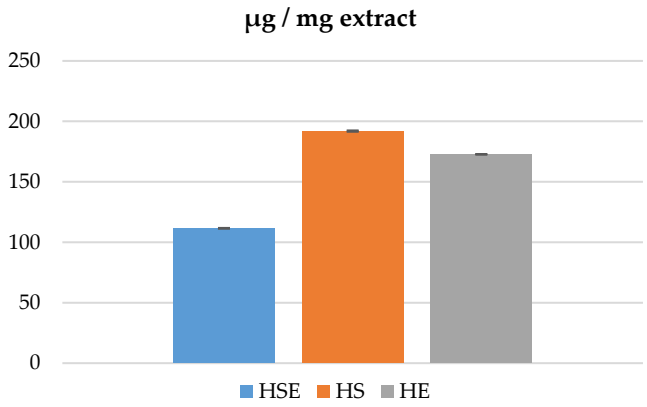


Figure 2. Total phenolic contents of extract of the water:ethanol (HSE), aqueous (HS), and ethanol (HE)

The total phenolic content of the all extracts were determined by taking quercetine as the standard (Fig. 3).

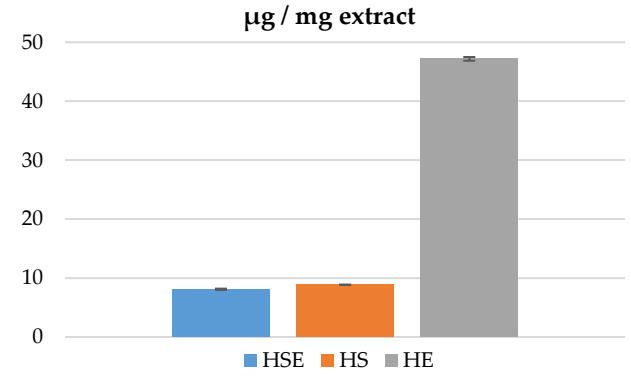
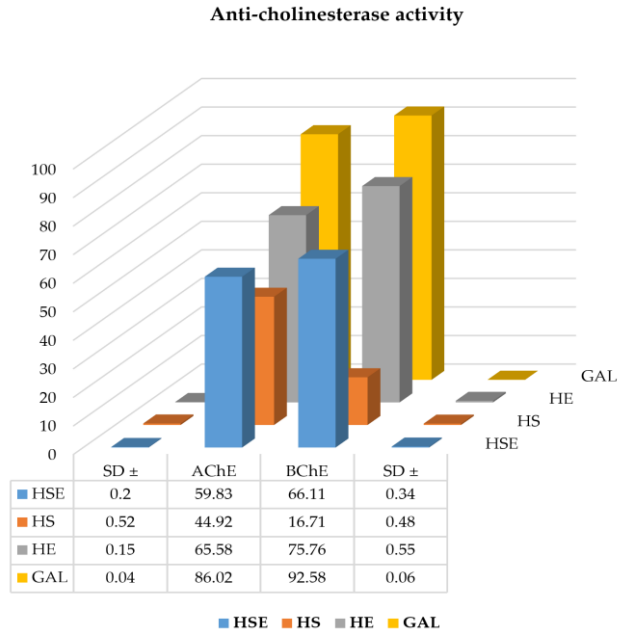


Figure 3. Total flavonoid contents of extract of the water:ethanol (HSE), aqueous (HS), and ethanol (HE)

Based on the results, the water extract was the highest total phenolic content, followed by the ethanol extract, while the water-ethanol extract was the lowest phenolic content. With regards to total flavonoid content, the ethanol extract was the richest, followed by the water extract, with the water-ethanol extract showing the lowest flavonoid level. In general, the ethanol extract was found to be rich in both phenolic and flavonoid content.

3.2. Results of anti-cholinesterase activity

Anticholinesterase activities of all extracts were determined by inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes. Galantamine was used as a standard. The results are shown in Fig. 4.



	SD ±	AChE	BChE	SD ±
HSE	0.2	59.83	66.11	0.34
HS	0.52	44.92	16.71	0.48
HE	0.15	65.58	75.76	0.55
GAL	0.04	86.02	92.58	0.06

Figure 4. Inhibition of AChE and BChE enzymes by extracts of the water:ethanol (HSE), aqueous (HS), and ethanol (HE), and its standard deviation values (SD)

When evaluating anticholinesterase activity, the ethanol extract showed the highest inhibition rate at 65% for AChE enzyme inhibition, followed by the water-ethanol extract with 59% inhibition. The aqueous extract demonstrated an enzyme inhibition rate of approximately 44%. For BChE enzyme inhibition, the ethanol extract again led with a 75% inhibition rate, while the water-ethanol extract exhibited 66% inhibition. The lowest inhibition rate, at 16%, was sighted in the aqueous extract. Generally, the ethanol extract presented a high inhibition rate for both enzymes.

3.3. Results of LC-HR/MS

Calibration equation, R^2 value, relative uncertainty ratio, linear range, LOD-LOQ values, relative standard deviation and recovery of 12 phenolic compounds in all extracts are shown. (Table 2). According to LC-HR/MS analysis of 3 different extracts, we obtained 12 major phenolic compounds (Table 3).

In the LC-HR-MS analysis, the water-ethanol extract demonstrated the highest concentration of Rhamnocitrin at 0.343 µg/mL, followed by Naringenin (0.169 µg/mL), and Kaempferol (0.123 µg/mL).

Table 2. All compounds were determined of all extracts by LC-HR/MS

COMPOUNDS	Relative Uncertainity (%)	Molecular Formula	m/z	Ionization	Lineer Range (µg/mL)	Lineer Regration Equation	LOD/ LOQ	R^2	Recovery	RSD (%)
Apigenin 7-glucoside	3.59	$C_{21}H_{20}O_{10}$	431.0984	Neg	0.3-7	y=0.0246x+0.00306	0.01/0.03	0.9962	96.07	4.61
Ellagic acid	4.20	$C_{14}H_6O_8$	300.9990	Neg	0.05-10	y=0.0085x-0.000612	0.3/1.0	0.9994	101.49	3.90
Quercitrin	3.78	$C_{21}H_{20}O_{11}$	447.0933	Neg	0.05-10	y=0.0179x-0.0003331	0.01/0.03	0.9990	97.00	4.76
Nepetin	2.19	$C_{16}H_{12}O_7$	315.0510	Neg	0.05-10	y=0.0853x+0.00269	0.01/0.03	0.9992	97.76	3.70
Isosakuranetine	3.98	$C_{16}H_{14}O_5$	285.0769	Neg	0.05-10	y=0.0235x+0.000561	0.01/0.03	0.9992	96.56	3.70
Nepetin-7-glucoside	3.07	$C_{22}H_{22}O_{12}$	479.1184	Pos	0.05-10	y=0.00629x-0.0001951	0.01/0.03	0.9997	102.18	3.51
Rhamnocitrin	3.16	$C_{16}H_{12}O_6$	301.0707	Pos	0.05-10	y=0.03122x-0.002136	0.01/0.03	0.9995	103.58	3.27
Hederagenin	1.80	$C_{30}H_{48}O_4$	473.3625	Pos	0.5-10	y=0.00131x+0.00146	0.1/0.3	0.9948	97.49	1.84
Naringenin	4.20	$C_{15}H_{12}O_5$	271.0612	Neg	0.1-10	y=0.0281x+0.00182	0.01/0.03	0.9995	86.65	4.20
Luteolin	3.42	$C_{15}H_{10}O_6$	285.0405	Neg	0.1-10	y=0.117x+0.00848	0.01/0.03	0.9981	96.98	3.42
Hispidulin	3.41	$C_{16}H_{12}O_6$	301.0707	Pos	0.05-10	y=0.02614x+0.0003114	0.01/0.03	0.9993	98.36	3.41
Kaempferol	3.56	$C_{15}H_{10}O_6$	285.0405	Neg	0.5-7	y=0.0827x+0.00953	0.01/0.03	0.9958	90.25	3.58

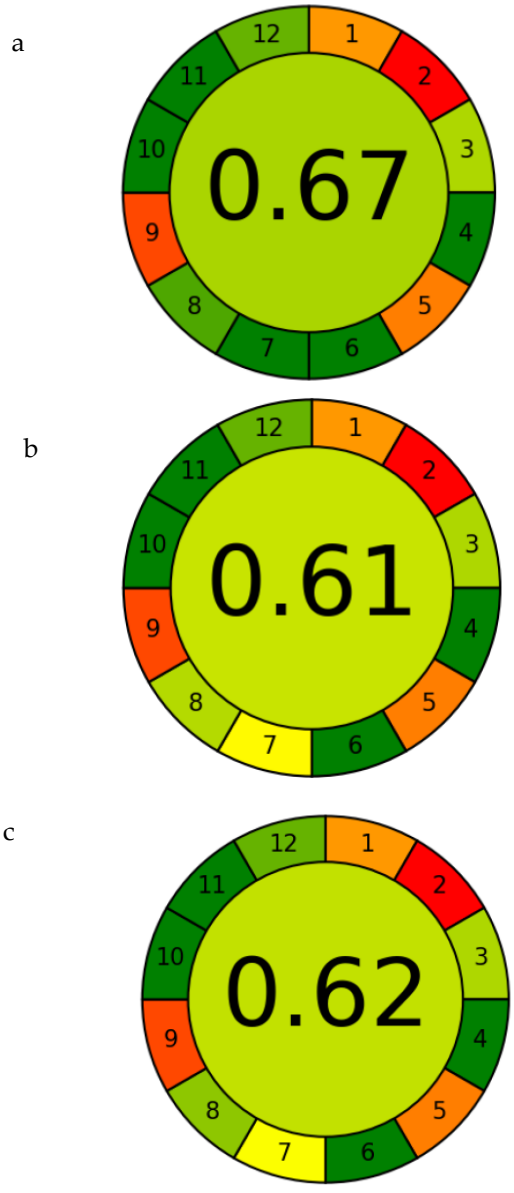


Figure 5. Greenness scores of all extracts by AGREE:Analytical Greennes Calculator software (a: HS, b:HSE, c:HE)

In the ethanol extract, Quercitrin was the most abundant compound at 0.333 µg/mL, followed by Hederagenin (0.183 µg/mL). The water extract included the highest levels of Quercitrin (0.302 µg/mL), whereas Nepetin (0.002 µg/mL) and Nepetin-7-glucoside (0.06 µg/mL) were found in the lowest amounts. Similarly, Nepetin was also the compound with the lowest concentration in the water-ethanol extract, with a value of 0.003 µg/mL.

3.4. Results of greenness

The results of greenness metrics were presented as below (Fig. 5). The software was evaluated on 12 principles. The weight of score of each principles is not different. The results were obtained for all extracts between 0.61 to 0.67, in terms of examined acceptable green metrics. The highest score (0.67) obtained from aqueous extract. The results were calculated from 11 metrics were same for the each extract, however, only the score of one criteria (criteria 7) was different. This criteria rely on the consumption and recycle of analytical waste. According to this, the extraction methods require large volume of solvent. In this technique, we used large amount of water, water:ethanol, and ethanol, respectively. Accordingly, compare to other solvents, water is more recyclable. Therefore, grenness score of aqueous extract is better than ethanolic and water:ethanolic extracts.

Table 3. 12 phenolic compounds determined in the all extracts by LC-HR/MS

COMPOUNDS	HE (µg/mL)	HS (µg/mL)	HSE (µg/mL)
Apigenin 7-glucoside	0.097	0.083	—
Ellagic Acid	—	0.073	—
Quercitrin	0.333	0.302	—
Naringenin	0.074	0.072	0.169
Nepetin	—	0.002	0.003
Hispidulin	—	0.013	0.054
Luteolin	0.065	—	—
Isosakuranetin	0.069	0.072	0.121
Nepetin-7-glucoside	—	0.06	—
Rhamnocitrin	—	—	0.343
Hederagenin	0.183	0.092	—
Kaempferol	—	—	0.123

Conclusion

In this study, the total phenolic-flavonoid capacities and the total phenolic content of three different extracts from *Hypericum perforatum* L. were investigated using LC-HR/MS, and their anticholinesterase activity was analyzed. Additionally, the sustainability of the extraction process was evaluated through green chemistry analysis of the solvents and extraction methods, using the AGREE: Analytical Greenness Calculator program.

The plant is widely used in Europe to treat anxiety and is also known for its various effects, including wound healing and antispasmodic properties. *H. perforatum* oil is commonly used for wound healing. Its tea has been shown to aid in the treatment of anxiety disorders. The plant is also existing in capsule form and has recently gained attention in the cosmetic industry due to its antioxidant effects. The naphthodianthrone derivatives, flavonoids, biflavonoids, and phloroglucinols it includes are potent chemical compounds with strong antioxidant properties. The aerial parts of *H. perforatum* L. were extracted using 100% water, a 50% ethanol-50% water mixture and 100% ethanol under reflux in a water bath at 60°C. The antioxidant properties and total phenolic-flavonoid content of the resulting extracts were examined using the Folin-Ciocalteu method, while their anticholinesterase activity was analyzed using the Ellman method. LC-HR-MS analysis disclosed that the water-ethanol extract contained the highest concentration of Rhamnocitrin, while the ethanol extract was rich in Quercitrin and Hederagenin. The water extract also indicated high Quercitrin levels, but Nepetin and Nepetin-7-glucoside were present in the lowest amounts across all extracts. The ethanol extract showed the highest levels of total flavonoid content and strong inhibition rates for both AChE (65%) and BChE (75%) enzymes. In spite of the water extract had the highest total phenolic content, its enzyme inhibition rates were lower, especially for BChE (16%). The water-ethanol extract demonstrated moderate phenolic and flavonoid content and intermediate enzyme inhibition. Overall, the ethanol extract exhibited the most potent activity across the assays. The greenness metrics analysis revealed that the aqueous extract achieved the highest score due to its superior recyclability compared to ethanol and water-ethanol extracts. Although most metrics were consistent across extracts, the aqueous extract's better performance in waste consumption and solvent recyclability resulted in a higher overall greenness score.

In conclusion, the results align well, showing that the ethanol extract exhibits strong antioxidant capacity and anticholinesterase activity. Continued chemical and biological studies on *Hypericum* species will be valuable for the discovery of natural bioactive compounds, contributing to the scientific field.

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