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A comparative exploration of the phytochemical profiles and bio-pharmaceutical potential of *Helichrysum stoechas* subsp. *barrelieri* extracts obtained *via* five extraction techniques

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A comparative exploration of the phytochemical profiles and bio-pharmaceutical potential of *Helichrysum stoechas* subsp. *barrelieri* extracts obtained *via* five extraction techniques

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Graphical_abstract

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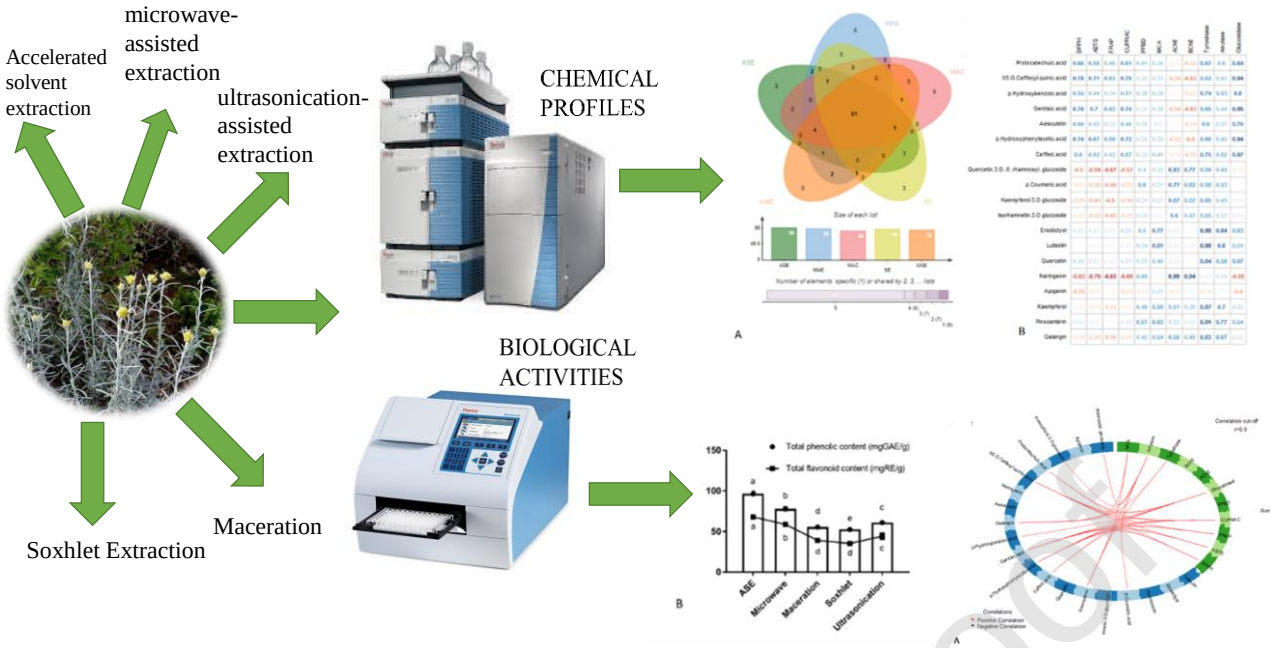

Highlights

- Biological and phytochemical profiles of *Helichrysum stoechas* subsp. *barrelieri* extracts were investigated
- Types of extraction techniques were found to influence the biological activities of the extracts
- Quercetin was the main component in the tested extracts
- Accelerated solvent extraction yielded the best antioxidant ability
- Results from this study could open new insights for designing novel pharmaceuticals

Abstract

We endeavoured to probe into and compare the possible effect(s) of different extraction techniques (accelerated solvent extraction (ASE), microwave-assisted extraction (MAE), ultrasonication-assisted extraction (UAE), maceration, and Soxhlet extraction (SE)) on the bioactivity (antioxidant and enzyme inhibitory activities) of the aerial parts of *Helichrysum stoechas* subsp. *barrelieri* (Ten.) Nyman. Total phenolic and flavonoid contents of the extracts obtained by different extraction methods followed the order of ASE>MAE>UAE>maceration>SE. Extract obtained by ASE was the most potent radical scavenger (219.92 and 313.12 mg Trolox equivalent [TE]/g, against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), respectively) and reducing agent (927.39 and 662.87 mg TE/g, for cupric reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant power (FRAP), respectively). *Helichrysum*

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stoechas extract obtained by UAE (18.67 mg ethylenediaminetetraacetic equivalent [EDTAE]/g) was the most active metal chelator and inhibitor of acetylcholinesterase (4.23 mg galantamine equivalent [GALAE]/g) and butyrylcholinesterase (6.05 mg GALAE/g) cholinesterase. Extract from maceration (183.32 mg kojic acid equivalent [KAE]/g) was most active against tyrosinase while ASE extract (1.66 mmol acarbose equivalent [ACAE]/g) effectively inhibited α-glucosidase. In conclusion, data amassed herein tend to advocate for the use of non-conventional extraction techniques, namely ASE and UAE, for the extraction of bioactive secondary metabolites from *H. stoechas* aerial parts.

Keywords: *Helichrysum stoechas*; antioxidant; enzyme inhibition; multivariate analysis; bioactive products

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1. Introduction

The origin of the word *Helichrysum* genus comes from the ancient words "helios" (sun) and "chrysos" (gold) and reflects the intensively yellow colour of most flowers of this genus. The *Helichrysum* genus (Asteraceae) comprises approximately 600 species widely distributed in the southern regions of the world [1]. "*Historia Plantarum*", one of the most important records of natural history written between the 2nd and 3rd century B.C. by the Greek Theophrastus of Eresos, described the use of *Helichrysum* genus for curative purposes. In addition, members of *Helichyrsum* genus have been documented to be used against snake bite and to treat burns. [2]. "*De Materia Medica*" written by Pedanius Dioscorides reported the application of decoction of floral filaments of *Helichrysum* in wine against different inflammatory complications related to snake bites, sciatica, urinary tract and hernias [3]. Later during Renaissance, the Dutch botanist Herman Boerhaave reported the use of herbs from this genus in

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South Africa for the treatment of hysteria and nervousness [4]. However, though medicinally used in many countries, there still a paucity of scientific information to validate such traditionally uses [2].

Helichrysum stoechas subsp. *barrelieri*, commonly known as everlasting, has a long traditional use in several cultures across the word.Ethnobotanical survey in the north-east of Portugal revealed that decoctions of *H. stoechas* have been used against cold, bronchitis, and fever [5]. In the Spanish folk medicine, *H. stoechas* is used for mitigation of inflammatory complications, for wound healing , to soothe toothache, manage urologic and digestive disorders [2]. Other traditional medicinal uses of *H. stoechas* includes the treatment of influenza, nervousness, and pancreatic problems [6]. Scientific studies have focused on the biological potential *H. stoechas* and reported its anti-α-glucosidase, anti-tyrosinase, anti-acetylcholinesterase, anti-dipeptidyl peptidase-4, and antioxidant activities [6]. Hydroalcoholic extract of *H. stoechas* has been identified as promising candidates for the cosmetic industry. Hydroalcoholic extract of *H. stoechas*, rich in 3,5-*O*-dicaffeoylquinic acid and myricetin *O*-acetylhexoside antioxidant properties, was successfully used for the development of polycaprolactone based microspheres which were incorporated into a moisturizer [5]. The ethanolic extract of *H. stoechas* exhibited analgesic effect *in vivo* [7]. Dichloromethane extract of *H. stoechas* aerial part demonstrated antibacterial (*Staphylococcus aureus* and *Mycobacterium phlei)* and antifungal (*Candida albicans)* properties. Arzanol, α-pyrone, helipyrone, *p*-hydroxybenzoic, caffeic acid, neochlorogenic acid, 5,7-dihydroxy-3,6,8-trimethoxyflavone, isoquercitrinand, quercetagetin-7-O-glucopyranoside, and santinol B have been isolated from the

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methanolic extracts of *H. stoechas*. [6]. The capitula of *H. stoechas* extracted with 70% ethanol () showed significant amounts of phenolic acids (chlorogenic and quinic acid and their derivatives) as well as other polyphenols such as quercetin, kaempferol, apigenin glucosides, and tetrahydroxychalcone-glucoside [8].

Increased concerns about the negative impact of chemicals on the environment has resulted in a paradigm shift whereby more ecologically friendly approach are been being favored. Tremendous efforts are being made to apply the principle of 'green chemistry and technology' in the area of phytochemistry and drug development from natural products. Scientists are investigating the effectiveness of novel extraction methods on the bioactivity of natural compounds compared to well-known and widely used conventional unsustainable and environmentally unfriendly extraction techniques [9, 10]. Recently, several scientific studies have reported multiple bioactivities of *H.stoechas*. However, the possible effects of extraction techniques on extraction of bioactive secondary metabolites from *H. stoechas* have not been explored.. Additionally, there are no reported studies in terms of assessment of extraction conditions on bioactive properties of obtained extracts. Therefore, the present study sets out to employ conventional and non-conventional extraction techniques (maceration and soxhlet extraction (SE), accelerated solvent extraction (ASE), microwave-assisted extraction (MAE), and ultrasonication-assisted extraction (UAE)) to extract bioactive compounds from *H. stoechas* aerial parts. Besides, the antioxidant and enzyme inhibitory activities of the different extracts will be gauged using standard *in vitro* bio-assays.

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2. Materials and Methods

2.1. Collection of plant material

Helichyrsum stoechas was cultivated at the Mugla area in Turkey and was collected during late spring in 2017. Identification and conformation of plant material as well as issuing of voucher specimen (MARE-19324) was done by botanist Dr. Gizem Bulut from the Marmara University (Istanbul, Turkey). Naturally dried plant material (aerial parts as mix) was minced and stored in the dark at the room temperature.

2.2. Extraction techniques

In order to get detailed insight in extraction influence on bioactives isolation from *H. stoechas*, five different techniques were applied. All extractions were performed with ethanol as a solvent, and except for the accelerated solvent extraction (ASE), in all other cases the plant:solvent ratio was 1:20. Microwave assisted extraction (MAE) was performed for half an hour at the microwave power of 600 W. Sonication of plant-ethanol mixture was done in ultrasonic bath for an hour at room temperature. For ASE, 1g of the samples was placed into an extraction cell together with diatomic earth and extracted with ethanol (20 mL) at 120 °C /1500 psi for 6 min. The process was performed in ASE 350 system Dionex Corporation (Sunnyvale, CA, USA). Traditional techniques (maceration and soxhlet extraction (SE)) were performed as prescribed by the pharmacopoeia. Briefly, plant materials (5 g) were stirred with ethanol (100 ml) for 24 h at the room temperature in maceration technique. In SE, the plant materials (5 g) were extracted with ethanol (100 ml) by using a soxhlet apparatus for 6 h. Obtained extracts were converted in a dry form by using vacuum evaporator and stored in a refrigerator.

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2.3. Assays for total phenolic and flavonoid contents

The content of two major groups of bioactives (phenols-TPC and flavonoids-TFC) in obtained extracts was determined spectrophotometrically using appropriate Folin-Ciocalteu and aluminium chloride methods [11]. Expression of obtained results was done by equivalents of standards - gallic acid (in case of TPC) and rutin (in case of TFC).

2.4. Chemical profiling

Separation, identification and quantification of polyphenols in tested extracts were done by using an UHPLC system (Accela 600) together with LTQ OrbiTrap MS (ThermoFisher Scientific, Bremen, Germany). All the method details were given in previous work [12]. Quantification was done using high resolution mass spectrometry (HRMS) method by comparison of exact masses and retention times of investigated compounds with available standards. Tentative identification of compounds was done by high resolution mass spectrometry (HRMS) and MS^n fragmentation using appropriate literature [8, 13-16].

2.5. Determination of antioxidant and enzyme inhibitory effects

To provide acomprehensive insights into bio-potential of the extracts and influence of extraction techniques on their bioactivity, the antioxidant, anti-α-amylase, anti-α-glucosidase, anti-cholinesterases, and anti-tyrosinase activities assays were performed. Estimation of anti-enzymatic activity of the extracts was done by *in vitro* assays previously described by Uysal et al. [11]. The data obtained by these assays

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were given as reference inhibitors equivalents: galantamine (GALAE) for acetylcholinesterase (AChE) and butrylcholinesterase (BChE), kojic acid (KAE) for tyrosinase, and acarbose (ACAE) for α-amylase and α-glucosidase. Measurement of the antioxidant and free radical scavenging properties of the extracts, ferric reducing antioxidant power (FRAP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), cupric reducing antioxidant capacity (CUPRAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), metal chelating and phosphomolybdenum tests were performed. The data were given as reference compounds (Trolox (TE) and ethylenediaminetetraacetic (EDTAE) equivalents). Detailed description of applied assays were given previously [11].

2.6. Statistical analysis

Any significantdifference ($p < 0.05$) between the extracts was calculated using the parametric One-way ANOVA test together with Tukey's test. Venn diagram analysis of identified phytochemicals in five methods of extraction was done with the online tool [17]. Then Pearson's coefficients were calculated between the studied biological activities and the quantified phytochemical content. Besides, the supervised modelling partial least squares discriminant analysis (PLS-DA) and heat map were applied to firstly explore the variation between the five techniques of extraction and secondly to identified the biological activities having the highest discrimination potential according to variable's important in projection (VIP score > 1.2). The R (v. 3.5.1.) packages mixOmics and XLSTAT v. 2018 software were used for all calculations.

3. Results and Discussion

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The total phenolic and flavonoid contents of the extracts of *H. stoechas* aerial parts obtained by different extraction techniques followed this order ASE > MAE > UAE > maceration > SE (Figure 1B). Previously it was reported that ASE was the most efficient method for the extraction of phenolics compared to shaking, vortex mixing, stirring, and sonication [18]. Accelerated solvent extraction involves the use of ordinary solvents under elevated temperature and pressure for extraction of bioactive compounds from plant materials. It has the key advantage of enhancing extraction, reducing extraction time, being reproducible, and require less solvent [19]. Moreover, in ASE all extraction conditions are completely monitored and controlled, thereby increasing the content of the desired compounds [20]. However, research conducted by Cai et al. [21], demonstrated that conventional extraction was more effective for the extraction of anthocyanins, a class of flavonoids, as compared to UAE and ASE. It is noteworthy to quote that conditions for conventional extraction from this study involved the application of heat under acidified conditions. In fact, acids are important for stabilizing the flavylium cation of anthocyanins [22] and might have contributed to the enhanced extraction of anthocyanins. Following ASE, MAE was the next technique which effectively extracted phenolics and flavonoids from *H. stoechas* aerial parts. Microwave-assisted extraction is regarded as a "green" extraction method as it involves the use of safe solvents such as water or ethanol. Additionally, it requires less solvent, shorter extraction time, thereby reducing energy consumption and also increases the extraction yield [23]. Due to the activity of microwaves, ionic conduction together with rotation of dipole molecules occur. During MAE, polar moleculesrotate in an attempt to align with the electric field, while the friction between the molecules

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generate heat energy, increasing medium temperature, causing cell lysis which facilitates extraction and increase mass diffusion, improving the extraction yield [24].

The UHPLC/MS characterization of phytochemicals in *H. stoechas* samples resulted in the detection of a total of 107 compounds (Table 1), of which 19 were confirmed using commercially available standards. Venn diagram showing the enrolment of overlapping and non-overlapping compounds between the five methods of extraction was performed (Figure 1A). It can be observed that a total of 81 compounds were involved in the overlap of the five methods of extraction. Nonetheless, the hydroxycinnamic acid (dicaffeoyl-succinylquinic acid) and the flavonoids (quercetagetin 3-*O*-(acetyl-hexosyl)-7-*O*-hexoside and quercetagetin 3-*O*-(coumaroyl)-hexoside isomer 2) were found exclusively in the ASE extracts. Similarly three hydroxycinnamic acids (dicaffeoyl-quinic acid isomer 2, dicaffeoyl-quinic acid hexoside isomer 2 and dicaffeoyl-shikimic acid) were identified only in the SE extracts. The largest number of identified compounds were derivatives of hydroxycinnamic acids (40 compounds), flavonoid aglycones and glycosides (55 compounds). Hydroxycinnamic acids were generally identified as esters with quinic acid and some were acylated with various carboxylic acids. As for flavonoids, quercetagetin derivatives were the most abundant, which has been reported to be common in *Helichrysum* spp [6]. Confirmation of quercetagetin in *H. stoechas* was done by high abundance of 167 *m/z* in MS spectra obtained by the specific retro Diels-Alder (RDA) fragmentation of flavonoids [25]. A large number of flavonoid glycosides were acylated with coumaroyl, malonyl or acetyl residue, and their identification and structure assignment is difficult due to the existence of many structural isomers that

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give identical MS spectra. Their proper identification requires isolation and characterization using advanced nuclear magnetic resonance (NMR) methods. Hence the exact position of the binding of these groups to glycoside is not indicated in the table. Glycosylation position (3-*O* or 7-*O*) was determined by the ratio of abundance of $[M–H]^-$ and the radical aglycone ions, which are very abundant for deprotonated flavonol 3-*O*-glycosides [26]. The exact position of the glycosylation can be assumed based on the relative intensity of the fragments, as each glycosylation site gives specific fragmentation at the optimum collision energy. Increasing collision energy yields fragments containing both the glycosidic moiety and the aglycone itself [23].

Four pyrone derivatives were identified in all five *H. stoechas* extracts. Some pyrone derivatives, previously isolated from *H. stoechas* showed antimicrobial properties [27]. In this study helipyrone (15.54 min and 319 *m/z*) was identified, as well as three structurally similar derivatives (Table 1).

Quantitative determination of 19 components (7 phenolic acids, 3 glucosides, 8 flavonoids and 1 coumarin derivative) was done using appropriate standards (Table 2). Information about calibration curves (regression equation parameters, correlation coefficient, limits of detection and quantification) are given in Table S1. Among these compounds, quercetin was dominant and its amount in the analyzed extracts was in the range from 285.50 mg/kg (in SE) to 1372.45 mg/kg (in MAE). On the other hand, low amounts of apigenin and naringenin were detected in *H. stoechas* extracts. Naringenin was in the range 0.851-3.949 mg/kg, while apigenin was detected in 3 of 5 analysed extracts. Generally, all extracts were much richer in phenolic acids and their extraction was enhanced by ASE and maceration as compared to other extraction

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techniques. Furthermore, it was observed that maceration enhanced glucosides extraction compared to the other studied extraction methods. In the case of quercetin 3-*O*-(6"-rhamnosyl)-glucoside its presence in MAE or ASE was not detected. This could be a consequence of high temperature which occurs in these processes. In case of aglycones, UAE was found to be the ideal extraction technique. Cavitation is the phenomenon which occurs during UAE. This process leads to high shear forces in the extraction media, then to macro-turbulences and to a micro mixing, thus improving extraction efficiency [28, 29]. The lowest extraction yield was observed for SE. Inferior extraction potential of SE in comparison to ASE or MAE has already been reported in the scientific literature [30].

The possible effect(s) of the selected extraction techniques on the antioxidant ability of *H. stoechas* was appraised using different *in vitro* assays (Table 3). The extract obtained by ASE was recognized as the most potent radical scavenger (219.92 and 313.12 mg TE/g, against DPPH and ABTS, respectively). This can be linked with the composition of the extract obtained by this technique. For instance, the extract obtained by ASE had the highest amount of 5-O-caffeoyl-quinic, p-hydroxybenzoic, gentisic, *p*-hydroxyphenylacetic and caffeic acids. In addition, to the above mentioned phenolic acids, the ASE extract contained the highest amount of aesculetin, eriodictyol and queretin which are well-known according to their antioxidant ability. The DPPH and ABTS assays are some of the common methods for evaluatingthe radical scavenging properties of natural compounds. In the presence of electron or hydrogen donating compounds, the stable DPPH radical decolorize by losing its purple colour [31]. The ABTS assay, regarded as a sensitive technique to assess antioxidant activity

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due to its rapid reaction kinetics [32], is based on the spectrophotometric monitoring of ABTS radical cation decay following the addition of antioxidants [33]. A group of researchers [34] described the possible mechanism for the scavenging of DPPH and ABTS (Figure 2) by chlorogenic acid identified from *H. stoechas* aerial part. As seen from Figure 2, the hydrogen atom of one hydroxyl group of caffeic acid is delocalised to stabilise free radical (DPPH or ABTS).

Likewise, extract of *H. stoechas* aerial part obtained by ASE showed highest reducing capacity (927.39 and 662.87 mg TE/g, for CUPRAC and FRAP, respectively). The CUPRAC method is based on the spectrophotometric measurement of copper (I)-neocuproine complex, as a consequence of the redox reaction of the antioxidant and copper (II)-neocuproine complex [35]. In the case of FRAP, reduction of Fe^{3+} to Fe^{2+}, in the presence of 2,4,6-trypyridyl-s-triazine, leads to the formation of an intense blue coloured complex [33, 36]. As shown in Figure 1C, DPPH, FRAP, CUPRAC and ABTS were correlated to phenolic and flavonoid contents, meaning that as phenolic and flavonoid content decreased the antioxidant activities of the extract was also reduced. This pattern was also reported in other studies [37, 38]. Moreover, as previously mentioned, phenolic acids such as gentisic, 5-*O*-caffeoyl-quinic, *p*-hydroxyphenylacetic and caffeic acids which were observed to be abundant in all extracts, seemed to be related to the antioxidant property of *H. stoechas* (Figure 3 A&B). Iron, a redox-active transition metal, catalyse several cellular reactions and is essential for the activity of iron and heme-containing proteins vital for life [39]. Iron toxicity relates to its role in the Fenton reaction [40], generating reactive oxygen species, namely hydroxyl radicals, which can damage lipids, proteins, and DNA, leading to

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possible mutagenic insult [41]. The present study showed that *H. stoechas* aerial part possessed metal chelating abilities. However, the chelating activity was dependant on the extraction method. Extract of aerial parts of *H.stoechas* obtained by UAE (18.67 mg EDTAE/g) was the most active metal chelator while the extract obtained by SE was the least active (2.91 mg EDTAE/g). Since the same solvent was employed for all extraction techniques, it might be argued that the extraction methods might have affected the extracted secondary metabolites. As opposed to SE, which is continuous extraction technique involving the use of heat, UAE was performed at lower temperature (30 °C). It might be suggested that the observed metal chelating activity was related to thermolabile secondary compounds which were degraded by the prolonged heating used for SE. It is noteworthy to mentionthat the other methods used, i.e., ASE (120 °C) and MAE (heating up of sample as a result of microwave radiation exposure) also involved high temperature, thereby explaining their lower activity. On the other hand, maceration is a non-cell disruptive method and thus limited the release of secondary compounds from the cell matrix. Similar results were obtained for some other plant matrices demonstrating the advantages of ASE over the other used extraction techniques [42].

Plant secondary metabolites possess a broad-spectrum of enzyme inhibitory capacity. Binding of the secondary metabolites to the enzymes, decreases their bioactivity, thereby modulating metabolic reactions. Interaction of plant secondary metabolites to enzymes can follow different kinetics, depending on the binding site of the inhibitor (secondary metabolite) to the enzyme [43-49]. The inhibitory activity of *H. stoechas* extracts on selected enzymes is summarized in Table 4. Extracts of *H.*

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stoechas, traditionally used against nervousness, inhibited cholinesterase enzymes. From Table 4, it is observed that the extract obtained by UAE was the most potent inhibitor of AChE (4.23 mg GALAE/g) and BChE (6.05 mg GALAE/g). As evidenced by several scientific reports, reduced level of acetylcholine in the brain has been associated to Alzheimer's disease (AD) [50]. The role of AChE in the pathogenesis of AD is clearly understood as compared to BChE. A group of researchers conducting experiments on rodents reported that brain-targeted BChE inhibitors increase acetylcholine levels and enhanced cognition in aged rats [51], supporting the role of BChE in cholinergic deficit.

A highest Pearson's coefficients between both cholinesterase enzymes and the flavanone compound naringenin was found (Figure 3A&B). In fact, the ability of naringenin to attenuate the impairment of learning and memory has been well ascertained [52]. The tested extracts also inhibited tyrosinase, a rate limiting enzyme responsible for the biosynthesis of melanin [53]. Tyrosinase inhibitors are claimed for the management of epidermal hyperpigmentation (excess of melanin) conditions, such as, freckles, melasma, and age spots. Data gathered revealed that *H. stoechas* extract obtained by maceration (183.32 mg KAE/g) was most active against tyrosinase. The ability of *H. stoechas* extracts to inhibit α-amylase and α-glucosidase, two enzymes targeted in the management of type II diabetes was also evaluated. *Helichrysum stoechas* extracts exhibited low inhibitory activity against α-amylase, with values ranging from 0.46 to 0.63 mmol ACAE/g. A more pronounced inhibition was observed against α-glucosidase, with values ranging from 1.59 to 1.66 mmol ACAE/g, the highest inhibition was recorded from the ASE extract. It is worth mentioning that the

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inhibition pattern for this enzyme was related to the phenolic/flavonoid content. In fact, the α-glucosidase inhibitory activity of *H. stoechas* was closely linked to phenolics and flavonoids content, as evidenced by the correlation coefficient of Pearson (Figure 1C). Besides, enzyme inhibition depends on the interaction of secondary metabolite(s) with the protein structure of the enzyme and thus depends on the type of inhibitor.

Univariate analysis provided further information on the relationship between the five extraction techniques and observed biological activities, taken independently. A significant difference between the extracts was obtained for all biological activities. Based upon this results obtained through the One-Way ANOVA and in order to evaluate the general similarities between the different extraction techniques and to classify them, a multivariate statistical analysis of the dataset was performed. Therefore, multivariate analysis including PLS-DA and agglomerative hierarchical clustering were successively performed. The factor named "Method of extraction", comprising of five modalities was considered as class membership. As we observed in Figure 4A, a clear separation between the extraction methods was noticed along the first two components of PLS-DA. The first component allowed to stand out the two green shorter extraction time methods (ASE and MAE) from the two conventional extraction methods (SE, maceration) and the other green extraction methods (UAE). The second component separated the two low temperature extraction methods (maceration and UAE) from the three high temperature extraction methods (SE, ASE, and MAE). Then Cluster heatmap analysis was applied on the result of PLS-DA by retaining the first component. Euclidean distance and Ward was adopted as the similarity measurement and the linkage rule respectively. Four clusters emerged with

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reference to the heat-map (Figure 4B). The first and second groups included the soxhlet and MAE extracts respectively, the third group was composed of maceration and ultrasonication extracts while the fourth one was represented by the ASE extract. Furthermore, the main biological activities responsible for this discrimination by evaluating the variables important in projection (VIP analysis) of the PLS-DA model were identified. Biological activities with VIP values were higher than 1.2 were noted to contribute significantly for the discrimination. Accordingly, among the eleven evaluated biological activities, antioxidant activities (DPPH, ABTS, FRAP, and CUPRAC) mostly contributing to separate ASE and MAE methods from SE, maceration and UAE along the first component while α-amylase allowed to discriminate the low temperature extraction methods from the high temperature extraction methods along the second component (Figure 4D).

As expected, PLS-DA achieved a better discrimination of the studied extraction methods. In fact, the robustness of the discriminant model was excellent, due to a very low classification error rate that was about 0.06 for the first two components (Figure 4C).

4. Conclusion

In line with advances in extraction technologies, advocating "green" extraction methods meeting sustainable development goals, the present study attempted to investigate the possible variances in bioactivity of *H. stoechas* subsp. *barrelieri* aerial parts extracted using conventional and non-conventional extraction techniques. ASE, using high temperature and high pressure, was the most efficient extraction method for phenolics and flavonoids. Likewise, *H. stoechas* extract obtained by ASE exhibited

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highest antioxidant capacities. Extracts of *H. stoechas* obtained from ASE and UAE showed potent enzyme inhibitory action. Scientific data collected from this study supported the use of non-conventional extraction techniques for the extraction of bioactive secondary metabolites from medicinal plants.

Compliance with Ethical Standards

Conflict of Interest

Gokhan Zengin declares that he has no conflict of interest. Aleksandra Cvetanović declares that she has no conflict of interest. Uroš Gašić declares that he has no conflict of interest. Živosla Tešić declares that he has no conflict of interest. Alena Stupar declares that she has no conflict of interest. Gizem Bulut declares that she has no conflict of interest. Kouadio Ibrahime Sinan declares he has no conflict of interest. Sengul Uysal declares that she has no conflict of interest. Marie Carene Nancy Picot-Allain that she has no conflict of interest. Mohamad Fawzi Mahomoodally declares he has no conflict of interest.

Conflict of Interest File

Declarations of interest: none.

Ethical Approval

This article does not contain any studies with human or animal subjects.

Informed Consent

Not applicable

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Author statements

Gokhan Zengin: Conceptualization; Data curation; Investigation: Writing - original draft

Aleksandra Cvetanović: Conceptualization; Data curation; Investigation; Writing - original draft

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Figure Captions

Figure 1. A: Venn graph showing the overlap of differentially identified phytochemical compounds in each extraction techniques. B&C: Total phenolic and flavonoid contents of *Helichrysum stoechas* subsp. *barrelieri* extracts (Letters indicate significant differences in the extracts ($p < 0.05$)) and their relationship with evaluated biological activities. Superscripts letters indicate significant differences in the extracts ($p < 0.05$).

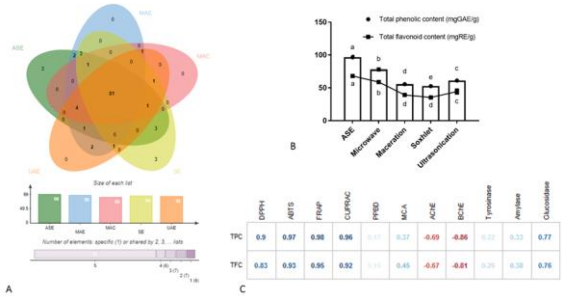

Figure 2. Proposed mechanism of chlorogenic acid and radical (DPPH and ABTS) scavenging. Adapted from Li et al. (2014).

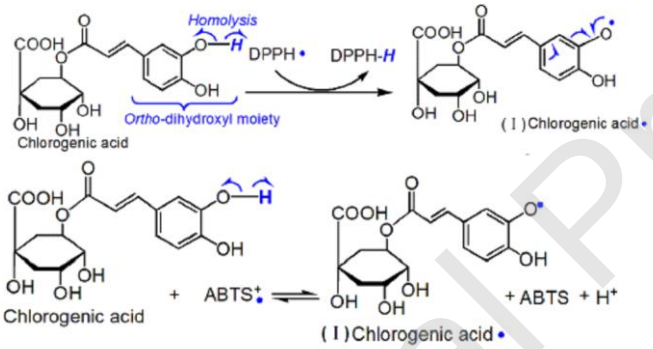

Figure 3. Circosplot and corrplot displaying the relationship among evaluated biological activities and quantified 19 phytochemical compounds of the tested extracts. Blue and Red edges of circosplot indicate negative and positive correlation respectively.

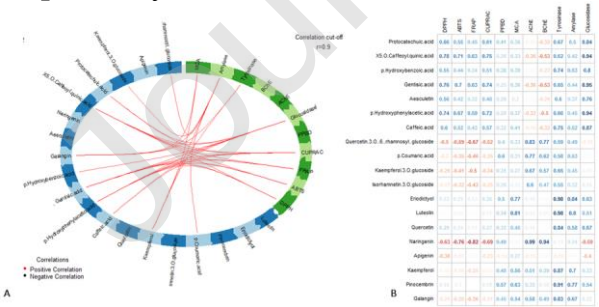

Figure 4. Partial Least Squares Discriminant Analysis (PLS-DA) and Cluster Analysis on *H. stoechas* samples biological activities according to five methods of extraction. A: Sample plot with confidence ellipse based on the methods of extraction. B: Clustered Image Map (Euclidean Distance, Ward linkage). C: The model robustness per

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component. D: Discriminant biological activities by Variable Importance in Projection (VIP) analysis.

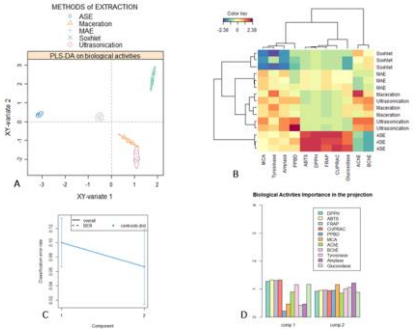

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Table 1. High resolution MS data and negative ion mode MS4 fragmentation of phenolics found in *Helichrysum stoechas* subsp. *barrelieri* extracts

No	Compound name	t_R, min	Molecular formula, [M–H]⁻	Calculated mass, [M–H]⁻	Exact mass, [M–H]⁻	Δ ppm	MS² Fragments, (% Base Peak)	MS³ Fragments, (% Base Peak)	MS⁴ Fragments, (% Base Peak)	ASE	MAE	MAC	SE	UAE
Hydroxybenzoic acids														
1	Dihydroxybenzoic acid hexoside	4,07	$C_{13}H_{15}O_9^-$	315,07216	315,07117	3,14	153(100), 152(50), 109(15), 108(10)	109(100)	–	+	+	+	+	+
2	Protocatechuic acid	4,48	$C_7H_5O_4^-$	153,01933	153,01883	3,27	109(100), 95(75), 79(20), 59(10)	81(100), 68(25), 65(15)	–	+	+	+	+	+
3	*p*-Hydroxybenzoic acid	5,39	$C_7H_5O_3^-$	137,02442	137,02399	3,14	109(10), 93(100)	–	–	+	+	+	+	+
4	Gentisic acid	5,54	$C_7H_5O_4^-$	153,01933	153,01889	2,88	136(5), 125(10), 109(100), 95(20), 79(10)	81(85), 67(100), 63(60)	–	+	+	+	+	+
5	*p*-Hydroxyphenylacetic acid	5,83	$C_8H_7O_3^-$	151,04007	151,03966	2,71	121(15), 107(100), 95(70), 79(15), 59(25)	123(10), 95(30), 79(100), 69(10), 51(20)	–	+	+	+	+	+
6	Dihydroxybenzoic acid ethyl ester	8,14	$C_9H_9O_4^-$	181,05063	181,05008	3,04	153(100), 109(10)	109(100)	–	+	+	+	+	+
Hydroxycinnamic acids														
7	Caffeoyl-quinic acid isomer 1	4,35	$C_{16}H_{17}O_9^-$	353,08781	353,08655	3,57	191(100), 179(5)	173(80), 127(85), 111(40), 93(70), 85(100)	–	+	+	+	+	+
8	Caffeoyl-quinic acid isomer 2	4,70	$C_{16}H_{17}O_9^-$	353,08781	353,08612	4,79	191(100), 179(30), 135(10)	173(80), 127(85), 111(40), 93(70), 85(100)	57(100)	+	+	+	+	+
9	Caffeic acid hexoside	5,18	$C_{15}H_{17}O_9^-$	341,08781	341,08633	4,34	179(100), 135(10)	135(100)	107(100), 79(20)	+	+	+	+	+
10	5-*O*-Caffeoyl-quinic acid	5,31	$C_{16}H_{17}O_9^-$	353,08781	353,08608	4,90	191(100), 179(5)	173(75), 127(100), 111(40), 93(60), 85(90)	109(30), 99(40), 85(100)	+	+	+	+	+
11	Caffeoyl-glycerol	5,78	$C_{12}H_{13}O_6^-$	253,07176	253,07117	2,33	179(35), 161(50), 135(100)	117(30), 107(100), 93(5), 78(20)	–	+	+	+	+	+
12	Caffeoyl-quinic acid isomer 3	5,76	$C_{16}H_{17}O_9^-$	353,08781	353,08612	4,79	191(100), 179(5)	173(80), 127(85), 111(40), 93(70), 85(100)	–	+	+	+	+	+
13	Caffeic acid	5,84	$C_9H_7O_4^-$	179,03498	179,03410	4,92	135(100)	135(60), 117(15), 107(100), 91(55), 79(15)	–	+	+	+	+	+
14	Dicaffeoyl-quinic acid isomer 1	5,92	$C_{25}H_{23}O_{12}^-$	515,11950	515,11698	4,89	353(100), 335(30), 191(10), 179(25)	191(100), 179(40), 135(10)	173(80), 127(85), 111(40), 93(70), 85(100)	+	+	+	+	+
15	Coumaroyl-quinic acid isomer 1	5,95	$C_{16}H_{17}O_8^-$	337,09289	337,09123	4,92	191(100), 179(5), 163(10)	173(75), 127(100), 111(40), 93(60), 85(90)	109(20), 99(30), 85(100)	+	+	+	+	+
16	Caffeoyl-shikimic acid	6,00	$C_{16}H_{15}O_8^-$	335,07724	335,07672	1,55	179(100), 135(25)	135(100)	107(40), 91(100)	+	+	+	+	+
17	Feruloyl-quinic acid isomer 1	6,21	$C_{17}H_{19}O_9^-$	367,10346	367,10164	4,96	191(100), 173(5)	173(65), 127(100), 111(35), 93(50), 85(90)	109(20), 99(30), 85(100)	+	+	+	+	+
18	Chrysanthemorimic acid isomer 1	6,26	$C_{31}H_{29}O_{15}^-$	641,15119	641,15033	1,34	479(100)	353(60), 305(40), 287(25), 257(45), 191(100)	173(75), 127(100), 111(40), 93(60), 85(90)	+	+	+	+	+
19	Coumaroyl-quinic acid isomer 2	6,34	$C_{16}H_{17}O_8^-$	337,09289	337,09155	3,98	191(100), 179(5), 163(10)	173(75), 127(100), 111(50), 93(60), 85(80)	109(40), 99(10), 85(100)	+	+	+	+	+

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20	Dicaffeoyl-quinic acid hexoside isomer 1	6,41	$C_{31}H_{33}O_{17}^-$	677,17232	677,17169	0,93	515(100), 497(30), 485(5), 353(20), 323(10)	353(100), 341(40), 323(90), 191(30), 179(20)	191(100), 179(25), 173(30), 135(5)	+	+	+	+	+
21	Chrysanthemorimic acid isomer 2	6,56	$C_{31}H_{29}O_{15}^-$	641,15119	641,14990	2,01	479(100), 335(10), 305(5)	353(5), 305(90), 261(100), 173(15)	243(30), 215(25), 203(100), 189(85)	+	+	+	+	+
22	Dicaffeoyl-quinic acid isomer 2	6,59	$C_{25}H_{23}O_{12}^-$	515,11950	515,11847	2,00	353(100), 191(10), 179(5)	191(100), 179(35), 173(10), 135(10)	173(25), 153(25), 127(100), 111(70), 93(85)	–	–	–	+	–
23	Dicaffeoyl-quinic acid hexoside isomer 2	6,63	$C_{31}H_{33}O_{17}^-$	677,17232	677,17249	-0,25	617(5), 587(15), 557(10), 515(100), 353(40)	353(100), 191(5), 179(5)	191(100), 179(45), 173(15), 135(10)	–	–	–	+	–
24	*p*-Coumaric acid	6,70	$C_9H_7O_3^-$	163,04007	163,03970	2,27	119(100)	119(60), 101(20), 93(25), 91(100), 72(10)	–	+	+	+	+	+
25	Dicaffeoyl-quinic acid isomer 3	6,97	$C_{25}H_{23}O_{12}^-$	515,11950	515,11701	4,83	353(100), 335(20), 203(30), 179(10), 173(10)	191(35), 179(65), 173(100), 135(10)	155(20), 137(10), 111(50), 93(100), 71(30)	+	+	+	+	+
26	Dihydroxybenzoyl-caffeoyl-hexoside	7,00	$C_{22}H_{21}O_{12}^-$	477,10385	477,10151	4,90	433(15), 323(5), 315(100)	153(100), 152(40), 109(10)	109(100), 108(15)	+	+	–	+	–
27	Acetyl-dicaffeoyl-quinic acid isomer 1	7,01	$C_{27}H_{25}O_{13}^-$	557,13006	557,12787	3,93	521(10), 485(100), 395(20), 323(15)	323(100), 221(5), 161(20)	263(15), 221(40), 203(20), 179(50), 161(100)	+	+	–	–	+
28	Caffeoyl-coumaroyl-quinic acid isomer 1	7,02	$C_{25}H_{23}O_{11}^-$	499,12404	499,12299	2,10	379(100), 353(25), 337(30), 295(60), 203(20)	335(15), 295(100), 269(15), 203(40), 175(15)	277(100), 267(25), 249(20)	+	+	+	+	+
29	Dicaffeoyl-quinic acid isomer 4	7,09	$C_{25}H_{23}O_{12}^-$	515,11950	515,11695	4,95	353(100), 335(5), 191(10), 179(5)	191(100), 179(40), 135(10)	173(80), 127(85), 111(40), 93(70), 85(100)	+	+	+	+	+
30	Caffeoyl-quinic acid isomer 5	7,16	$C_{16}H_{17}O_9^-$	353,08781	353,08609	4,87	191(100), 179(10)	173(50), 127(100), 111(20), 93(40), 85(55)	109(10), 99(15), 85(100)	+	+	+	+	+
31	Malonyl-dicaffeoyl-quinic acid isomer 1	7,21	$C_{28}H_{25}O_{15}^-$	601,11989	601,11768	3,68	557(100), 515(80), 439(70), 395(50), 377(10)	395(100), 377(10), 335(5), 233(30)	353(5), 335(10), 233(100), 173(15)	+	+	+	+	+
32	Dicaffeoyl-quinic acid isomer 5	7,31	$C_{25}H_{23}O_{12}^-$	515,11950	515,11711	4,64	353(100), 335(5), 299(10), 203(10), 173(5)	191(60), 179(70), 173(100), 135(10)	155(20), 137(10), 111(50), 93(100), 71(30)	+	+	+	+	+
33	Malonyl-dicaffeoyl-quinic acid isomer 2	7,45	$C_{28}H_{25}O_{15}^-$	601,11989	601,11817	2,86	557(60), 515(60), 439(60), 395(100), 377(10)	335(5), 233(100), 173(15)	173(100)	+	+	+	–	+
34	Rosmarinic acid	7,54	$C_{18}H_{15}O_8^-$	359,07724	359,07545	4,98	223(10), 197(15), 179(15), 161(100), 133(5)	133(100)	105(100)	+	+	+	+	+
35	Caffeoyl-coumaroyl-quinic acid isomer 2	7,62	$C_{25}H_{23}O_{11}^-$	499,12404	499,12286	2,36	463(40), 353(90), 337(100), 319(10), 191(20)	191(100), 163(10)	173(25), 153(25), 127(100), 111(70), 85(95)	+	+	+	+	+
36	Acetyl-dicaffeoyl-quinic acid isomer 2	7,76	$C_{27}H_{25}O_{13}^-$	557,13006	557,12761	4,40	395(100), 377(15), 335(5), 233(30)	335(5), 233(100), 173(15)	173(100)	+	+	+	+	+
37	Feruloyl-quinic acid isomer 2	7,84	$C_{17}H_{19}O_9^-$	367,10346	367,10190	4,25	349(60), 193(15), 191(100)	173(70), 127(100), 111(30), 93(35), 85(90)	99(15), 85(100)	+	+	+	+	+
38	Feruloyl-caffeoyl-quinic acid	7,85	$C_{26}H_{25}O_{12}^-$	529,13515	529,13318	3,72	367(100), 353(10)	191(100)	173(80), 127(85), 111(40), 93(70), 85(100)	+	+	+	+	+

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39	Acetyl-dicaffeoyl-quinic acid isomer 3	8,03	$C_{27}H_{25}O_{13}^-$	557,13006	557,12799	3,72	395(100), 377(10), 233(30), 173(5)	335(5), 233(100), 173(15)	173(100)	+	+	+	+	–
40	Tricaffeoyl-quinic acid	8,35	$C_{34}H_{29}O_{15}^-$	677,15119	677,15045	1,09	515(100), 497(5), 485(5), 353(15), 323(10)	353(100), 335(15), 299(5), 191(5), 179(10)	191(55), 179(65), 173(100), 135(10)	+	+	+	+	+
41	Acetyl-dicaffeoyl-quinic acid isomer 4	8,38	$C_{27}H_{25}O_{13}^-$	557,13006	557,12854	2,73	395(100), 233(5)	335(5), 233(100), 173(30)	173(100)	+	+	–	+	–
42	Feruloyl-quinic acid isomer 3	8,53	$C_{17}H_{19}O_9^-$	367,10346	367,10211	3,68	323(70), 167(60), 233(30), 191(100)	173(50), 127(40), 109(40), 93(65), 85(100)	–	+	+	+	+	+
43	Diferuloyl-quinic acid	8,54	$C_{27}H_{27}O_{12}^-$	543,15080	543,14929	2,78	549(10), 367(100), 349(70), 193(15), 191(10)	193(15), 191(100), 173(10), 134(5)	173(80), 127(85), 111(40), 93(70), 85(100)	+	+	+	+	+
44	Dicaffeoyl-shikimic acid	8,70	$C_{25}H_{21}O_{11}^-$	497,10894	497,10721	3,48	335(100), 179(5)	225(35), 211(35), 179(70), 173(100), 161(60)	155(50), 137(15), 127(25), 111(70), 93(80)	–	–	–	+	–
45	Dicaffeoyl-succinylquinic acid	9,04	$C_{29}H_{27}O_{14}^-$	599,14063	599,13971	1,54	437(100), 419(10), 275(70)	275(100)	233(15), 215(100), 173(15)	+	–	–	–	–
46	Caffeic acid ethyl ester	9,20	$C_{11}H_{11}O_4^-$	207,06628	207,06575	2,56	179(100), 161(15), 135(15)	135(100)	107(100), 91(10), 79(50)	+	+	+	+	+
Coumarins														
47	Aesculin	4,95	$C_{15}H_{15}O_9^-$	339,07216	339,07123	2,74	177(100)	149(10), 133(100), 105(10), 89(5)	89(100)	+	+	+	+	+
48	Aesculetin	5,82	$C_9H_5O_4^-$	177,01933	177,01881	2,94	149(10), 133(100), 105(10), 89(5)	89(100)	–	+	+	+	+	+
Flavonoids														
49	Quercetagetin 3,7-di-*O*-hexoside	4,96	$C_{27}H_{29}O_{18}^-$	641,13594	641,13440	2,40	479(100), 317(30)	359(5), 317(100), 316(30)	299(100), 271(85), 231(40), 195(50), 167(60)	+	+	+	+	+
50	Quercetin 3,7-di-*O*-hexoside	5,16	$C_{27}H_{29}O_{17}^-$	625,14102	625,13947	2,48	505(10), 463(100), 301(30)	343(10), 301(100), 300(10), 271(5)	271(100), 255(50), 179(60), 151(90)	+	+	+	+	+
51	Quercetagetin 3-*O*-(acetyl-hexosyl)-7-*O*-hexoside	5,67	$C_{29}H_{31}O_{19}^-$	683,14650	683,14453	2,88	521(30), 479(100), 317(40)	359(5), 317(100), 316(30)	299(100), 271(85), 231(40), 195(50), 167(60)	+	–	–	–	–
52	Quercetagetin 3-*O*-hexoside	5,91	$C_{21}H_{19}O_{13}^-$	479,08311	479,08145	3,46	317(100), 316(30)	299(100), 271(95), 195(50), 167(40), 167(30)	271(100), 255(25), 243(10), 231(30), 199(10)	+	+	+	+	+
53	Luteolin 8-*C*-hexoside	6,09	$C_{21}H_{19}O_{11}^-$	447,09329	447,09125	4,56	429(10), 357(40), 327(100)	299(100), 284(10)	271(30), 255(100), 213(60), 199(40), 175(30)	+	+	+	+	+
54	Quercetagetin 7-*O*-hexoside isomer 1	6,12	$C_{21}H_{19}O_{13}^-$	479,08311	479,08093	4,55	317(100)	299(40), 271(100), 243(40), 195(70), 167(50)	243(100), 227(15), 215(10), 199(55)	+	+	+	+	+
55	Quercetagetin 3-*O*-(malonyl-coumaroyl)-hexoside-7-*O*-hexoside	6,29	$C_{39}H_{37}O_{23}^-$	873,17311	873,17426	-1,32	829(15), 625(100)	479(100), 317(20)	359(5), 317(100), 316(35)	+	+	–	–	–

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56	Quercetagetin 3-*O*-(coumaroyl)-hexoside isomer 1	6,31	$C_{30}H_{25}O_{15}^-$	625,11989	625,11884	1,68	479(100), 317(20)	359(5), 317(100), 316(35)	299(100), 271(85), 231(40), 195(50), 167(60)	+	+	–	–	–
57	Quercetagetin 7-*O*-hexoside isomer 2	6,46	$C_{21}H_{19}O_{13}^-$	479,08311	479,08124	3,90	317(100)	299(100), 271(50), 243(20), 195(40), 167(30)	271(100), 255(25), 243(10), 231(30), 199(10)	+	+	+	+	+
58	Quercetin 3-*O*-(6"-rhamnosyl)-glucoside	6,50	$C_{27}H_{29}O_{16}^-$	609,14611	609,14380	3,79	343(10), 301(100), 300(35), 271(10), 255(5)	283(10), 271(60), 255(40), 179(100), 151(75)	151(100)	–	–	+	+	+
59	Quercetagetin 7-*O*-(acetyl)-hexoside isomer 1	6,55	$C_{23}H_{21}O_{14}^-$	521,09368	521,09216	2,92	318(10), 317(100)	287(30), 271(100), 179(30), 167(75)	271(10), 243(100), 227(40), 215(15)	+	+	+	+	+
60	Naringenin 7-*O*-hexoside isomer 1	6,58	$C_{21}H_{21}O_{10}^-$	433,11402	433,11188	4,94	341(10), 313(30), 272(10), 271(100), 151(5)	177(10), 165(5), 151(100), 119(5), 107(5)	107(100), 83(10), 65(5)	+	+	+	+	+
61	Quercetagetin 3-*O*-(coumaroyl)-hexoside isomer 2	6,66	$C_{30}H_{25}O_{15}^-$	625,11989	625,11902	1,39	479(100), 317(20)	359(5), 317(100), 316(35)	299(100), 271(85), 231(40), 195(50), 167(60)	+	–	–	–	–
62	Luteolin 7-*O*-hexoside	6,71	$C_{21}H_{19}O_{11}^-$	447,09329	447,09146	4,09	285(100)	257(30), 241(100), 217(75), 199(85), 175(95)	241(5), 226(15), 213(30), 197(100)	+	+	+	+	+
63	Quercetin 7-*O*-hexoside isomer 1	6,72	$C_{21}H_{19}O_{12}^-$	463,08820	463,08598	4,79	302(10), 301(100)	273(10), 255(15), 179(70), 151(100), 107(15)	107(100)	+	+	+	+	+
64	Patuletin 7-*O*-hexoside	6,79	$C_{22}H_{21}O_{13}^-$	493,09876	493,09680	3,97	477(5), 373(5), 331(100), 316(5)	316(100), 209(5), 181(5), 166(5)	287(100), 270(60), 244(10), 166(15)	+	+	+	+	+
65	Quercetagetin 7-*O*-(acetyl)-hexoside isomer 2	6,84	$C_{23}H_{21}O_{14}^-$	521,09368	521,09222	2,80	318(10), 317(100)	287(30), 271(100), 179(30), 167(80)	271(10), 243(100), 227(40), 215(15)	+	–	–	+	–
66	Quercetin 3-*O*-(acetyl)-hexoside	6,95	$C_{23}H_{21}O_{13}^-$	505,09876	505,09756	2,38	463(15), 445(5), 301(100), 300(30)	283(10), 271(60), 255(40), 179(100), 151(75)	151(100)	+	+	+	–	+
67	Kaempferol 3-*O*-glucoside	7,13	$C_{21}H_{19}O_{11}^-$	447,09329	447,09158	3,82	327(20), 285(80), 284(100), 255(10)	255(100), 227(10)	227(100), 211(60)	+	+	+	+	+
68	Quercetin 7-*O*-(acetyl)-hexoside isomer 1	7,20	$C_{23}H_{21}O_{13}^-$	505,09876	505,09756	2,38	445(5), 343(5), 302(5), 301(100)	283(5), 273(15), 255(20), 179(65), 151(100)	107(100), 83(5), 65(5)	+	+	+	+	+
69	Isorhamnetin 3-*O*-glucoside	7,23	$C_{22}H_{21}O_{12}^-$	477,10385	477,10202	3,84	357(20), 315(50), 314(100), 300(5), 285(10)	300(30), 285(100), 271(75), 257(10), 243(25)	270(100)	+	+	+	+	+
70	Kaempferol 7-*O*-hexoside	7,25	$C_{21}H_{19}O_{11}^-$	447,09329	447,09167	3,62	327(5), 286(5), 285(100)	257(40), 241(30), 229(30), 151(100), 107(10)	107(100), 83(10), 65(5)	+	+	+	+	+
71	Quercetagetin	7,35	$C_{15}H_9O_8^-$	317,03029	317,02879	4,73	299(100), 287(95), 27190), 195(50), 167(60)	271(100), 255(25), 243(10), 231(30), 199(10)	243(100), 227(10), 215(10), 199(95)	+	+	+	+	+
72	Eriodictyol 7-*O*-hexoside	7,40	$C_{21}H_{21}O_{11}^-$	449,10894	449,10699	4,34	287(100), 151(10)	151(100)	107(100)	+	+	+	+	+
73	Quercetin 7-*O*-hexoside isomer 2	7,52	$C_{21}H_{19}O_{12}^-$	463,08820	463,08602	4,71	302(5), 301(100)	273(10), 257(15), 179(100), 151(70), 107(5)	151(100)	+	+	+	+	+
74	Naringenin 7-*O*-hexoside isomer 2	7,71	$C_{21}H_{21}O_{10}^-$	433,11402	433,11264	3,19	313(10), 285(5), 272(10), 271(100), 151(5)	177(10), 165(5), 151(100), 119(5), 107(5)	107(100), 83(10), 65(5)	+	+	+	+	+
75	Quercetagetin 7-*O*-(diacetyl)-rhamnoside	7,72	$C_{25}H_{23}O_{14}^-$	547,10933	547,10822	2,03	318(10), 317(100)	287(30), 271(100), 179(30), 167(75)	271(10), 243(100), 227(40), 215(15)	+	–	+	+	+
76	Quercetin 7-*O*-(acetyl)-hexoside isomer 2	7,78	$C_{23}H_{21}O_{13}^-$	505,09876	505,09756	2,38	445(5), 302(10), 301(100)	273(15), 257(10), 179(100), 151(80)	151(100)	+	+	+	–	+

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77	Quercetagetin 7-*O*-(acetyl-caffeoyl)-hexoside isomer 1	7,86	$C_{32}H_{27}O_{17}^-$	683,12537	683,12469	1,00	537(5), 521(5), 365(10), 317(100)	299(100), 271(85), 231(40), 195(50), 167(60)	271(100), 255(25), 243(10), 231(30), 199(10)	–	+	–	–	+
78	Quercetin 3-*O*-(coumaroyl)hexoside isomer 1	7,95	$C_{30}H_{25}O_{14}^-$	609,12498	609,12421	1,26	463(100), 301(15)	301(100), 300(30)	271(30), 255(20), 179(100), 151(80)	+	+	+	+	+
79	Quercetin 7-*O*-(acetyl)-hexoside isomer 3	8,02	$C_{23}H_{21}O_{13}^-$	505,09876	505,09756	2,38	463(20), 445(80), 323(25), 302(15), 301(100)	273(15), 257(15), 179(100), 151(75)	151(100)	–	+	+	–	+
80	Quercetin 3-*O*-(coumaroyl)hexoside isomer 2	8,09	$C_{30}H_{25}O_{14}^-$	609,12498	609,12421	1,26	463(100), 301(15)	301(100), 300(20)	271(50), 255(30), 179(100), 151(80)	+	+	+	+	+
81	Quercetagetin 3-*O*-(acetyl-coumaroyl)-hexoside	8,19	$C_{32}H_{27}O_{16}^-$	667,13046	667,12964	1,23	545(30), 521(100), 505(50), 487(30), 461(10)	479(10), 461(70), 317(100), 316(90)	299(100), 271(85), 231(40), 195(50), 167(60)	+	+	+	–	+
82	Quercetin 7-*O*-(caffeoyl)-hexoside	8,21	$C_{30}H_{25}O_{15}^-$	625,11989	625,11847	2,27	463(40), 445(20), 323(20), 302(15), 301(100)	273(25), 257(20), 179(100), 151(75)	151(100)	+	+	+	+	+
83	Quercetin 7-*O*-(coumaroyl)hexoside	8,32	$C_{30}H_{25}O_{14}^-$	609,12498	609,12421	1,26	573(10), 463(40), 445(5), 302(10), 301(100)	271(20), 255(15), 179(100), 151(80), 107(10)	151(100)	+	+	+	+	+
84	Quercetin 7-*O*-(acetyl)-hexoside isomer 4	8,36	$C_{23}H_{21}O_{13}^-$	505,09876	505,09756	2,38	445(5), 302(10), 301(100)	273(15), 257(10), 179(100), 151(85)	151(100)	+	+	+	+	+
85	Quercetin 3-*O*-(acetyl-coumaroyl)-hexoside isomer 1	8,37	$C_{32}H_{27}O_{15}^-$	651,13554	651,13495	0,91	505(100), 301(10)	463(15), 445(25), 301(100), 300(80)	273(25), 257(20), 179(100), 151(75)	–	+	–	–	+
86	Kaempferol 7-*O*-(coumaroyl)-hexoside	8,39	$C_{30}H_{25}O_{13}^-$	593,13006	593,12799	3,49	447(15), 307(10), 286(15), 285(100)	257(100), 241(50), 229(35), 213(40), 151(90)	256(10), 239(25), 229(100), 213(20), 163(35)	+	+	+	+	+
87	Isorhamnetin 7-*O*-(coumaroyl)-hexoside	8,52	$C_{31}H_{27}O_{14}^-$	623,14063	623,14063	0,00	447(5), 316(10), 315(100), 300(20)	300(100)	271(100), 255(50), 227(20), 151(45)	+	+	+	+	+
88	Quercetagetin 7-*O*-(acetyl-caffeoyl)-hexoside isomer 2	8,57	$C_{32}H_{27}O_{17}^-$	683,12537	683,12476	0,89	537(5), 521(5), 365(10), 317(100)	299(100), 271(85), 231(40), 195(50), 167(60)	271(100), 255(25), 243(10), 231(30), 199(10)	+	–	–	+	–
89	Eriodictyol	8,62	$C_{15}O_{11}O_6^-$	287,05611	287,05510	3,52	151(100)	107(100)	65(100)	+	+	+	+	+
90	Luteolin	8,69	$C_{15}H_9O_6^-$	285,04046	285,03961	2,98	257(40), 241(100), 217(50), 199(70), 175(70)	223(40), 213(45), 197(100), 153(15)	–	+	+	+	+	+
91	Quercetin	8,74	$C_{15}H_9O_7^-$	301,03537	301,03408	4,29	283(15), 271(60), 257(25), 179(100), 151(80)	151(100)	107(100), 83(10)	+	+	+	+	+
92	Quercetin 7-*O*-(acetyl-caffeoyl)-hexoside	8,79	$C_{32}H_{27}O_{16}^-$	667,13046	667,12946	1,50	625(20), 505(70), 487(50), 365(20), 301(100)	273(25), 257(20), 179(100), 151(75)	151(100)	+	–	–	+	–
93	Quercetin 3-*O*-(acetyl-coumaroyl)-hexoside isomer 2	9,03	$C_{32}H_{27}O_{15}^-$	651,13554	651,13446	1,66	505(100), 301(10)	463(15), 445(25), 301(100), 300(80)	273(25), 257(20), 179(100), 151(75)	+	+	–	+	–
94	Naringenin	9,52	$C_{15}H_{11}O_5^-$	271,06120	271,06036	3,10	225(5), 177(10), 151(100)	107(100)	65(100)	+	+	+	+	+
95	Apigenin	9,55	$C_{15}H_9O_5^-$	269,04554	269,04492	2,30	225(100), 201(30), 183(20), 151(30), 149(50)	197(25), 181(100), 169(15)	–	–	+	–	+	+
96	Quercetin 7-*O*-(methylbutyryl)-hexoside	9,58	$C_{26}H_{27}O_{13}^-$	547,14571	547,14571	0,00	445(10), 302(20), 301(100)	273(25), 257(20), 179(100), 151(75)	151(100)	+	+	+	+	+
97	Kaempferol	9,73	$C_{15}H_9O_6^-$	285,04046	285,03940	3,72	257(60), 241(70), 229(100), 213(80), 185(90)	201(100), 185(20), 141(35)	–	+	+	+	+	+

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98	Pinobanksin	9,86	$C_{15}H_{11}O_5^-$	271,06120	271,06055	2,40	253(100), 225(20), 215(15), 209(10), 197(15)	235(10), 225(85), 209(50), 197(100), 181(15)	179(100), 169(35), 151(45)	+	+	+	+	+
99	Isorhamnetin	9,91	$C_{16}H_{11}O_7^-$	315,05103	315,04947	4,95	301(20), 300(100)	283(40), 271(80), 255(30), 227(30), 151(100)	243(100), 227(45)	+	+	+	+	+
100	Pinocembrin	11,80	$C_{15}H_{11}O_4^-$	255,06628	255,06512	4,55	213(100), 187(15), 151(30), 145(10), 107(5)	185(100), 169(20), 145(20)	157(30), 143(50), 141(100), 117(20), 115(30)	+	+	+	+	+
101	Dimethoxy-trihydroxyflavone	11,89	$C_{17}H_{13}O_7^-$	329,06668	329,06537	3,98	314(100), 299(10)	299(100), 286(5), 285(5), 271(90)	271(100)	+	+	+	+	+
102	Galangin	11,90	$C_{15}H_9O_5^-$	269,04554	269,04477	2,86	241(40), 227(80), 213(100), 197(90), 169(50)	198(10), 195(20), 185(100), 169(50), 143(35)	157(20), 143(100)	+	+	+	+	+
103	Trihydroxy-methoxyflavone	12,2	$C_{16}H_{11}O_6^-$	299,05611	299,05536	2,51	284(100), 256(10)	256(100), 255(15), 239(5)	239(100), 238(40), 227(25), 211(10), 199(5)	+	+	+	+	+
Pyrones														
104	Pyrone derivative 1	13,40	$C_{15}H_{15}O_6^-$	291,08741	291,08676	2,23	139(100), 95(5)	95(100)	55(100), 53(70)	+	+	+	+	+
105	Pyrone derivative 2	14,5	$C_{16}H_{17}O_6^-$	305,10306	305,10229	2,52	153(100), 139(40), 109(5), 95(5)	109(100)	93(70), 55(100)	+	+	+	+	+
106	Pyrone derivative 3	14,6	$C_{26}H_{31}O_7^-$	455,20753	455,20605	3,25	343(100)	301(100), 273(10), 244(10), 204(50)	283(100), 231(60), 161(25), 149(70)	+	+	+	+	+
107	Helipyrone	15,5	$C_{17}H_{19}O_6^-$	319,11871	319,11838	1,03	153(100), 209(10)	109(100)	93(70), 55(100)	+	+	+	+	+
	standard													

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Table 2. Amount (mg/kg) of individual phenolics found in *Helichrysum stoechas* subsp. *barrelieri* extracts.

Compounds	ASE	MAE	Maceration	SE	UAE
Protocatechuic acid	137.897	43.622	118.789	37.376	73.624
5-*O*-Caffeoyl-quinic acid	865.075	351.715	668.398	233.956	362.150
p-Hydroxybenzoic acid	416.444	139.752	416.784	108.031	232.923
Gentisic acid	81.917	48.626	69.735	38.499	47.796
Aesculetin	43.616	21.039	43.077	24.068	28.308
p-Hydroxyphenylacetic acid	121.266	54.078	99.514	36.044	56.462
Caffeic acid	263.428	107.528	253.201	66.141	133.582
Quercetin 3-*O*-(6"-rhamnosyl)-glucoside	–	–	19.430	2.109	13.689
p-Coumaric acid	34.039	18.095	54.895	28.390	50.388
Kaempferol 3-*O*-glucoside	7.016	0.830	30.497	4.950	16.025
Isorhamnetin 3-*O*-glucoside	37.208	4.687	91.649	30.655	47.912
Eriodictyol	15.936	12.510	20.356	4.818	15.075
Luteolin	3.952	4.465	5.998	1.329	4.190
Quercetin	1021.636	473.324	1372.451	285.508	708.984
Naringenin	0.888	0.851	2.929	2.413	3.949
Apigenin	–	1.943	–	0.553	0.937
Kaempferol	81.705	57.076	149.901	36.249	105.012
Pinocembrin	76.001	41.816	103.433	22.353	76.421
Galangin	17.236	13.679	38.086	8.698	26.520
TIPC	3225.26	1395.636	3559.123	972.14	2003.947

-not detected. TIPC: Total Individual Phenolic Content

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Table 3. Antioxidant properties of *Helichrysum stoechas* subsp. *barrelieri* extracts*.

Extraction methods	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	Metal chelating (mg EDTAE/g)	Phosphomolybdenum (mmol TE/g)
ASE	219.92±3.21^{a}	313.12±8.42^{a}	927.39±11.19^{a}	662.87±20.41^{a}	14.42±0.68^{b}	2.00±0.11^{a}
Microwave	107.74±0.26^{b}	204.14±2.48^{b}	531.74±8.80^{b}	459.50±0.42^{b}	17.11±0.69^{a}	1.74±0.04^{ab}
Maceration	92.53±0.27^{c}	144.28±5.03^{c}	382.89±2.73^{c}	271.62±1.96^{c}	15.12±1.85^{ab}	1.85±0.04^{ab}
Soxhlet	90.05±0.92^{c}	138.68±1.20^{c}	335.97±9.89^{d}	286.11±1.76^{c}	2.91±0.43^{c}	1.65±0.07^{b}
Ultrasonication	91.72±0.62^{c}	146.11±3.82^{c}	400.59±2.78^{c}	285.14±5.25^{c}	18.67±1.33^{a}	2.27±0.20^{a}

* Values expressed are means ± S.D. of three parallel measurements. DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; CUPRAC: Cupric reducing antioxidant capacity; FRAP: Ferric reducing antioxidant power; TE: Trolox equivalent; EDTAE: Ethylenediaminetetraacetic equivalent. Superscripts letters indicate significant differences in the extracts ($p < 0.05$).

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Table 4. Enzyme inhibitory properties of *Helichrysum stoechas* subsp. *barrelieri* extracts*.

Extraction methods	AChE inhibition (mg GALAE/g)	BChE inhibition (mg GALAE/g)	Tyrosinase inhibition (mg KAE/g)	Amylase inhibition (mmol ACAE/g)	Glucosidase inhibition (mmol ACAE/g)
ASE	3.56±0.04^{c}	5.42±0.01^{d}	180.64±0.46^{a}	0.58±0.01^{ab}	1.66±0.01^{a}
Microwave	3.63±0.07^{c}	5.64±0.02^{c}	180.09±0.60^{a}	0.57±0.03^{b}	1.62±0.01^{bc}
Maceration	4.07±0.37^{b}	5.91±0.09^{b}	183.32±0.78^{a}	0.59±0.01^{a}	1.63±0.01^{bc}
Soxhlet	3.92±0.19^{b}	5.82±0.05^{b}	174.50±2.71^{b}	0.46±0.01^{c}	1.59±0.01^{d}
Ultrasonication	4.23±0.07^{a}	6.05±0.03^{a}	180.95±0.47^{a}	0.63±0.02^{a}	1.60±0.01^{b}

*Values expressed are means ± S.D. of three parallel measurements. AChE: Acetylcholinesterase; BChE: Butyrylcholinesterase; GALAE: Galantamine equivalent; KAE: Kojic acid equivalent; ACAE: Acarbose equivalent. Superscripts letters indicate significant differences in the extracts ($p < 0.05$).

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