European Journal of Integrative Medicine 37 (2020) 101171

Contents lists available at ScienceDirect

European Journal of Integrative Medicine

journal homepage: www.elsevier.com/locate/eujim

Research paper

Bioactivities of iridoids and flavonoids present in decoctions from aerial parts of *Verbascum betonicifolium*

Sezan R. Fadel[a], Hamdi Bendif[b,c], Laura Guedes[d], Rebeca André[d], Rita Pacheco[d,e], Rita Guedes[d], Karim Merabti[b], Mohamed Djamel Miara[f], Maria Luísa Serralheiro[d,g,*]

[a] Technical University of Dresden, Bergstrasse 66, 01069 Dresden, Germany
[b] Natural and Life Sciences Department, Faculty of Sciences, University of M'sila, 28000 Msila, Algeria
[c] Laboratoire d'Ethnobotanique et des Substances Naturelles, Département des Sciences Naturelles, Ecole Normale Supérieure (ENS), Kouba, 16308 Alger, Algeria
[d] University of Lisbon, Faculty of Sciences, BioISI - Biosystems & Integrative Sciences Institute, Campo Grande, 1749-016 Lisboa, Portugal
[e] Área Departamental de Engenharia Química, Instituto Superior de Engenharia de Lisboa, Av. Conselheiro Emídio Navarro, 1959-007 Lisboa, Portugal
[f] Department of Nature and Life Sciences, Faculty of Nature and Life Sciences, University Ibn Khaldoun, Tiaret, Algeria
[g] University of Lisbon, Faculty of Science, Department of Chemistry and Biochemistry, Campo Grande, 1749-016 Lisboa, Portugal

ARTICLE INFO

Keywords:
Verbascum betonicifolium
HRMS
Phytochemical composition
Antioxidant activity
Acetylcholinesterase inhibitory activity
Cytotoxicity

ABSTRACT

Introduction: *Verbascum betonicifolium* (*V. betonicifolium*) is a plant used in traditional medicine for several ailments. The objective of this study was to determine the antioxidant activity and acetylcholinesterase inhibitory activity of the aqueous extract together with the metabolites responsible for these activities. This paper presents the first phytochemical characterization and bioactivities of aqueous extracts from aerial parts.

Methods: The compounds present in the aerial part aqueous extract were identified by high performance liquid chromatography, coupled to a diode-array detector (HPLC-DAD) and by high-resolution mass spectrometry (HRMS), using LC–MS/MS analyses. Antioxidant activity was measured as the capacity to scavenge the free radical DPPH and the AChE activity was determined using the Ellman test. The cytotoxicity was determined used HepG2 cell lines.

Results: Several types of metabolites were found, primary metabolites (malic, citric and gluconic acids), phenolic compounds (verbascoside, luteolin), terpenoids (iridoid glycoside, unedide), among others. The total phenol content of 28 μg of gallic acid equivalents/mg of extract was determined. The aqueous extract antioxidant activity had and EC_{50} of 70 μg/mL and the AChE inhibitory activity an IC_{50} of 750 μg/mL. No cytotoxicity towards HepG2 cells was detected, even using a concentration of 1 mg/mL.

Conclusions: The phenolic compounds present in the extract may be the main contributors to the bioactivities of *V. betonicifolium*. These results show for the first time the richness of phytochemicals and the strong bioactivities of *V. betonicifolium* and that the aqueous extract could be used as new natural sources of bioactive molecules.

1. Introduction

Verbascum L. is the largest genus of the Scrophulariaceae family, which comprises more than 300 species of wild growing plants [1]. Several preparations, such as decoctions and infusions of *Verbascum* L., have been used in traditional medicines, for centuries, in almost all parts of the world [2]. They are traditionally consumed to relieve abdominal pains [3], for the treatment of inflammatory diseases, asthma, spasmodic coughs and other respiratory problems [4–6]. Constipation and inflammation are other illnesses which have shown improvement having been treated with *Verbascum* species decoctions and infusions [7].

Many other internal and external uses of *Verbascum* L. species have been documented in several societies in Europe, Asia, Africa and northern America [8]. During explorative research concerning various plants of *Verbascum* genus, several biological activities have been reported, such as antioxidant [1] and anti-cholinesterase [6,9], anti-inflammatory, antinociceptive and wound-healing, cytotoxicity activity against several cell lines [10], antitumor activity [11], immunomodulatory, antimicrobial, antimalarial and anthelmintic, antiviral [12], anti-ulcerogenic, antihyperlipidemic and hepatoprotective effect [13].

Acetylcholinesterase (AChE, E.C. 3.1.1.7) is an enzyme that catalyses the hydrolysis of the neurotransmitter acetylcholine. This enzyme

* Corresponding author at: Departamento de Química e Bioquímica, Faculdade de Ciências Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.
E-mail address: mlserralheiro@fc.ul.pt (M.L. Serralheiro).

https://doi.org/10.1016/j.eujim.2020.101171
Received 8 May 2020; Received in revised form 3 July 2020; Accepted 4 July 2020

is mainly localized in the synaptic gaps [14] and in the neuromuscular junctions [15,16]. The inhibition of AChE has been used to alleviate the symptoms of several diseases, like Alzheimer Disease [17,18] and severe constipation [19,20].

Alzheimer and inflammatory bowel diseases may also be associated with the existence of free radicals inside the cells [21]. The formation of these radicals may be due to the normal oxidative metabolism. Although there are endogenous cellular compounds to neutralize free radicals, this auto-controlling process sometimes is not enough. The existence of endogenous free radicals together with other factors can originate diseases like intestinal inflammation, Alzheimer and several others [22,23]. The antioxidant activity is an important biological activity since it is a natural defence mechanism against oxidations processes that may occur inside the cell. To help the natural defence mechanism it maybe important to consume beverages and other type of food containing antioxidants [21].

Some of the drugs used in clinic like galantamine, a reversible enzyme inhibitor with clinical applications, were found in plants used for natural medicine [23,24]. The search for antioxidants and enzyme inhibitors from natural origin is a matter of continuous research [23,25].

Several medicinal plants rich in phenolic compounds are known to exhibit antioxidant activity and AChE inhibitory capacity [25–27]. Phytochemical studies on *Verbascum* species have revealed the presence of many compounds which can be classified into eight main groups: saponins, iridoid glycosides, phenylethanoid glycosides, neolignan glucosides, flavonoids, acetophenone glucoside, phenolic acids and fatty acids [2,13].

The existence of several phytochemical compounds with phenolic structure in *Verbascum* L. species can hypothesize *V. betonicifolium* to also exhibit some of these biological activities.

Seeing that there are no reports about the detailed phytochemical profile and potential biological properties of *V. betonicifolium*, this work has as main objective contribute to the knowledge about this plant and explores the composition of *V. betonicifolium* aqueous extracts, its antioxidant and AChE inhibitory activities, as a possible contribution to the knowledge at molecular level of activities described previously as alleviating constipation and inflammation associated diseases together with its potential cytotoxicity against human liver cancer cells.

2. Materials and methods

2.1. Reagents and chemicals

All chemicals were analytical grade. Methanol, acetonitrile (ACN), water and formic acid are from Fisher Scientific, Optima™. Trifluoroacetic acid (TFA), potassium dihydrogen phosphate, sodium chloride and tris(hydroxymethyl)aminomethane (Tris) were bought from Merck. Ethanol 96 % was bought from Carlo Erba and potassium chloride from Fluka. Folin-Ciocalteu Reagent and sodium carbonate decahydrate were obtained from Fluka (Steinheim, Germany). Acetylcholinesterase (AChE), acetylcholine iodide (AChI), 5 − 5'-dithiobis (2-nitrobenzoic acid) (DTNB), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma. Magnesium chloride hexahydrate and sodium hydrogen phosphate were bought to Panreac. RPMI 1640 (RPMI), glutamine, Pen-Strep (penicillin and streptomycin mixture), foetal bovine serum (FBS) and trypsin were bought from Lonza (Verviers, Belgium).

2.2. Plant material

Aerial part (leaves, flowers and stems) of *V. betonicifolium sf.*family of Scrophulariacea were harvested in the Djurdjura medal of Algeria (from the vicinity of Bouira province, 800–1200 m a.s.l., N 36°27′03″, E 3° 56′03″), in May 2018. The botanical identification and authentication of the plant specimen has been confirmed by Dr. Merabti K. at the Department of Biology, M'sila University, Algeria using flora of Algeria [28].The reference specimen of the plant collected, namely (Msila univ. 01-2018-Bouira), was deposited in the herbarium of the Department of Biology of M'sila University, Algeria.

2.3. Aqueous extract

Before undergoing extraction, plant material was washed, dried at room temperature, protected from light for seven days and powdered to a fine grade by using a laboratory scale mill. The decoction was obtained by boiling the plant material in water for 20 min. After filtration an opalescent brown extract was obtained, freeze-dried and stored at − 20 °C. Yield of 11 %

2.4. Preparation of mucilage-free extract

To separate mucilages from the extract, 100 mg of the dried extract were macerated with 10 mL of distilled water and 40 mL ethanol and kept on ice for 2 h. After this time the mixture was centrifuged for 30 min at 4 °C and 5000 rpm and the upper phase was kept. The obtained pellet was mixed with 10 mL distilled water and 40 mL of ethanol and the procedure was repeated. The collected upper phase, designated as Extract without mucilages, was freeze-dried and stored at − 20 °C. The yield was 57 %.

2.5. HPLC-DAD analysis

The chromatographic analyses were carried out on a VWR-Hitachi Elite LaChrom (VWR, Hitachi, Japan) with a LiChroCART RP-18, 100 Å, 250 × 4 mm, 5 µm column (Merck), a L-2200 automatic injector, a L-2300 column oven and a L-2455 photodiode detector (DAD). The software in the data acquisition was the EZChrom Elite (Hitachi-VWR International). For each run were injected 25 µL of the solution to analyse (1 mg dried extract/mL), using the elution process described in [29].

2.6. Total phenolic content (TPC)

Total phenolic content (TPC) was determined according to Folin-Ciocalteu method with some minor modifications as described in [30]. For the analysis of the TPC, 10 mg dried extract/mL solutions of the plant aqueous extract and the extract without mucilages were prepared in water. The method was carried out and the absorbance of the solutions was recorded on an UV–vis recording spectrometer (UV-160U-Schimadzu) at 760 nm. TPC was quantified in a calibration curve prepared by the same method using a set of standard gallic acid solutions of different concentrations. The TPC was expressed as µg gallic acid equivalents (GA)/mg extract.

2.7. LC–MS/MS analysis

The chromatographic analyses were carried out in an Elute autosampler UHPLC (Bruker, Bremen, Germany), using an Intensity Solo 2 RP-18, 100 × 2.1 mm, 1.8 µm column (Bruker, Bremen, Germany). A volume of 5 µL was injected (auto injector) into the system using a gradient composed by water with 0.1 % formic acid (eluent A) and ACN with 0.1 % formic acid (eluent B) as follows: 0 min – 95 % A; 1.5 min – 95 % A; 13.5 min – 25 % A; 18.5 min – 0 % A; 21.5 min – 0 % A; 23.5 min – 95 % A; 30 min – 95 % A. The flow rate was set at 0.250 mL/min and the column was kept at 35 °C.

For mass spectrometry, an Impact II QTOF (Bruker, Bremen, Germany) was used and the data was acquired through the DataAnalysis 4.4 software as described in [29] and in more detail in Data in Brief [31].

2.8. Evaluation of antioxidant activity

The ability of the extracts to scavenge DPPH radicals was determined following the procedure described in [32]. The test was carried out in triplicate. Inhibition of DPPH in percent (I, %) was calculated as given below:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100 \quad (1)$$

A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the extract.

2.9. Determination of acetylcholinesterase activity

The AChE activity of the extracts was determined with DTNB absorption test, according to [32]. The result of the hydrolysis of AChI was the formation of the yellow 5-thio-2-nitrobenzoate anion which is the reaction of DTNB with thiocholines, catalysed by enzymes which were recorded with a UV–vis recording spectrometer (UV-160U Schimadzu) at a wavelength of 405 nm.

2.10. Cytotoxicity

The cytotoxicity of the plant extracts on HepG2 was determined by the MTT assay [33]. The positively charged MTT penetrate the viable cells that convert MTT to formazan crystals, which are soluble in methanol. Their absorbance values can be recorded at 595 nm with a reference wavelength of 630 nm in an automated microplate reader. The HepG2 cell culture was grown according to the methodology described in [32]. The percentage of cells viability C_V (%) was calculated by the following equation:

$$C_V\ (\%) = (\text{sample/control}) \times 100 \quad (2)$$

2.11. Statistical analysis

All the values are replicate of at least 3 experiments and comparison between the mean values was carried using tests F in ANOVA analysis at a confidence level of 95 % (a = 0.05), using Excell Microsoft™ program.

3. Results

The aerial part of *V. betonicifolium* was analyzed by preparing a plant decoction (using boiling water) in a concentration similar to the one that may be consumed in an herbal drink (10 g/100 mL, a cup of tea). Since this process co-extracts mucilages and non-starch polysaccharides [34] an ethanol precipitation of these compounds (extract free of mucilages) was carried out [35]. Both extracts were compared during the bioactivity study.

3.1. Phytochemical profile

In order to have some insight about the phenolic compounds that might be responsible for the bioactivities, the extracts were analyzed by HPLC-DAD, Fig. 1. Fig. 1(a) shows the complete plant extract and Fig. 1(b) the mucilage-free extract. The chromatograms indicate that the compounds are the same however there was an increase of approximately 63 % in the amount of almost all the compounds after the removal of mucilages. This is in accordance with the TPC values determined in the extracts and may explain why the extract without mucilages always indicated a higher activity.

Several compounds are eluted in the beginning of the chromatogram indicating the presence of hydrophilic compounds (highly water soluble). The UV–vis analysis (Fig. 2) indicates the presence of several caffeic acid derivatives, probably chlorogenic acid and verbascoside, usually described in *Verbascum* genus. The UV–vis spectrum of

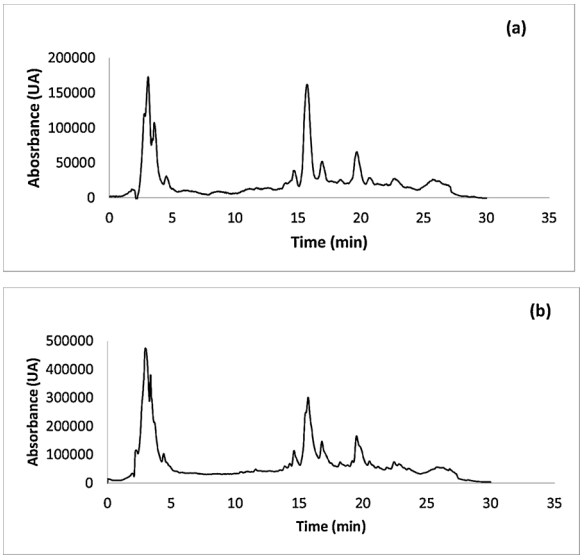

Fig. 1. RP-HPLC-DAD of decoction from *V. betonicifolium*: (a) complete decoction; (b) mucilage-free extract.

compounds eluted at retention time 14.72 and 16.93 min show resemblance with the derivatives of caffeic acid [36], Fig. 2a–c and with flavonoid UV–vis spectrum Fig. 2d [36].

3.2. Compound identification in V. betonicifolium mucilage-free extract

The compounds present in *V. betonicifolium* mucilage-free extract were identified by using an LC–MS/MS. The extract without mucilages was analyzed in positive and negative mode, but the identification was carried out only in negative mode where a higher number of compounds was found, also with higher intensity. The chromatogram in the negative mode is shown in Data in Brief [31].

The compounds were tentatively identified by using the exact mass and the formula suggestions by the data acquired in the DataAnalysis™. PubChem and Metlin databases were also used to propose a chemical structure and mass fragmentation was applied to confirm the hypotheses. The tentative identification of some of the compounds present in the mucilage free extract can be seen in [31]. Six types of plant metabolites were found: primary metabolites like malic, gluconic and citric acids, representing 36 % of the identified compounds; secondary metabolites like flavonoid derivatives (apigenin, luteolin) and several glycosylated derivatives, representing 27 % of the identified compounds; caffeic acid derivatives (chlorogenic acid) and several flavonoid derivatives representing 9 % of the identified compounds; iridoids like methylscutelloside, scropheanoside, saccatoside and so on, representing 30 % of the identified compounds; one lignan and a lactone sesquiterpenoid were also tentatively suggested. This type of chemical structures has been detected in other *Verbascum* species [2,13]. Recently other *Verbascum* species were reported to have verbascoside and chlorogenic acid together with other phenolic compounds as major compounds in an ethanol-aqueous extract [37]. The most intense compounds are in fact the primary metabolites, representing 36 % of the total, followed by iridoids and flavonoids, as shown in the heatmap in Fig. 3.

The compounds identified in other *Verbascum* sp. are mainly iridoids, caffeic acid derivatives and flavonoids. Verbascoside and chlorogenic acid can be referred as caffeic acid derivatives while apigenin, luteolin and their glycosidic derivatives as members of the flavonoid type of compounds. These compounds were also detected in other *Verbascum* sp., mainly verbascoside [38]. Iridoids like unedide, shanzhiside, mehylscutelloside, saccatoside [39], sauroposide and scropheanoside II were detected. Iridoids have been found in several

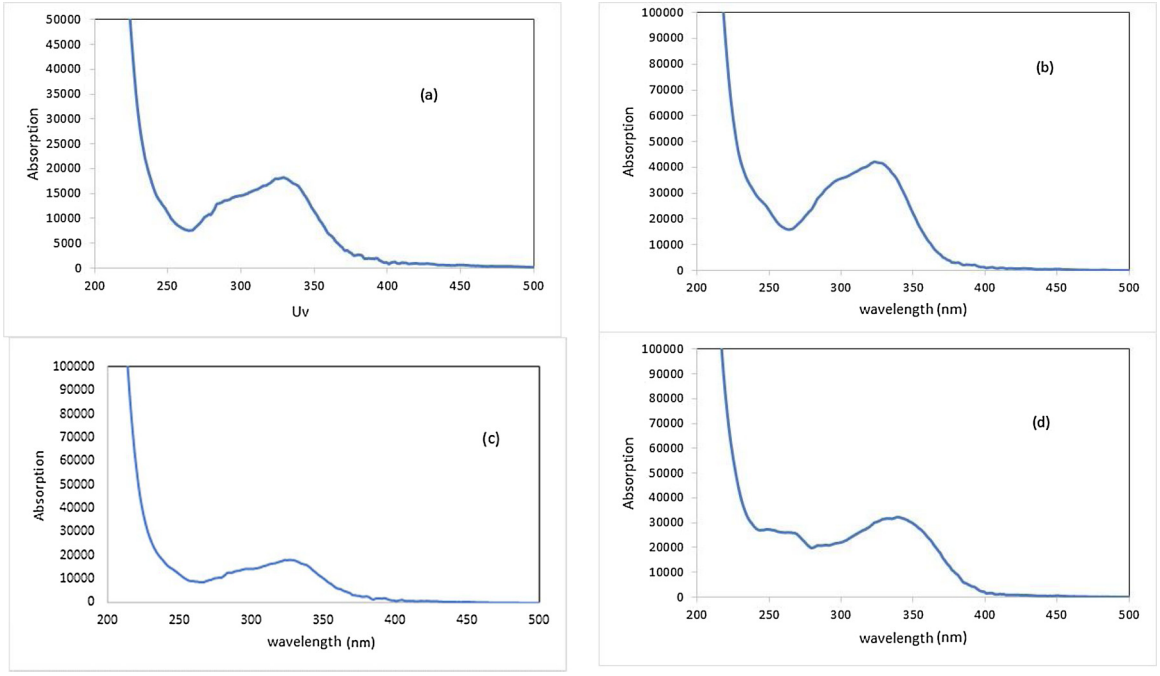

Fig. 2. UV–vis spectrum of 4 compounds appearing on the RP-HPLC-DAD; (a) Rt = 14.7 min; (b) Rt = 16.9 min; (c) Rt = 20.7 min; (d) Rt = 19.7 min.

Verbascum species [13] but these reported here have also been reported by other researchers in *V. betonicifolium.*

3.3. Total phenolic content (TPC)

The *V. betonicifolium* aqueous extract contains several phenolic compounds. In order to compare the amount of phenolics in the extract TPC was determined by the conventional spectrophotometric method using Folin-Ciocalteu. Both extracts were analysed. TPC was determined and presented in Table 1.

TPC of the extract without mucilages is higher than the complete extract. There was an increase in 52 % in the phenolic content by the

Rt	Compound	Intensities	Metabolites
1.1	malic acid		primary
1.3	gluconic acid		primary
1.8	citric acid		primary
1.8	6-*O*-acetylascorbic acid		primary
1.9	unedide		iridoid
2.5	shanzhiside		iridoid
3.9	glycoside derivative		primary
4.1	mehylscutteloside		iridoid
5.1	chlorogenic acid		caffeoyl derivative
5.7	luteolin 7-diglucoronide		flavonoid
5.8	clerodendrin		flavonoid
6.1	flavonoid derivative		flavonoid
6.5	verbascoside		caffeoyl derivative
6.6	luteolin 7-O-glucoside		flavonoid
6.6	luteolin 7-O-glucuronide		flavonoid
6.6	saccatoside		iridoid
6.7	sauroposide		lignan
6.8	scropheanoside II		iridoid
7.1	apigenin 7-O-glucoside		flavonoid
7.2	apigenin derivative		flavonoid
8.4	luteolin		flavonoid
9.2	apigenin		flavonoid
11.7	phytuberin		sesquiterpenoid

Fig. 3. Heatmap representing the intensities of each compound in the aqueous *V. betonicifolium* extract (represents intensity 137316; represents intensity 1672).

Table 1
Total phenolic content and biological activities of V. betonicifolium extracts. The upper letters for each row indicated values different at α = 0.05.

Tests	Extract	Extract without mucilages
Total phenolic content (μg GA eq/mg)	18.9 ± 0.5[a]	28.7 ± 0.9[b]
Antioxidant activity (DPPH test) EC_{50} (μg/mL)	184.1 ± 2.9[a]	70.6 ± 2.1[b]
AChE inhibitory activity IC_{50} (μg/mL)	1200 ± 3[a]	750 ± 6[b]
HepG2 viability (1 mg/mL)	100 % AChE	– DPPH
Galantamine IC_{50} (μg/mL)	0.22 [34]	–
BHT IC_{50} (μg/mL)	–	15 [29]

withdrawal of polysaccharides from the aqueous extract. TPC in the mucilage-free extract represents 2.8 %, a value similar to that indicated in another *Verbascum* sp [9].

3.4. Antioxidant activity

The antioxidant activity was evaluated in the present work using the DPPH test. The DPPH test indicates the capacity of the compounds present in the extractions to scavenge free radicals that may exist in the biological systems.

Several concentrations of the extracts were used to determine if a relationship existed between the concentration of the polyphenols and their antioxidant activity. Fig. 4 shows the effect of increasing both extracts concentration on the capacity to scavenge the DPPH free radical. A correlation of 0.984 and 0.964 for plant extract and mucilage-free extract was obtained. The EC_{50} value for each extract was calculated and indicated in Table 1. The withdrawal of mucilages increased the antioxidant activity in 62 %. For the mucilage-free extract an EC_{50} of 70.6 μg/mL was determined, a value of the same magnitude as the standard BHT 15.7 μg/mL [30], meaning a high antioxidant activity. This aqueous extract is similar to the antioxidant activities of other *Verbascum* sp. [9,40,41].

3.5. Acetylcholinesterase activity

The decoction from *V. betonicifolium* was used to determine its AChE inhibition capacity of the complete extract and the mucilage-free extract. There was a correlation between the amount of the extract and the inhibition activity of AChE, 0.995 and 0.984 for the complete decoction and decoction without mucilages. IC_{50} values were calculated from figures relating to the inhibitory activity with the extract concentration. The extract concentration providing 50 % inhibition (IC_{50}) was calculated from the regression analysis between the inhibitory activity of the decoctions and their concentration, indicated in Table 1. Once again the mucilage free extract has a higher inhibitory activity than the complete one. The IC_{50} value determined for *V. betonicifolium* is of the same magnitude as those obtained with other plants [32,36,42–44]. Other *Verbascum* species tested for AChE inhibitory activities either used methanolic extract [9] or, when using aqueous extract, did not test its activity, but instead the isolated compounds [6].

3.6. Cytotoxicity

The complete extract cytotoxicity was studied using liver simulating cells. HepG2 cell line obtained from a cancer liver has the biochemical metabolism of liver cells and is used to study cytotoxicity of compounds towards liver cancer cells [45]. Different concentrations from the decoction were tested on what concerns the capacity to kill these cancer cells. Concentrations of the decoction were used from 0.1 mg/mL to 1 mg/mL and the cells were able to keep their viability even under the highest concentration (Table 1). The cells viability in the presence of the plant extract is in all concentrations around 100 % demonstrating that the cells are not cytotoxic and do not contain compounds able to kill liver cancer cells. In fact, other *Verbascum* species were not cytotoxic [46].

4. Discussion

Secondary metabolites are usually considered bioactive molecules, that is compounds with no nutritional value but biochemically active inside the human body, what may justify some of the properties described by medicinal plant users. *V. betonicifolium* extract showed an antioxidant activity measured as the capacity to scavenge free radicals similar to the BHT used in Food industry [47]. This antioxidant activity although being a result of all the metabolites present in the extract, is mainly due to flavonoids as they possess double bonds adjacent to hydroxyl groups. Flavonoids have been shown to possess high antioxidant activity, for instance other natural products like honey, containing flavonoids have demonstrated high antioxidant activity [48,49]. Iridoids and natural organic acids lack in their chemical structure a double bond to stabilize the radical formed by the loss of a hydrogen atom to the free radical DPPH. Flavonoids have shown antioxidant activity, mainly having an −OH at position C_3 [50] and hydroxyl groups in 3′, 4′ carbon in ring B of the flavonoid structure [51] as is the case of the flavonoids detected in the extract of *V. betonicifolium*.

The capacity of flavonoids to inhibit AChE has been well documented [42,52–55]. The aglycon has a higher AChE inhibitory activity than the glycosylated derivatives [56] because it fits better inside the enzyme active site [57]. In *V. betonicifolium* extract, luteolin and apigenin have low intensities but the number of total flavonoids is high (nine compounds detected) being seven of them glycosylated and all together can account for the IC_{50} value determined. Apigenin and luteolin, although in a low amount have IC_{50} values oscillating between 2 μg/mL and 25 μg/mL [58,59]. Caffeic acid derivatives like chlorogenic acid and verbascoside, also detected in the aqueous *V. betonicifolium* extract, although with very low intensities, they also have capacity to

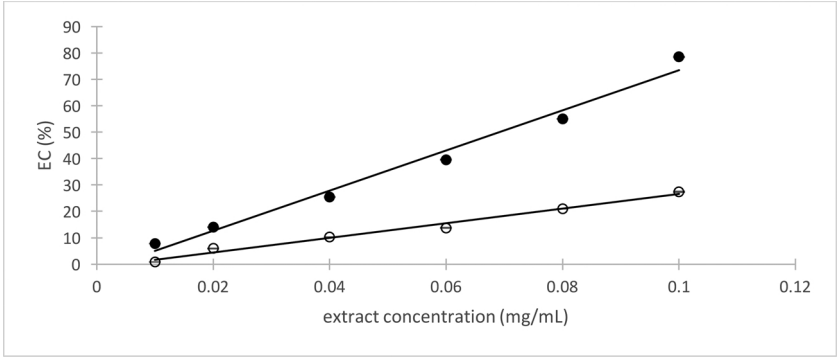

Fig. 4. Antioxidant activity (%) of *V. betonicifolium* aqueous extract: (○)- with mucilages; (●)-mucilage-free extract.

inhibit the activity of AChE. Chlorogenic acid has IC_{50} of 196 μg/mL [36] and verbascoside approximately 48 μg/mL [9].

The capacity of iridoids to inhibit AChE is not high, although the bibliography refers high activity for acetylcholinesterase inhibition by iridoids [60], gentiopicroside, an iridoid detected in another genus, *Centaurium erythraea* [29] was devoided of enzyme inhibitory activity. This was explained by theoretical docking studies carried out with gentiopicroside and AChE active site. These studies showed that the inhibition constant is higher for gentiopicroside comparatively to the polyphenolic compounds and it does not establish strong interactions with the groups of the amino acid residues in the enzyme active site [29] leading to higher inhibition values.

The *V. betonicifolium* extract, although containing iridoids or primary metabolites with low antioxidant and inhibition of acetylcholinesterase activity, contains flavonoids and other phenolic compounds with reported AChE inhibition and antioxidant potential and therefore mostly on account of the later compounds, showed to have an IC_{50} of the same order of magnitude of extracts containing mixtures of flavonoids and phenolic acids reported in the literature [32,36,44].

BHT was shown to have adverse effects in laboratory animals [61], so aqueous extract of this plant seems to be a good alternative to chemical antioxidants as they have been consumed for centuries with no adverse registered effects and having simultaneously other health benefits like digestive improvement.

The present study had the main limitation of not doing a better purification of the secondary metabolites. The future work will be to purify the extract through an SPE column where several hydrophilic primary metabolites that do not have either antioxidant activity or acetylcholinesterase inhibitory capacity can be discarded. This purification will increase the concentration of secondary metabolites per mg of extract, leading to higher bioactivities. To get a better insight into the class of compounds that present the antioxidant or the enzyme inhibitory activity, a separation step between the two types of chemical structures, iridoids and flavonoids, could also be introduced in the purification method, mainly by preparative RP-HPLC-DAD.

5. Conclusions

Systematic studies of medicinal plants namely *V. betonicifolium* are reported here for the first time. Aqueous extract from aerial part of *V. betonicifolium* are a rich source of antioxidant, also showing capacity to inhibit acetylcholinesterase. These activities are mainly due to the polyphenols, especially flavonoids and phenolic acids present in the decoction of this herb, which seems to be a good promising source of bioactive molecules. The antioxidant activities displayed by aqueous *V. betonicifolium* extracts may justify a future inclusion of this species in the list of natural additives to be used in foodstuffs for their preservative properties. This is a plant worthwhile to explore as inhibitor of AChE activity facilitating the digestion and also on what concerns antioxidant activity, leading to the development of new process of aromatizing and giving nutritional value to oils and other foods.

Funding sources

This work was funded by the Portuguese Foundation for Science and Technology, FCT, through the Research Unit grant UIDB/04046/2020 and UIDP/04046/2020 to BioISI; This work was also funded by FCT research contract to project PTDC/BIA-BQM/28355/2017; This project was also funded by the BioISIMassSPEC Facility of BioISI.

Data availability

All the data presented here and in the Data in Brief [31] will be given upon request to the corresponding author

CRediT authorship contribution statement

Sezan R. Fadel: Methodology. **Hamdi Bendif:** Writing - original draft. **Laura Guedes:** . **Rebeca André:** . **Rita Pacheco:** . **Rita Guedes:** . **Karim Merabti:** . **Mohamed Djamel Miara:** . **Maria Luísa Serralheiro:** Conceptualization, Formal analysis, Funding acquisition, Supervision.

Declaration of Competing Interests

There were no commercial involvement in the conduct of the study.

Acknowledgments

Sezan Fadel thanks the ERASMUS exchange of students Program between the Technical University of Dresden, Dresden, Germany and Faculty of Science of the University of Lisbon, Portugal; Rita Guedes thanks BioISI for the Master Grant; Laura Guedes thanks a grant from FCT project; Rebeca André thanks FCT for a PhD grant PD/BD/142861/2018.

References

[1] N. Aligiannis, S. Mitaku, E. Tsitsa-Tsardis, C. Harvala, I. Tsaknis, S. Lalas, S. Haroutounian, Methanolic extract of *Verbascum macrurum* as a source of natural preservatives against oxidative rancidity, J. Agric. Food Chem. 51 (2003) 7308–7312, https://doi.org/10.1021/jf034528+.
[2] I.I. Tatli, Z.S. Akdemir, Chemical constituents of *Verbascum* L. species, FABAD J. Pharm. Sci. 29 (2004) 93–107.
[3] A.U. Turker, E. Gurel, Common mullein (*Verbascum thapsus* L.): recent advances in research, Phyther. Res. 19 (2005) 733–739, https://doi.org/10.1002/ptr.1653.
[4] M. Şengul, H. Ögutcu, A. Adiguzel, F. Şahin, A.A. Kara, İ. Karaman, M. Gulluce, Antimicrobial effects of *Verbascum georgicum* Bentham extract, Turk. J. Biol. 29 (2005) 105–110.
[5] I.I. Tatli, T.F. Erdogan, C. Kahraman, B. Kivcak, Z.S. Akdemir, Brine shrimp lethality bioassay of some *Verbascum* species growing in Turkey, FABAD J. Pharm. Sci. 33 (2008) 193–198.
[6] C. Kahraman, I. Tatli, I.E. Orhan, Z.S. Akdemir, Cholinesterase inhibitory and antioxidant properties of *Verbascum mucronatum* Lam. And its secondary metabolites, Zeitschrift Fur Naturforsch. C 65 (2010) 667–674, https://doi.org/10.1515/znc-2010-11-1206.
[7] M. Ghoshal, Mulling Over Mullein Leaf, (2020) (Accessed 5 June 2020), https://www.healthline.com/health/mullein-leaf.
[8] B. Meurer-Grimes, D.L. McBeth, B. Hallihan, S. Delph, Antimicrobial activity in medicinal plants of the Scrophulariaceae and Acanthaceae, Int. J. Pharmacogn. 34 (1996) 243–248, https://doi.org/10.1076/phbi.34.4.243.13220.
[9] M. Georgiev, K. Alipieva, I. Orhan, R. Abrashev, P. Denev, M. Angelova, Antioxidant and cholinesterases inhibitory activities of *Verbascum xanthophoeniceum* Griseb. and its phenylethanoid glycosides, Food Chem. 128 (2011) 100–105, https://doi.org/10.1016/j.foodchem.2011.02.083.
[10] W.H. Talib, A.M. Mahasneh, Antiproliferative activity of plant extracts used against cancer in traditional medicine, Sci. Pharm. 78 (2010) 33–45, https://doi.org/10.3797/scipharm.0912-11.
[11] M. Riaz, M. Zia-Ul-Haq, H.Z.E. Jaafar, Common mullein, pharmacological and chemical aspects, Rev. Bras. Farmacogn. 23 (2013) 948–959, https://doi.org/10.1590/S0102-695X2013000600012.
[12] M. Rajbhandari, R. Mentel, P.K. Jha, R.P. Chaudhary, S. Bhattarai, M.B. Gewali, N. Karmacharya, M. Hipper, U. Lindequist, Antiviral activity of some plants used in Nepalese traditional medicine, evidence-based complement, Altern. Med. 6 (2009) 517–522, https://doi.org/10.1093/ecam/nem156.
[13] C. Kahraman, Z.S. Akdemir, I.I. Tatli, Promising cytotoxic activity profile, biological activities and phytochemical screening of *Verbascum* L. species, Med. Aromat. Plant Sci. Biotechnol. 6 (2012) 63–75.
[14] J.O. Rinne, V. Kaasinen, T. Järvenpää, K. Någren, A. Roivainen, M. Yu, V. Oikonen, T. Kurki, Brain acetylcholinesterase activity in mild cognitive impairment and early Alzheimer's disease, J. Neurol. Neurosurg. Psychiatry 74 (2003) 113–115, https://doi.org/10.1136/jnnp.74.1.113.
[15] R. Gašperšič, B. Koritnik, N. Črne-Finderle, J. Sketelj, Acetylcholinesterase in the neuromuscular junction, Chem. Biol. Interact. 119–120 (1999) 301–308, https://doi.org/10.1016/S0009-2797(99)00040-X.
[16] E. Blotnick-Rubin, L. Anglister, Fine localization of acetylcholinesterase in the synaptic cleft of the vertebrate neuromuscular junction, Front. Mol. Neurosci. 11 (2018) 1–11, https://doi.org/10.3389/fnmol.2018.00123.
[17] M. Heinrich, H. Lee Teoh, Galanthamine from snowdrop-the development of a modern drug against Alzheimer's disease from local Caucasian knowledge, J. Ethnopharmacol. 92 (2004) 147–162, https://doi.org/10.1016/j.jep.2004.02.012.
[18] NIA: National Institute of Aging, Alzheimer's Disease Fact Sheet, https://www.nia.nih.gov/health/alzheimers-disease-fact-sheet.

[19] E.M. Jarvie, S. Cellek, G.J. Sanger, Potentiation by cholinesterase inhibitors of cholinergic activity in rat isolated stomach and colon, Pharmacol. Res. 58 (2008) 297–301, https://doi.org/10.1016/j.phrs.2008.09.001.

[20] K. Nagahama, Y. Matsunaga, M. Kawachi, K. Ito, T. Tanaka, Y. Hori, H. Oka, M. Takei, Acotiamide, a new orally active acetylcholinesterase inhibitor, stimulates gastrointestinal motor activity in conscious dogs, Neurogastroenterol. Motil. 24 (2012) 566–574, https://doi.org/10.1111/j.1365-2982.2012.01912.x.

[21] P. Arulselvan, M.T. Fard, W.S. Tan, S. Gothai, S. Fakurazi, M.E. Norhaizan, S.S. Kumar, Role of antioxidants and natural products in inflammation, Oxid. Med. Cell. Longev. 2016 (2016), https://doi.org/10.1155/2016/5276130.

[22] E. Niki, Do free radicals play causal role in atherosclerosis? Low density lipoprotein oxidation and vitamin E revisited, J. Clin. Biochem. Nutr. 48 (2011) 3–7, https://doi.org/10.3164/jcbn.11-007FR.

[23] Y. Ma, M.W. Yang, X.W. Li, J.W. Yue, J.Z. Chen, M.W. Yang, X. Huang, L.L. Zhu, F.F. Hong, S.L. Yang, Therapeutic effects of natural drugs on Alzheimer's disease, Front. Pharmacol. 10 (2019) 1–20, https://doi.org/10.3389/fphar.2019.01355.

[24] H.A. Mucke, The case of galantamine: repurposing and late blooming of a cholinergic drug, Futur. Sci. OA 1 (2015) 1–6, https://doi.org/10.4155/fso.15.73.

[25] D.P. Xu, Y. Li, X. Meng, T. Zhou, Y. Zhou, J. Zheng, J.-J. Zhang, H.-B. Li, Natural antioxidants in foods and medicinal plants: extraction, assessment and resources, Int. J. Mol. Sci. 18 (2017) 1–32, https://doi.org/10.3390/ijms18010096.

[26] R. Tundis, M. Bonesi, F. Menichini, M.R. Loizzo, Recent knowledge on medicinal plants as source of cholinesterase inhibitors for the treatment of dementia, Mini Rev. Med. Chem. 16 (2016) 605–618, https://doi.org/10.2174/1389557515666150709104731.

[27] T.C. Dos Santos, T.M. Gomes, B.A.S. Pinto, A.L. Camara, A.M. De Andrade Paes, Naturally occurring acetylcholinesterase inhibitors and their potential use for Alzheimer's disease therapy, Front. Pharmacol. 9 (2018) 1–14, https://doi.org/10.3389/fphar.2018.01192.

[28] P. Quézel, S.A. Auteus-Santa, Nouvelle flore de l'Algérie et des régions désertiques méridionales, Paris, (1962) https://bibliotheques.mnhn.fr/medias/doc/exploitation/HORIZON/493163/nouvelle-flore-de-l-algerie-et-des-regions-desertiques-meridionales.

[29] L. Guedes, P. Reis, M. Machuqueiro, A. Ressaissi, R. Pacheco, M.L. Serralheiro, Bioactivities of *Centaurium erythraea* (Gentianaceae) decoctions: antioxidant activity, enzyme inhibition and docking studies, Molecules 24 (2019) 1–18, https://doi.org/10.3390/molecules24203795.

[30] A.T. Mata, C. Proença, A.R. Ferreira, M.L.M. Serralheiro, J.M.F. Nogueira, M.E.M. Araújo, Antioxidant and antiacetylcholinesterase activities of five plants used as Portuguese food spices, Food Chem. 103 (2007) 778–786, https://doi.org/10.1016/j.foodchem.2006.09.017.

[31] M.L. Serralheiro, R. Guedes, S.R. Fadel, H. Bendif, Data on identification of primary and secondary metabolites in aqueous extract of *Verbascum betonicifolium*, Data in Brief, submitted (2020).

[32] P.L. Falé, C. Ferreira, A.M. Rodrigues, P. Cleto, P.J.A. Madeira, M.H. Florêncio, F.N. Frazão, M.L.M. Serralheiro, Antioxidant and anti-acetylcholinesterase activity of commercially available medicinal infusions after in vitro gastrointestinal digestion, J. Med. Plants Res. 7 (2013) 1370–1378, https://doi.org/10.5897/jmpr13.4438.

[33] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63, https://doi.org/10.1016/0022-1759(83)90303-4.

[34] J.M. Cruz-Rubio, R. Loeppert, H. Viernstein, W. Praznik, Trends in the use of plant non-starch polysaccharides within food, dietary supplements, and pharmaceuticals: beneficial effects on regulation and wellbeing of the intestinal tract, Sci. Pharm. 86 (2018) 1–13, https://doi.org/10.3390/scipharm86040049.

[35] M. Edmond Ghanem, R.-M. Han, B. Classen, J. Quetin-Leclerq, G. Mahy, C.-J. Ruan, P. Qin, F. Pérez-Alfocea, S. Lutts, Mucilage and polysaccharides in the halophyte plant species *Kosteletzkya virginica*: localization and composition in relation to salt stress, J. Plant Physiol. 167 (2010) 382–392, https://doi.org/10.1016/j.jplph.2009.10.012.

[36] M. Hernandez, P. Falé, M. Araújo, M. Serralheiro, Acetylcholinesterase inhibition and antioxidant activity of the water extracts of several *Hypericum* species, Food Chem. 120 (2010) 1076–1082, https://doi.org/10.1016/j.foodchem.2009.11.055.

[37] A. Dalar, A.S. Bengu, O. Allahverdiyev, Analysis of phytochemical composition and biological activities of *Verbascum cheiranthifolium* var. cheirenathifolium stem and flowers, Int. J. Second. Metab. 5 (2018) 233–242, https://doi.org/10.21448/ijsm.401127.

[38] V. Mihailović, S. Kreft, E.T. Benković, N. Ivanović, M.S. Stanković, Chemical profile, antioxidant activity and stability in stimulated gastrointestinal tract model system of three *Verbascum* species, Ind. Crops Prod. 89 (2016) 141–151, https://doi.org/10.1016/j.indcrop.2016.04.075.

[39] S.V. Luca, M.E. Czerwińska, A. Miron, A.C. Aprotosoaie, L. Marcourt, J.-L. Wolfender, S. Granica, K. Skalicka-Woźniak, High-performance countercurrent chromatographic isolation of acylated iridoid diglycosides from *Verbascum ovalifolium* Donn ex Sims and evaluation of their inhibitory potential on IL-8 and TNF-α production, J. Pharm. Biomed. Anal. 166 (2019) 295–303, https://doi.org/10.1016/j.jpba.2019.01.031.

[40] G. Öztürk, H.G. Ağalar, G. Yildiz, F. Göger, N. Kirimer, Biological activities and luteolin derivatives of *Verbascum eskisehirensis* Karavel., Ocak Ekici, J. Res. Pharm. 23 (2019) 532–542, https://doi.org/10.12991/jrp.2019.160.

[41] G. Paun, E. Neagu, C. Albu, G.L. Radu, *Verbascum phlomoides* and *Solidago virgaureae* herbs as natural source for preventing neurodegenerative diseases, J. Herb. Med. 6 (2016) 180–186, https://doi.org/10.1016/j.hermed.2016.08.001.

[42] A. Ferreira, C. Proença, M.L.M. Serralheiro, M.E.M. Araújo, The in vitro screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal, J. Ethnopharmacol. 108 (2006) 31–37, https://doi.org/10.1016/j.jep.2006.04.010.

[43] A.A. Arantes, P.L. Falé, L.C.B. Costa, R. Pacheco, L. Ascensão, M.L. Serralheiro, Inhibition of HMG-CoA reductase activity and cholesterol permeation through Caco-2 cells by caffeoylquinic acids from *Vernonia condensata* leaves, Braz. J. Pharmacogn. 26 (2016) 738–743, https://doi.org/10.1016/j.bjp.2016.05.008.

[44] A. Ressaissi, N. Attia, P.L.V. Falé, R. Pacheco, V.H. Teixeira, Aqueous extracts from Nopal (Opuntia ficus-indica): antiacetylcholinesterase and antioxidant activity from phenolic bioactive compounds, Int. J. Green Herb. Chem. 5 (2016) 337–348.

[45] S. Sefried, H.-U. Häring, C. Weigert, S.S. Eckstein, Suitability of hepatocyte cell lines HepG2, AML12 and THLE-2 for investigation of insulin signalling and hepatokine gene expression, Open Biol. 8 (2018), https://doi.org/10.1098/rsob.180147.

[46] İ.İ. Tatli, Z.S. Akdemir, Cytotoxic activity on some *Verbascum* species growing in Turkey, Hacettepe Univ. J. Fac. Pharm. 26 (2006) 77–85.

[47] M. Kebede, S. Admassu, Application of antioxidants in food processing industry: pptions to improve the extraction yields and market value of natural products, Adv. Food Technol. Nutr. Sci. 5 (2019) 38–49, https://doi.org/10.17140/aftnsoj-5-155.

[48] M.B. Bahadori, G. Zengin, L. Dinparast, M. Eskandani, The health benefits of three Hedgenettle herbal teas (*Stachys byzantina*, *Stachys inflata*, and *Stachys lavandulifolia*) - profiling phenolic and antioxidant activities, Eur. J. Integr. Med. 36 (2020) 101134, , https://doi.org/10.1016/j.eujim.2020.101134.

[49] H. Zaidi, S. Ouchemoukh, N. Amessis-Ouchemoukh, N. Debbache, R. Pacheco, M.L. Serralheiro, M.E. Araujo, Biological properties of phenolic compound extracts in selected Algerian honeys - the inhibition of acetylcholinesterase and α-glucosidase activities, Eur. J. Integr. Med. 25 (2019) 77–84, https://doi.org/10.1016/j.eujim.2018.11.008.

[50] M.M. Silva, M.R. Santos, G. Caroço, R. Rocha, G. Justino, L. Mira, Structure-antioxidant activity relationships of flavonoids: a re-examination, Free Radic. Res. 36 (2002) 1219–1227, https://doi.org/10.1080/198-1071576021000016472.

[51] D. Amić, D. Davidović-Amić, D. Bešlo, N. Trinajstić, Structure-radical scavenging activity relationships of flavonoids, Croat. Chem. Acta 76 (2003) 55–61, https://doi.org/10.1016/j.phytochem.2006.07.002.

[52] I. Uriarte-Pueyo, M.I. Calvo, Flavonoids as acetylcholinesterase inhibitors, Curr. Med. Chem. 18 (2011) 5289–5302, https://doi.org/10.2174/092986711798184325.

[53] P.L.V. Falé, P.J.A. Madeira, M.H. Florêncio, L. Ascensão, M.L.M. Serralheiro, Function of *Plectranthus barbatus* herbal tea as neuronal acetylcholinesterase inhibitor, Food Funct. 2 (2011) 130–136, https://doi.org/10.1039/c0fo00070a.

[54] A. Khadhri, I. Bouali, S. Belkhir, R. Mokded, S. Smiti, P. Falé, M.E.M. Araújo, M.L.M. Serralheiro, In vitro digestion, antioxidant and antiacetylcholinesterase activities of two species of ruta: *ruta chalepensis* and *Ruta montana*, Pharm. Biol. 55 (2017) 101–107, https://doi.org/10.1080/13880209.2016.1230634.

[55] J.T.C. Júnior, S.M. de Morais, L.G. Vieira, J. de B. Alexandre, M. do S. Costa, M.F.B. Morais-Braga, J.E.G.L. Júnior, M.M.O. Silva, L.M. Barros, H.D.M. Coutinho, Phenolic composition and anticholinesterase, antioxidant, antifungal and antibiotic modulatory activities of *Prockia crucis* (Salicaceae) extracts collected in the Caatinga biome of Ceará State, Brazil, Eur. J. Integr. Med. 7 (2015) 547–555, https://doi.org/10.1016/j.eujim.2015.04.006.

[56] Y. Xie, W. Yang, X. Chen, J. Xiao, Inhibition of flavonoids on acetylcholine esterase: binding and structure-activity relationship, Food Funct. 5 (2014) 2582–2589, https://doi.org/10.1039/c4fo00287c.

[57] M.T.H. Khan, I. Orhan, F.S. Şenol, M. Kartal, B. Şener, M. Dvorská, K. Šmejkal, T. Šlapetová, Cholinesterase inhibitory activities of some flavonoid derivatives and chosen xanthone and their molecular docking studies, Chem. Biol. Interact. 181 (2009) 383–389, https://doi.org/10.1016/j.cbi.2009.06.024.

[58] A. Balkis, K. Tran, Y.Z. Lee, K. Ng, Screening flavonoids for inhibition of acetylcholinesterase identified baicalein as the most potent inhibitor, J. Agric. Sci. 7 (2015) 26–35, https://doi.org/10.5539/jas.v7n9p26.

[59] R. Tundis, M. Bonesi, F. Menichini, M.R. Loizzo, F. Conforti, G. Statti, F.M. Pirisi, F. Menichini, Antioxidant and anti-cholinesterase activity of *Globularia meridionalis* extracts and isolated constituents, Nat. Prod. Commun. 7 (2012) 1015–1020, https://doi.org/10.1177/1934578X1200700814.

[60] A.O. Aremu, S.O. Amoo, A.R. Ndhlala, J.F. Finnie, J. Van Staden, Antioxidant activity, acetylcholinesterase inhibition, iridoid content and mutagenic evaluation of *Leucosidea sericea*, Food Chem. Toxicol. 49 (2011) 1122–1128, https://doi.org/10.1016/j.fct.2011.02.003.

[61] R. Lanigan, T. Yamarik, Final report on the safety assessment of BHT, Int. J. Toxicol. 21 (2002), https://doi.org/10.1080/10915810290096513.