Benzophenones from *Hypericum elegans* with antioxidant and acetylcholinesterase inhibitory potential

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ABSTRACT

Background: *Hypericum elegans* is used in Bulgarian folk medicine for treatment of wounds, depression, gastrointestinal and bacterial diseases. **Objective:** Recently, new natural benzophenones: Elegaphenone and O-glycosides: Hypericophenonoside, Neoannulatophenonoside and Elegaphenonoside as well as already known 7-Epiclusianone were isolated from the titled species. The aim of the present study was to evaluate the antioxidant and acetyl cholinesterase inhibitory potential of the isolated compounds. **Materials and Methods:** 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS) free radicals, ferric reducing antioxidant power (FRAP) assay as well as inhibition of lipid peroxidation in linoleic acid system were used for determination of antioxidant activity. Modified Ellman's colorimetric method was carried out to assess the acetyl cholinesterase inhibition potential. Hyperoside and Galantamine hydrobromide were used as positive controls. **Results:** Hypericophenonoside was found to possess the strongest DPPH radical scavenging activity ($IC_{50} = 181.85 \pm 6.82$ μM), while Neoannulatophenonoside showed the highest ABTS ($IC_{50} = 0.25 \pm 0.005$ μM) and lipid peroxidation inhibitor activity. FRAP activity was demonstrated only by prenylated aglycones – Elegaphenone [942.16 ± 4.03 μM Trolox Equivalent (TE)] and 7-Epiclusianone (642.95 ± 3.95 μM TE) and was stronger compared to the control Hyperoside (421.75 ± 9.29 μM TE). Elegaphenone and 7-Epiclusianone were found to possess moderate acetyl cholinesterase inhibitory potential with IC_{50} values of 192.19 ± 3.54 μM and 142.97 ± 4.62 μM, respectively. **Conclusion:** The results obtained revealed that *H. elegans* is a potential natural source of bioactive compounds and benzophenones could be useful in therapy of free radical pathologies and neurodegenerative disorders.

Key words: Acetylcholinesterase inhibition, antioxidant activity, benzophenones, *Hypericum elegans*

INTRODUCTION

The formation of reactive oxygen species (ROS), such as peroxyl radical, superoxide anion radical, and hydroxyl radical may cause oxidative stress and destruction of unsaturated lipids also DNA, proteins, and other essential molecules leads to ageing. ROS are also involved in the pathogenesis of degenerative or chronic diseases such as arteriosclerosis and cancer.[1,2] However, the use of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are widely used nowadays in processed food products is in doubt due to safety concerns related to their potential toxicity and adverse side effects.[3,4] Thus, attention is increasingly being focused on the development and utilization of natural sources as antioxidants.[5]

Acetylcholinesterase (AChE) inhibitors are widely used for the symptomatic treatment of Alzheimer's disease (AD) to enhance central cholinergic transmission.[6] Oxidative stress is directly related to neurodegenerative diseases. Therefore, the antioxidant potentials of various plant extracts can be helpful in neuroprotection.[7]

Hypericum species (Clusiaceae) are distributed widely in temperate regions, and have been used as traditional

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medicines in different parts of the world.[8] Extracts from various *Hypericum* species have been shown to possess antibacterial, antistaphylococcal, antiviral, antiinflammatory, antioxidant,[9] antiproliferative[10] and cytotoxic[11] activities. Recently, a detailed investigation on the phytochemistry of *Hypericum elegans* established antioxidant activity of the methanol extract[12] as well as presence of new natural benzophenones and flavonoids.[13,14] In order to discover new natural sources for treatment of neurodegenerative disorders the antioxidant and anticholinesterase potential of prenylated benzophenones and benzophenone-O-glycoside*s* from *H. eleg*ans was evaluated using different *in vitro* methods.

MATERIALS AND METHODS

Chemicals and reagents

The standards of the benzophenones (1–5) were isolated from *Hypericum elegans* and identified in the Department of Pharmacognosy, Faculty of Pharmacy, Medical University Sofia as previously described.[13,14]

2,2′-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazine-6-sulfonic acid) (ABTS), sulfanilamide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride x 6H$_2$O, sodium acetate, potassium persulphate, acetylcholinesterase (AChE) type VI-S, from electric eel 349 U/mg solid, 411 U/mg protein, acetylthiocholine iodideacetylthiocholine iodide (AChI), 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB), were purchased from Sigma-Aldrich. All the others chemicals including the solvents were of analytical grade.

Measurement of antioxidant activity
DPPH radical scavenging activity
Free radical scavenging activity was measured by using DPPH method.[15] Different concentrations (1 ml) of compounds in MeOH were added to 1 ml methanolic solution of 2,2′-diphenyl-1-picrylhydrazyl (DPPH) (2 mg/ml). The absorbance was measured at 517 nm after 30 min. Results were evaluated as percentage scavenging of radical:

$$\text{DPPH radical scavenging activity } (\%) = \frac{\text{Abs}_{control} - \text{Abs}_{sample}}{\text{Abs}_{control}} 100,$$

where Abs$_{control}$ is the absorbance of DPPH radical in MeOH, Abs$_{sample}$ is the absorbance of DPPH radical solution mixed with sample. IC$_{50}$ value (concentration of sample where absorbance of DPPH decreases 50% compared to absorbance of blank) of the sample was determined. BHT was used as positive control. All determinations were performed in triplicate (n=3).

ABTS radical scavenging assay
For ABTS assay, the procedure followed the method of Arnao *et al,* 2001[16] with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulphate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 2ml ABTS solution with 50 ml methanol to obtain an absorbance of 0.305 ± 0.01 units at 734 nm using a spectrophotometer. A fresh ABTS solution was prepared for each assay. Different concentrations (1 ml) of compounds were allowed to react with 2 ml of the ABTS solution and the absorbance was taken at 734 nm after 5 min. The ABTS scavenging capacity of the compound was compared with that of BHT and percentage inhibition calculated as

$$\text{ABTS radical scavenging activity, } (\%) = \frac{\text{Abs}_{control} - \text{Abs}_{sample}}{\text{Abs}_{control}} 100,$$

where Abs$_{control}$ is the absorbance of ABTS radical in methanol; Abs$_{sample}$ is the absorbance of an ABTS radical solution mixed with sample. IC$_{50}$ value (concentration of sample where absorbance of ABTS decreases 50% with respect to absorbance of blank) of the sample was determined. BHT was used as positive control. All determinations were performed in triplicate (n=3).

Ferric reducing/antioxidant power (FRAP)
The FRAP assay was done according to the method described by Benzie and Strain[17] with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g C$_2$H$_3$NaO$_2$×3H$_2$O and 16ml C$_2$H$_4$O$_2$), *p*H 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM FeCl$_3$×6H$_2$O solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml FeCl$_3$×6H$_2$O solution and then warmed at 37 °C before using. 0.15 ml of compound in MeOH was allowed to react with 2.8 ml of the FRAP solution for 30 min in a dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. Results are expressed in μg Trolox equivalent (μg TE/mg de). BHT was used as reference. All determinations were performed in triplicate (*n*=3).

Determination of antioxidant activity in linoleic acid system by the FTC method
The antioxidant activity of studied compounds against lipid peroxidation was measured through ammonium thiocyanate assay, as described by Takao *et al.*, with some modifications.[18] The reaction solution, containing 0.2 ml of compound (0.1 mM in MeOH), 0.2 ml of linoleic acid emulsion (25 mg/ml in 99 % ethanol) and 0.4 ml of 50 mM phosphate buffer (pH 7.4), was incubated in a dark at 40 °C. A 0.1 ml aliquot of the reaction solution

was then added to 3 ml of 70 % (v/v) ethanol and 0.2 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.2 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance of the resulting red color was measured at 500 nm. Aliquots were assayed every 24 h until the day after the absorbance of the control solution (without compound) reached maximum value. BHT (1 mg mL^{-1}) was used as positive control. All determinations were performed in triplicate (n=3).

AChE inhibition assay

The enzyme inhibition activities for AChE was evaluated according to the spectrophotometric method previously reported by Ellman *et al*, in 1961 with minor modifications.[19] The benzophenones (**1-5**) were tested in different concentrations. Galanthamine hydrobromide (Nivalin^{TR}) was used as positive control. The compounds and the control were tested in a concentration range between 0.01 and 100 µg mL^{-1}. Briefly, to a 1 cm path length glass cell, 1500 µL phosphate buffer (pH 8), 200 µL AChE solution (0.3 U ml^{-1}), 200 µL test sample and 1000 µL DTNB (3 mM) were mixed and incubated at 37°C for 15 min. Then, 200 µl ATCI (15 mM) was added to the reaction mixture and the absorbance of the produced yellow 5-thio-2-nitrobenzoate anion was measured at a wavelength of 412 nm using a Shimatzu spectrophotometer (Japan) every 10 sec for 10 min. A control mixture was performed with addition of methanol instead of compound. Results were expressed as the average of triplicate. The enzyme inhibition (%) was calculated from the rate of absorbance change with time (V = Abs/t) data as follows: Inhibition

$$(\%) = \frac{V_{control} - V_{sample}}{V_{control}} 100$$, where $V_{control}$ was the change

of control absorbance; V_{sample} was the change of sample absorbance. Data were expressed as mean ± standard error (SEM) and the results were taken from at least three independent experiments performed in duplicate. The IC$_{50}$ values (concentration of test compounds that inhibits the hydrolysis of substrates by 50%) were determined by spectrophotometric measurement of the effect of increasing concentrations of test compounds on the AChE activity. IC$_{50}$ values were obtained from dose-effect curves by linear regression. All determinations were performed in triplicate (*n*=3).

RESULTS AND DISCUSSION

The five benzophenones tested in the present study were (E)-(2-(3,7-dimethylocta-2,6-dienyloxy)-4, 6-dihydroxyphenyl)(phenyl)methanone (Elegaphenone **1**), 7-Epi-clusianone **2**), 2′-*O*-β-d-glucopyranosyl-2,4,5′, 6-tetrahydroxybenzophenone (Hypericophenonoside **3**),

3′,5′,6-trihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucopyranoside (Neoannulatophenonoside **4**), and 3′,5′,6-trihydroxy-4-methoxybenzophenone-2-*O*-α-L-rhamnopyranoside (Elegaphenonoside **5**) [Figure 1].

Radical scavenging activity against DPPH, ABTS and FRAP of the benzophenones were compared with those of butylated hydroxytoluene (BHT) and Hyperoside and were expressed as IC$_{50}$ µM L^{-1}of inhibition and mM TE mM^{-1}, respectively [Table 1]. The DPPH-radical scavenging activity decreased in following order: Hyperoside (IC$_{50}$ 141.52 µM ± 6.54 µM) > **3** (IC$_{50}$ 181.85 µM ± 6.82 µM) > BHT (IC$_{50}$ 307.50 µM ± 2.45 µM) > **4** (IC$_{50}$ 917.215 µM ± 8.43 µM) > **5** (IC$_{50}$ 1855.085 µM ± 9.52 µM) > **1** (IC$_{50}$ 2329.83 µM ±10.45 µM) > **2** (IC$_{50}$ 2840.206 µM ±12.63 µM). The IC$_{50}$ values for ABTS activity of all compounds were lower than BHT (IC$_{50}$ 0.08 µM ± 0.004 µM) and ranged from 0.25 µM ± 0.005 µM (Compound **4**) to 3748.38 µM ± 25.56 µM (Compound **1**).

Generally, it is known that to characterize the properties of antioxidant compounds, different validated methods were employed. In our investigation three different simple redox-based assays for measuring the reducing capacity of the test compounds were used. Another assay used in this study has been selected to evaluate the inhibition of lipid peroxidation activity of the benzophenones under investigation in linoleic acid system by ferric thiocyanate (FTC) method.

It is generally accepted that antioxidant activity depends on the structure and substitution pattern of hydroxyl groups.
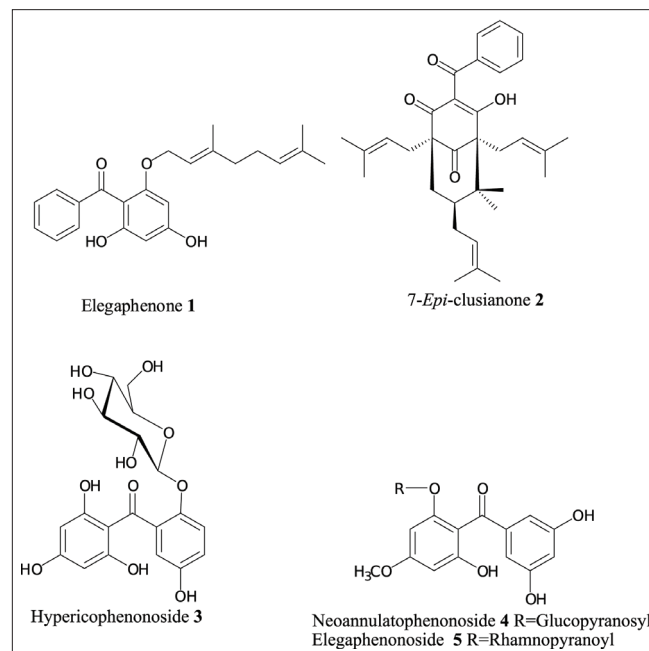
Figure 1: Structures of the studied benzophenones **1-5**

Table 1: DPPH, ABTS-radical scavenging, FRAP and AChE inhibitory potential of the studied compounds.

| Compound	DPPH IC$_{50}$ μM L^{-1}	ABTS IC$_{50}$ μM L^{-1}	FRAP μM TE mM^{-1}	AchE Inhib IC$_{50}$ μM L^{-1}
1	2329.83 ±10.45	379.85 ± 8.45	942.16 ± 4.03	192.19 ± 3.54
2	2840.206 ±12.63	3748.38 ± 25.56	642.95 ± 3.95	142.97 ± 4.62
3	181.85 ± 6.82	41.515 ± 0.85	ND	ND
4	917.215 ± 8.43	0.25 ± 0.005	ND	ND
5	1855.085 ± 9.52	87.17 ± 6.11	ND	ND
Hyperoside	141.52 ± 6.54	6.61 ± 0.31	7837.42 ±18.25	-
BHT	307.50 ± 2.45	0.08 ± 0.004	26.88 ± 2.41	-
Galantamine hydrobromide	-	-	-	0.43 ± 0.02

ND – Not Detectable
Results are represented as means ± standard deviation, *n* = 3

The essential requirement for effective radical scavenging is the orthodihydroxy configuration as well as double bond configured with a keto arrangement.[20] Considering the study results, we found a correlation between the DPPH radical scavenging and the number of free hydroxyl groups in structures of the benzophenones. The strong effect of compound **4** against ABTS free radical probably is related to the presence of methoxyl group in the aglycone moiety. However, compound **5** (rhamnoside) demonstrated lower DPPH and ABTS activity compared to compound **4**. The results revealed that glucose moiety probably exerts an influence on radical scavenging and antioxidant capabilities of the tested compounds.

In FRAP assay reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue color) at low pH can be monitored by measuring the change in absorption at 593 nm. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture. Among the isolated compounds only benzophenone aglycons **1** and **2** manifested FRAP activity. These results correspond with previous investigation of the benzophenones (Annulatophenonoside and Acetylannulatophenonoside) isolated from *H. maculatum*[20] and unambiguously proved that benzophenone-O-glucosides did not manifest any FRAP activity probably because of the impossibility to break the free radical chain by donating a hydrogen atom.

In the present study, the inhibition of lipid peroxidation of compounds (0.1 mM) was determined in linoleic acid system using the FTC method [Figure 2]. The highest significant diminution was demonstrated by compound **4** and it hindered the oxidation of linoleic acid for five days, whereas the outer benzophenones retained the lipid peroxidation for four days [Figure 2]. However, all tested
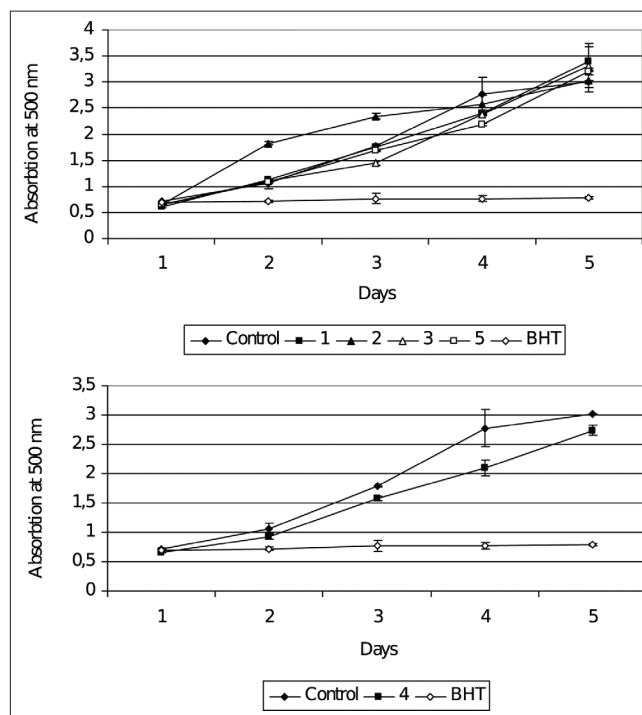
Figure 2: Antioxidant activity of benzophenones **1-5** in linoleic acid system

compounds demonstrated lower antioxidant activity compared to BHT.

The AChE inhibitory activity of the benzophenones was assayed by the method of Ellman *et al*. on AChE from electric eel. Concentration-inhibition curves were obtained from inhibitory concentration (IC$_{50}$) calculated by linear regression [Table 1]. The highest AChE inhibitory potency was displayed by compounds **1** and **2**, whose mean IC$_{50}$ values (192.19 μM L^{-1} ± 3.54 μM L^{-1} and 142.97 μM L^{-1} ± 4.62 μM L^{-1}) are lower than the IC$_{50}$ value of Galantamine hydrobromide.

Several studies have reported moderate anticholinesterase activity of the plant extracts and drugs.[21-23] In our study, although compounds **1** and **2** did not inhibit AChE to a great extent, their inhibitory effect is of interest due to the fact that these substances belong to the group of prenylated benzophenones. Although, alkaloids are considered to be the major anticholinesterase compounds found in plants,[22] recently investigation reported a significant positive correlation between the total phenolic content and anticholinesterase activity of methanol extracts of *Acorus calamus* and *Nardostachys jatamansi*.[24] The results obtained confirmed the potential of several groups of phenolic compounds for antioxidants and aetylcholinesterase inhibitors.

Further in vivo investigations are required for a better understanding of the antioxidant mechanisms involved and for the possible application as a food supplement or in the pharmaceutical industry.

CONCLUSION

Prenylated benzophenones and benzophenone-O-glycosides isolated from *Hypericum elegans* were investigated for their antioxidant and AChE inhibitory potential for the first time. The results revealed that *H. elegans* provide: A potential natural source of bioactive compounds; benzophenones could be useful in therapy of free radical pathologies and neurodegenerative disorders.

ACKOWLEDGMENTS

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