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Cellular reactive oxygen species inhibitory constituents of *Hypericum thasium* Griseb

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ABSTRACT

The phytochemical investigation of the ethyl acetate extract of *Hypericum thasium* has led to the characterization of four benzophenone derivatives **1–4**, a known benzophenone **5** and four known flavonoids, quercetin (**6**), quercitrin (**7**), isoquercetin (**8**), and 3, 8″-biapigenin (**9**). Lucigenin- and luminal-based chemiluminescence assays were employed to monitor the inhibitory activity of these compounds towards the production of reactive oxygen species (ROS) by human polymorphoneutrophils (PMNs). The assay results showed that benzophenones **1** and **3** are extracellular inhibitors of ROS production, while flavonoids **6**, **8**, and **9** can modulate intracellular ROS production.

1. Introduction

The *Hypericum* genus of family Guttiferae is represented in Turkey by 89 species, of which 43 are endemic. These are shrubs or herbs generally with translucent oil glands on their aerial parts (Guner et al., 2000; Davis, 1988). This genus is widespread in Turkey, and its species are used for the treatment of various ailments such as ulcer, the common cold and diabetes mellitus. It is also used as an antimicrobial, an antiviral, and an antidepressant in Turkish folk medicine and other parts of the world (Baytop, 1999; Bombardelli and Morazonni, 1994; Bisset, 1994). Various species of *Hypericum* of Turkish origin have been investigated for volatile compounds (Ozen et al., 2005; Erken et al., 2001). In our search for secondary metabolites from *Hypericum* species, we have carried out a phytochemical investigation on *Hypericum thasium* Griseb., and which has resulted in isolation of flavonoids and benzophenones.

H. thasium is a small-sized herb growing in the Balkan Peninsula and found usually in rocky and sandy places. No phytochemical study has been carried out on *H. thasium* previously.

The phytochemical investigation of an ethyl acetate extract of *H. thasium* has led to the isolation of four new benzophenones **1–4**, along with a known benzophenone **5** see (Fig. 1) and four known flavonoids **6–9**. Their structures were established on the basis of their physical and spectral features. The ability of compounds **1–9** to inhibit oxidative burst production by polymorphonuclear neutrophils (PMNs) was analyzed by a chemiluminescence assay. Measurement of chemiluminescence (CL) is an efficient and sensitive method to investigate the presence of different kinds of reactive oxygen species (HO, O_2^-, H_2O_2, $^1O_2$). Luminol- and lucigenin-dependent chemiluminescence are methods of choice to monitor the effects of drugs on ROS during the oxidative burst in a cell-based system. Luminol reacts in its univalently oxidized form and detects intracellularly-produced H_2O_2, $HO^{\cdot}$, and HOCl, whereas lucigenin is unable to enter the phagocytic cells and can react only with extracellular superoxide radicals (Dahlgren and Brihein, 1985; Meneses et al., 2005; Allen, 1986).

Compounds **1–9** were screened over a wide range of concentrations (3.1–100 μg/mL) for their modulatory effects on the oxidative burst from isolated phagocyte cells (Neutrophils) (Hadjimitova et al., 2004). The results of the assays signify that compounds **1**, **3**, and **6–9** possess a suppressive effect against the ROS production.

2. Results and discussion

The air dried aerial parts of *H. thasium* were collected from Edirne, Turkey, in 2000 and partitioned by using various solvents (see Experimental). The ethyl acetate-soluble fractions of *H. thasium* were subjected to silica gel, polyamide, and Sephadex LH-20 column chromatography and preparative recycling HPLC which led to the isolation of five benzophenones **1–5** and four flavonoid derivatives **6–9**. Among them, compounds **1–4** were found to be new, while compounds **5–9** were known constituents, identified

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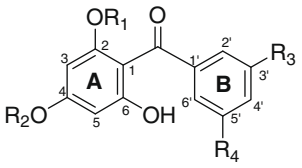


1 R_1= β-Xyl, R_2 = H, R_3 = R_4 = OH
2 R_1= β-Xyl, R_2 = CH_3, R_3 = R_4 = OH
3 R_1= 4-OAc-β-Xyl, R_2 = H, R_3 = R_4 = OH
4 R_1= 3-OAc-α-Ara, R_2 = H, R_3 = R_4 = OH
5 R_1= β-Glc, R_2 = R_3 = R_4 = H

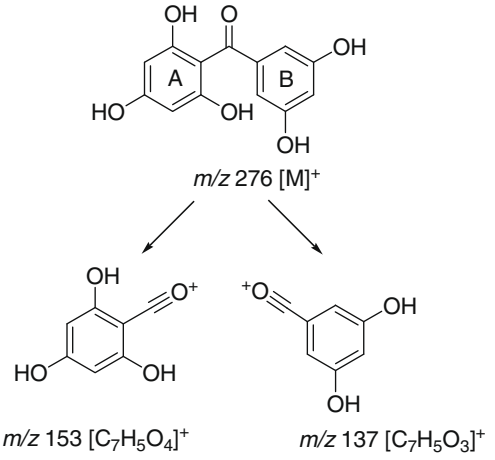
Fig. 1. Structures of compounds **1**–**5**, isolated from *Hypericum thasium*.

as garcimangosone D (**5**), quercetin (**6**), quercitrin (**7**), isoquercetin (**8**), and 3, 8″-biapigenin (**9**).

HRFAB-MS (−ve) of compound **1** exhibited the $[M-H]^-$ ion at *m/z* 393.0827, consistent with the formula $C_{18}H_{18}O_{10}$ (calcd. 394.0906). The presence of a hexose sugar was inferred from a fragment ion of *m/z* 262.0481 $[M-Xyl]^+$, in agreement with the formula $C_{13}H_{10}O_6$ in a HREI-MS spectrum (calcd. 262.0477). The EI-MS showed a fragmentation pattern characteristic for benzophenones containing phloroglucinol and resorcinol rings (Scheme 1). A fragment ion at *m/z* 153 $[C_7H_5O_4]^+$ derived from ring A, and another fragment ion at *m/z* 137 $[C_7H_5O_3]^+$ arising from ring B were observed in EI-MS.

The ^{1}H NMR spectrum of **1** displayed the presence of *meta* coupled protons at δ 6.02 (*d*, $J_{3,\ 5}$ = 2.0 Hz, C-5) and 6.12 (*d*, $J_{3,\ 5}$ = 2.0 Hz, C-3) for the unsymmetrically-substituted phloroglucinol ring A. The ^{1}H NMR spectrum also showed three *meta* coupled protons of ring B, resonating at δ 6.61 (2 H, *d*, $J_{2',\ 4'}$ = $J_{6',\ 4'}$ = 2.0 Hz, H-2′ and -6′) and 6.41 (1 H, *t*, $J_{4',\ 2'}$ = $J_{4',\ 6'}$ = 2.0 Hz, H-4′), which indicated a symmetrical resorcinol structure. The ^{1}H NMR spectrum showed signals for an anomeric proton at δ 4.78 (1 H, $J_{1'',\ 2''}$ = 7.2 Hz, H-1″) and other sugar protons at δ 2.97–3.82. The sugar was identified as xylose with a β-configured anomeric proton. The ^{13}C NMR signals corresponding to the sugar moiety (δ 102.1, 74.2, 77.1, 70.7, 66.7) were characteristic of a xylopyranoside (Gorin and Mazurek, 1975). The β-configuration of the xylose unit was deduced from its $J_{1'',\ 2''}$ coupling constants (7.3–8.0 Hz) (Beier et al., 1980).

In the HMBC spectrum of **1**, the aromatic protons of ring B at δ 6.61 (H-2′ and H-6′) showed cross-peaks with the carbonyl carbon (δ 199.1). While aromatic protons of ring A, H-3 (δ 6.12) and H-5 (δ 6.02), were correlated with a quaternary C-1 (δ 107.2) and also an oxygenated carbon C-4 (δ 164.0). The H-5 (δ 6.02) resonance showed a cross-peak with an oxygenated carbon signal at δ 161.6 (C-6), whereas (H-3) showed a cross-peak with another oxygenated carbon signal at δ 159.2 (C-2), that correlated also with the anomeric proton of xylose at δ 4.78 (H-1″). On this basis, xylose was placed at C-2, and compound **1** was identified as 3′,4,5′,6-tetrahydroxy-2-*O*-β-D-xylopyranosyl-benzophenone.



Scheme 1. General mass fragmentation of benzophenones containing phloroglucinol (ring A), and resorcinol (ring B) rings.

The (−)ve HRFAB-MS of compound **2** showed the $[M-H]^-$ peak at *m/z* 407.0977 in agreement with molecular formula $C_{19}H_{20}O_{10}$ (calcd. 408.1056). Moreover, the EI-MS indicated the presence of a sugar moiety by ion at *m/z* 276 $[M-xyl]^+$. The EI-MS also displayed fragment ions corresponding to rings A and B of benzophenones at *m/z* 167 $[C_8H_7O_4]^+$ and 137 $[C_7H_5O_3]^+$, respectively.

The ^{1}H NMR spectrum of compound **2** showed aromatic proton signals at δ 6.66 (2 H, d, $J_{2',\ 4'}$ = $J_{4',\ 6'}$ = 1.8 Hz, H-2′ and H-6′), and 6.45 (1 H, *t*, $J_{2',\ 4'}$ = $J_{4',\ 6'}$ = 1.8 Hz, H-4′), showing the presence of a symmetrical resorcinol ring B. Two *meta* coupled proton doublets resonated at δ 6.25 (H-3), and 6.18 (H-5) ($J_{3,\ 5}$ = 1.2 Hz). The ^{1}H, and ^{13}C NMR spectra suggested the presence of a methoxy group (^{1}H: δ 3.77 and ^{13}C: δ 55.9). The OCH_3 was placed at C-4 on the basis of HMBC correlations of the methoxyl group (δ_H 3.77) with δ_C 164.9 (Ferrari et al., 2000). The signal at δ 198.7 for the carbonyl group and 12 other signals of aromatic carbons at δ 111.0 (C-1), 158.6 (C-2), 95.2 (C-3), 164.9 (C-4), 96.2 (C-5), 160.5 (C-6), 142.8 (C-1′), 108.5 (C-2′), 159.4 (C-3′), 107.7 (C-4′), 159.4 (C-5′), and 107.7 (C-6′) were characteristic of a benzophenone structure containing phloroglucinol and resorcinol rings. The ^{1}H and ^{13}C NMR spectra also showed the presence of a hexose sugar which was identified as *O*-β-xyloside by the characteristic resonances for the anomeric proton at δ 4.85 ($J_{1'',\ 2''}$ = 7.1 Hz) and other protons. Its linkage at C-2 was deduced by HMBC (Fig. 2) and COSY experiments. Thus compound **2** was identified as 3′,5′,6-trihydroxy-4-methoxy-2-*O*-β-D-xyloside benzophenone, a new natural product. Compound **2** is a 2-*O*-β-xyloside derivative of annulatophenone, earlier isolated from *Hypericum annulatum* (Kitanov and Nedialkov, 2001).

The HRFAB-MS (−ve) spectrum of compound **3** showed an $[M-H]^-$ ion at *m/z* 435.0921, consistent with the formula $C_{20}H_{20}O_{11}$ (calcd. 436.1000) and a peak for $[M-Xyl-H]^-$ at *m/z* 261.0394 for the aglycon moiety, corresponding to the formula $C_{13}H_9O_6$ (calcd. 261.0399). The ^{1}H and ^{13}C NMR spectra were nearly identical to those of compound **1**. The only difference was in the sugar moiety in which the β-xyloside had an acetyl substitution at the C-4″ position. The acetyl group results in a downfield shift of H-4″, which appears as a doublet of triplet at δ 4.63 (1 H, $J_{4'',\ 3''}$ = $J_{4'',\ 5''ax}$ = 8.9 Hz and $J_{4'',\ 5''eq}$ = 5.2 Hz). The comparison of ^{13}C NMR spectral data with that of xylose showed a 2.3 ppm downfield shift of the C-4″ signal and also a *ca.* 3 ppm upfield shift of the C-3″ and C-5″ signals (Agrawal, 1992; Wende and Fry, 1997; Gorin and Mazurek, 1975).

The molecular formula of compound **4** was deduced to be $C_{20}H_{20}O_{11}$ by HRFAB-MS (−ve) at *m/z* 435.0922. In EI-MS, the frag-

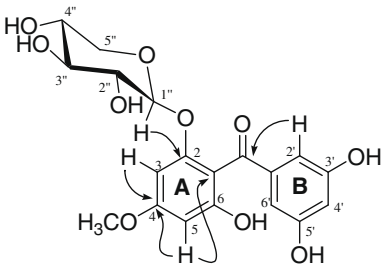


Fig. 2. Key HMBC correlations for compound **2**.

ment ions at *m/z* 262 $[M-Xyl]^+$, 153 $[C_7H_5O_4]^+$, and 137 $[C_7H_5O_3]^+$ were corresponded to phloroglucinol and resorcinol aglycone moieties, respectively.

The signals of three protons of ring B in the ^1H NMR spectrum of compound **4** were observed as a triplet at δ 6.41 (1 H, *t*, $J_{2',4'} = J_{4',6'}$ = 2.2 Hz, H-4′) and a doublet at δ 6.56 (2 H, d, $J_{2',4'} = J_{4',6'}$ = 2.2 Hz, H-2′ and H-6′). The two doublets at δ 6.03 (1 H, d, $J_{3,5}$ = 1.9 Hz, H-5) and 6.20 (1 H, d, $J_{3,5}$ = 1.9 Hz, H-3) were indicative of unsymmetrical substitution of ring A. The ^1H NMR spectrum showed some additional signals which were attributed to acetoxy methyls at δ 1.97 (s) and to a sugar moiety at δ 3.58 (1 H, *d*, $J_{2'',3''}$ = 0.7 Hz, H-2″), 3.62 (1 H, *dd*, $J_{5''\alpha,5''\beta}$ = 11.7 Hz and $J_{5''\beta,4''}$ = 4.1 Hz, H-5″β), 3.68 (1 H, *dd*, $J_{5''\alpha,5''\beta}$ = 11.7 and $J_{5''\alpha,4''}$ = 4.1 Hz, H-5″α), 3.96 (1 H, *ddd*, $J_{4'',5\alpha''} = J_{4'',5\beta''} = J_{4'',3''}$ = 4.1 Hz, H-4″) and 4.69 (1 H, *d*, $J_{3'',4''}$ = 4.1, H-3″, see Table 1). The anomeric proton appeared as a singlet at δ 5.45. The detailed analysis of ^{13}C NMR spectra revealed the sugar as α-L-arabinose (Agrawal, 1992). The anomeric proton signal at δ 5.45 exhibited HMBC cross-peaks with a C-2 quaternary carbon at δ 159.2. The H-3 at δ 6.20 also displayed correlation with C-2 as well as C-4. Therefore C-2 was identified as the linkage position of arabinose (Fig. 3). The signals for the sugar were resolved by detailed analysis of ^1H–^1H COSY and HMQC spectra. It was observed that the signal of H-3″ at δ 4.69 (1 H, br *d*, $J_{3'',4''}$ = 4.1 Hz) was shifted *ca.*1 ppm downfield in the ^1H NMR spectrum as compared to a free arabinose moiety (Agrawal, 1992). This was because of the deshielding effect of the attached acetyl moiety. The comparison of ^{13}C NMR spectral data with those of arabinose revealed a 3.3 ppm downfield shift of the C-3″ signal and 1.7 and 0.3 ppm upfield shifts of the C-2″ and C-4″ signals, respectively (Agrawal, 1989; Gorin and Mazurek, 1975). This observation unambiguously indicated C-3″ as site of acetyl substitution. Thus compound **4** was identified as 3′,4,5′,6-tetrahydroxy-2-*O*-α-L-arabinofuranoside, which is a 4-hydroxy derivative of acetyl annulatophenonoside, a constituent of *H. annulatum* (Nedialkov and Kitanov, 2002; however the structure is wrongly drawn with β-arabinose in this reference).

The molecular formula $C_{19}H_{20}O_9$ of compound **5** was established on the basis of its HRFAB-MS (−ve) spectrum which showed two main peaks at *m/z* 391.1021 for the $[M-H]^-$ and 229.0505 $[M-glc]^-$ for the aglycone fragment. The loss of a sugar moiety was also observed in the EI-MS spectrum with a peak at *m/z* 229 $[M-Glc]^+$. The other peaks, originating from rings B and A, were observed at *m/z* 105 $[C_7H_5O]^+$ and 153 $[C_7H_5O_3]^+$, respectively.

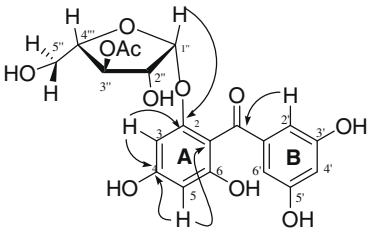


Fig. 3. Key HMBC correlations for compound **4**.

The ^1H NMR spectrum contained signals for a phenyl group at δ 7.69 (2 H, d, $J_{2',3'} = J_{5',6'}$ = 7.3 Hz, H-2′ and H-6′), 7.51 (1 H, *t*, $J_{4',3'} = J_{4',5'}$ = 7.3 Hz, H-4′) and 7.40 (2 H, d, $J_{2',3'} = J_{5',6'}$ = 7.3 Hz, H-3′ and H-5′) for ring B and at δ 6.21 (1 H, d, $J_{5,3}$ = 1.7 Hz, H-5) and 6.06 (1 H, d, $J_{3,5}$ = 1.7 Hz, H-3) for ring A. The anomeric proton signal indicated a β-linkage (δ 4.8, d, $J_{1'',2''}$ = 7.6 Hz). Other signals in the $_1H$ NMR spectrum at δ 2.81–3.86 and signals in the ^{13}C NMR spectrum corresponding to 6 sugar carbons at δ 101.5, 78.1, 77.7, 74.4, 70.9, and 62.4 were characteristic of a β-glucosyl residue. Finally compound **5** was identified as 4,6-dihydroxy-2-*O*-β-D-glucosylbenzophenone, on the basis of the similarities in spectral and the physical data with those of garcimangosone D, previously isolated from *Garcinia mangostana* (Huang et al., 2001).

The known compounds **6-9** were previously reported from other species of the genus *Hypericum*. The structures were elucidated by comparison with reported data (Markham et al, 1978; Agrawal, 1989).

Lucigenin- and luminal- enhanced chemiluminescence detect superoxide (O_2^-) production and the myeloperoxidase (MPO) system, respectively. The results of these assays in response to oxidative burst activators (zymosan or phorbol 12-myristate 13-acetate [PMA]) are shown in Fig. 4A and B, respectively. The IC_{50} inhibitory dose effect of the tested compounds on human PMNs are presented in Table 3. With the use of PMA, a protein kinase C activator, and the lucigenin based assay, strong inhibitory activity was found for benzophenones **1** and **3**, while moderate activity was observed for compounds **4**, **5**, and **6**. In another set of experiments, employing luminol and zymosan for the intracellular oxidative burst studies, compounds **6**, **8**, and **9** exhibited strong inhibitory activities with IC_{50} ranges between 10 and 16 μg/mL, while moderate activity was observed for compounds **1**, and **7** with IC_{50} ranges between 20 and 30 μg/mL (Table 3).

Table 1
^1H NMR values of the isolated new benzophenone compounds.

Protons	1	2	3	4
1	–	–	–	–
2	–	–	–	–
3	6.12, *d*, *J* = 2.0 Hz	6.25, *d*, *J* = 1.2 Hz	6.12, *d*, *J* = 1.9 Hz	6.20, *d*, *J* = 1.9 Hz
4	–	–	–	–
5	6.02, *d*, *J* = 2.0 Hz	6.18, *d*, *J* = 1.2 Hz	6.03, *d*, *J* = 1.9 Hz	6.03, *d*, *J* = 1.9 Hz
6	–	–	–	–
1′	–	–	–	–
2′	6.61, *d*, *J* = 2.0 Hz	6.66, *d*, *J* = 1.8 Hz	6.60, *d*, *J* = 1.8 Hz	6.56, *d*, *J* = 2.2 Hz
3′	–	–	–	–
4′	6.41, *t*, *J* = 2.0 Hz	6.45, *t*, *J* = 1.8 Hz	6.40, *t*, *J* = 1.8 Hz	6.41, *t*, *J* = 2.2 Hz
5′	–	–	–	–
6′	6.61, *d*, *J* = 2.0 Hz	6.66, *d*, *J* = 1.8 Hz	6.60, *d*, *J* = 1.8 Hz	6.56, *d*, *J* = 2.2 Hz
1″	4.78, *d*, *J* = 7.2 Hz	4.85, *d*, *J* = 7.1 Hz	4.85, *d*, *J* = 7.0 Hz	5.45, s
2″	2.97, *t*, *J* = 7.2 Hz	3.04, *t*, *J* = 7.1 Hz	3.05, *t*, *J* = 7.0 Hz	3.58, *d*, *J* = 0.7 Hz
3″	3.31, *t*, *J* = 9.3 Hz	3.33, *t*, *J* = 8.8 Hz	3.55, *t*, *J* = 8.9 Hz	4.69, *d*, *J* = 4.1 Hz
4″	3.44, *dt*, *J* = 9.3, 5.2 Hz	3.45, *dt*, *J* = 8.8, 5.1 Hz	4.63, *dt*, *J* = 8.9, 5.2 Hz	3.96, *ddd*, *J* = 4.1 Hz
5″	3.82, *dd*, *J* = 11.5, 5.2 Hz, (H-5α) 3.27, *dd*, *J* = 11.5, 1.8 Hz (H-5β)	3.83, *dd*, *J* = 11.5, 5.1 Hz, (H-5α) 3.27, *dd*, *J* = 11.5, 1.8 Hz (H-5β)	3.92, *dd*, *J* = 11.6, 5.2 Hz (H-5α) 3.33, *dd*, *J* = 11.6, 1.8 Hz (H-5β)	3.68, *dd*, *J* = 11.7, 4.1 Hz (H-5α) 3.62, *dd*, *J* = 11.7, 4.1 Hz (H-5β)
COCH₃	–	–	2.04, s	1.97, s
OCH₃		3.77 (s)		

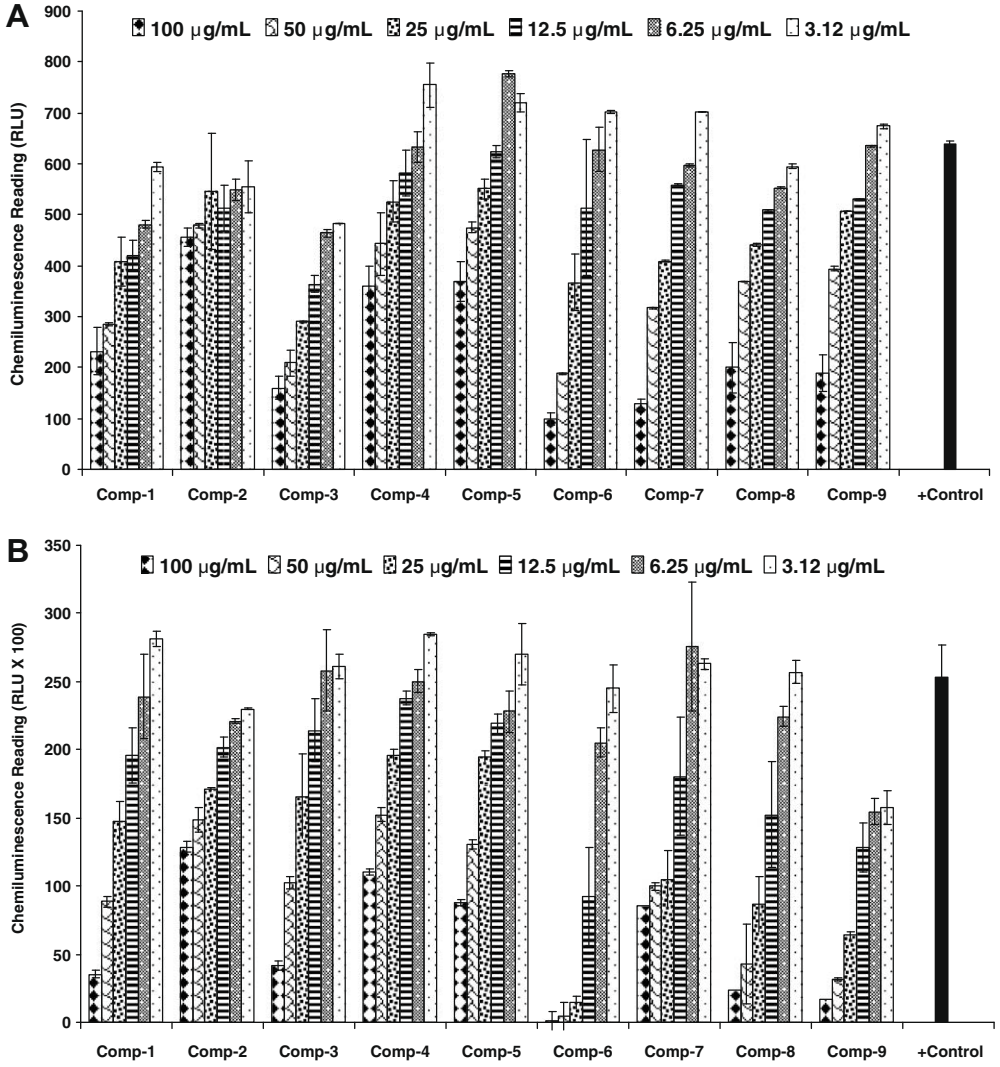


Fig. 4. Chemiluminescence effect of compounds **1**–**9** on neutrophil oxidative burst. Various concentrations of compounds **1**–**9** were tested for their modulatory effects on oxidative burst ROS production of neutrophils by using two luminescence enhancer Lucigenin (A) and Luminol (B). The activity of compounds was compared with the untreated samples (Filled bar). Each plot and error bar represents readings ± SD of three repeats.

This work presents the screening and identification of potential immunosuppresive activities of the benzophenones and flavonoids. Whilst some flavonoids have already been evaluated for their immunosuppresive properties (Pandey et al., 2005). This report constitutes the first study of immunosuppresive activity of benzophenone glycosides. Previously, many prenylated benzophenones were found to be cytotoxic (Chaturvedula et al., 2002), and anti-HIV active (Gustafson et al., 1992; Rao et al., 1980).

2.1. Conclusions

By the use of intra – and extracellular oxidative burst assays, compounds **1** and **3** were identified as selective extracellular inhibitors of superoxide radicals, while compounds **6**, **8**, and **9** demonstrated a selective inhibitory activity towards intracellular reactive oxygen species production.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a digital polarimeter JASCO DIP-360 in methanol. Infrared spectra were obtained on a Vector 22 Bruker spectrophotometer on KBr pellets. FAB Mass spectra were recorded on a Varian MAT 312 mass spectrometer. Accurate mass measurements were carried out with a FAB source by using glycerol as a matrix, and high-resolution-fast-atom bombardment mass spectra (HRFAB MS) were recorded with a JEOL HX 110 mass spectrometer in *m/z* (rel.%). The ^{1}H NMR, ^{13}C NMR, HMQC, and HMBC spectra were recorded on a Bruker AV-400 spectrometer, operating at 400 (^{1}H NMR) and 100 (^{13}C NMR) MHz. The chemical shift values were reported in δ (ppm), referenced with respect to the residual solvent signal from CD_3OD, and coupling constants (J) were measured in Hz. Thin-layer chromatography (TLC) was performed on precoated silica gel plates (DC-Alugram 60 UV_{254} of E. Merck), detection was by spraying with ceric sulphate reagent until coloration developed. Purification was carried out on ODS C-18 (63–212 µm, Wako Pure Chemical Industries Ltd., Japan), Polyamide-6 DF (Riedel-De Haen AG), Sephadex LH-20 and silica gel (E. Merck, 230–400 µm mesh) columns. Recycling preparative HPLC (LC-908 W, Japan Analytical Industry Co. Ltd.) was used for final purification with a column YMC-Pack J′sphere ODS L-80 and H-80 (8 nm pore size, 4 µm particle size, 150 × 4.6 mm), Japan. Chemiluminescence was recorded with a luminometer (Luminoskan RS Labsystem, Finland).

3.2. Plant material

The aerial parts of *Hypericum thasium* Griseb., were collected from the Edirne region of Turkey in June 2000. Voucher specimen (EDTU 9504) was deposited at the herbarium of Trakya University, Biology Department, Edirne, Turkey.

3.3. Extraction and Isolation

Dried and powdered aerial parts of *H. thasium* (288 g) were macerated in methanol at room temperature. The extract was filtered and concentrated under reduced pressure. The concentrated extract (70 g) was diluted with water and then successively extracted with hexane, chloroform, ethyl acetate, and *n*-butanol. The ethyl acetate extract (20 g) was fractionated by on silica gel and eluted with mixtures of hexane/EtOAc and EtOAc/MeOH to obtain 5 subfractions (A–E).

Subfractions A and B were subjected to silica gel column chromatography using a gradient of hexane-EtOAc. Quercetin (**6**, 32 mg) and 3, 8″-biapigenin (**9**, 15 mg) were obtained from fractions A and B, respectively. A Polyamide column was used for the purification of fraction C. Elution was carried out with 100% $CHCl_3$ to 20% MeOH/$CHCl_3$. Subfractions of this column were applied to RP-HPLC (ODS, L-80 column, MeOH:H_2O; 1:1, flow rate 4 mL/min) to obtain compounds **3** (23 mg) and **4** (16 mg). Subfractions of fraction D were applied to RP-HPLC (L-80 column, MeOH:H_2O; 1:1, flow rate 4 mL/min) after the polyamide column using 0–50% MeOH/$CHCl_3$ to obtain compounds **1** (18 mg) and **2** (21 mg). Fraction E was also loaded to the polyamide column using 0–50% MeOH/$CHCl_3$. RP HPLC was used for purification of the first subfraction of fraction E from the polyamide column. From this fraction, compound **5** (24 mg) was isolated by using an ODS column in RP-HPLC (L-80 column, MeOH:H_2O; 1:1, flow rate 4 mL/min). The later fractions from the polyamide column were subjected to Sephadex LH-20 column chromatography using H_2O and increasing amounts of MeOH to obtain quercitrin (**7,** 46 mg), and isoquercetin (**8,** 50 mg).

3.3.1. 3′,4,5′,6-Tetrahydroxy-2-O-β-D-xylosylbenzophenone (*1*)

A brown gum, $[\alpha]_D^{25}$: −22 (MeOH, *c* 0.18). UV λ_{max} (MeOH) nm (log ε): 224 (3.175), 227 (1.395), 305 (1.357). IR ν_{max} (nujol) cm^{-1}: 3333 (chelated OH), 1601 (>C=O), 1597 (>C=C<, aromatic). ^1H and ^{13}C NMR see Tables 1 and 2. EI-MS *m/z* (rel. int.): $[M-xyl]^+$ 262 (90), 245 (90), 229 (68), $[C_7H_5O_4]^+$ 153 (100), $[C_7H_5O_3]^+$ 137 (25), 126 (32), 73 (24), 69 (20), 57 (22). FAB MS *m/z* (rel. int.): 393 $[M-H]^-$ (5), 275 $[M-xyl]^-$ (90), 165 (25), 153 $[C_7H_5O_4]^-$ (22), 137 $[C_7H_5O_3]^-$ (10), 109 (10). HRFAB MS (−ve) $C_{18}H_{18}O_{10}$–H (calcd. 393.0821 found 393.0827).

3.3.2. 3′,5′,4-Trihydroxy-4-methoxy, 2-O-β-D-xylosylbenzophenone (*2*)

A brown gum, $[\alpha]_D^{25}$: −39 (MeOH, *c* 0.8). UV λ_{max} (MeOH) nm (log ε): 233 (0.431), 290 (3.073). IR ν_{max} (nujol) cm^{-1}: 3352 (chelated OH), 1617 (>C=O), 1591 (>C=C<, aromatic). ^1H and ^{13}C NMR see Tables 1 and 2. EI-MS *m/z* (rel. int.): $[M-xyl]^+$ 276 (96), 259 (80), $[C_8H_7O_4]^+$ 167 (70), $[C_7H_5O_3]^+$ 137 (12), 69 (15). FAB MS (−ve) *m/z* (rel. int.): 407 $[M-H]^-$ (20), 275 $[M-xyl]^-$ (100), 257 (10), 164 (10). HRFAB MS (−ve) $C_{19}H_{20}O_{10}$–H (calcd. 407.0978 found 407.0977)

3.3.3. 3′,4,5′,6-Tetrahydroxy-2-O-(4-O-acetyl-β-D-xylosyl)benzophenone (*3*)

A brown gum, $[\alpha]_D^{25}$: −63 (MeOH, *c* 0.2). UV λ_{max} (MeOH) nm (log ε): 221 (2.169), 263 (0.694), 277 (0.865), 305 (0.759). IR ν_{max} (nujol) cm^{-1}: 3318 (chelated OH), 1724 (>C=O, ester), 1601 (>C=O), 1596 (>C=C<, aromatic). ^1H and ^{13}C NMR see Tables 1 and 2. EI-MS *m/z* (rel. int.): $[M-xyl]^+$ 262 (90), 245 (90), 229 (68), $[C_7H_5O_4]^+$ 153 (100), $[C_7H_5O_3]^+$ 137 (30), 126 (32), 73 (24), 69 (20), 57 (22). FAB MS *m/z* (rel. int.): 435 $[M-H]^-$ (70), 327 (30), 275 $[M-xyl]^-$ (80), 261 (60), 165 (22). HRFAB MS (−ve) $C_{20}H_{20}O_{11}$–H (calcd. 435.0927 found 435.0921)

Table 2
^{13}C NMR values of isolated new benzophenones **1–4**.

Carbons	1	2	3	4
1	107.2	111.0	108.2	107.1
2	159.2	158.6	159.1	159.2
3	95.5	95.2	95.5	95.2
4	164.0	164.9	163.8	164.0
5	98.0	96.2	98.0	97.7
6	161.6	160.5	161.6	162.3
1′	143.5	142.8	143.5	143.9
2′	108.2	108.5	108.2	107.9
3′	159.2	159.4	159.3	159.5
4′	107.2	107.7	107.2	107.3
5′	159.2	159.4	159.3	159.5
6′	108.2	107.7	108.2	107.9
1″	102.1	102.4	102.1	107.7
2″	74.2	74.3	74.1	80.4
3″	77.1	77.7	74.1	80.5
4″	70.7	70.7	73.0	85.9
5″	66.7	66.7	63.6	62.8
6″	–	–	–	–
C = O	199.1	198.7	199.1	199.4
COOCH₃	–	–	172.0	172.4
COOCH₃	–	–	20.6	20.7
OCH₃	–	55.9	–	–

3.3.4. 3′,4,5′,6-Tetrahydroxy-2-O-(3-O-acetyl-α-L-arabinosyl)benzophenone (*4*)

A brown gum, $[\alpha]_D^{25}$: −6 (MeOH, *c* 0.5). UV λ_{max} (MeOH) nm (log ε): 221 (1.214), 263 (0.413), 277 (0.527), 308 (0.489). IR ν_{max} (nujol) cm^{-1}: 3322 (chelated OH), 1720 (>C=O, ester), 1601 (>C=O), 1595 (>C=C<, aromatic). ^1H and ^{13}C NMR see Tables 1 and 2. EI-MS *m/z* (rel. int.): $[M-ara]^+$ 262 (42), 244 (60), 216 (28), $[C_7H_5O_4]^+$ 153 (36), $[C_7H_5O_3]^+$ 137 (30), 126 (48), 97 (20), 69 (32). FAB MS *m/z* (rel. int.): 435 $[M-H]^-$ (100), 261 $[M-ara]^-$ (100), 243 (35). HRFAB MS (−ve) $C_{20}H_{20}O_{11}$–H (calcd. 435.0927 found 435.0922) (see Table 3).

3.4. Biological assays

3.4.1. Reagents and chemicals

Luminol (3-aminophthalhydrazide) was purchased from Research Organics (U.S.A), lucigenin (bis-*N*-methylacridinium nitrate) and Hanks Balance Salts Solution (HBSS) were purchased

Table 3
The IC_{50} of all tested compounds with each reading represents readings ± SD of three repeats.

Compounds	PMNs oxidative burst activity IC_{50} (μg/mL)	
	Using luminol	**Using lucigenin**
1	31.57 ± 3.28	18.50 ± 1.74
2	>50	>50
3	37.35 ± 6.36	26.05 ± 1.97
4	>50	39.73 ± 5.90
5	>50	36.08 ± 1.74
6	10.27 ± 1.31	30.0 ± 7.50
7	20.32 ± 3.74	49.02 ± 6.32
8	16.24 ± 3.97	>50
9	12.5 ± 3.40	>50
Sodium diethyldithiocarbamate Trihydrate	1.27 ± 0.23	8.16 ± 1.9
Ibuprofen	15.09 ± 5.06	–

from Sigma, Germany. Lymphocytes separation medium (LSM) was purchased from MP Biomedicals, Inc., Germany. Zymosan-A (*Saccharomyces cerevisiae* origin) and phorbol 12-myristate 13-acetate (PMA) were purchased from Fluka (BioChemika). Dimethyl sulphoxide (DMSO), ethanol and ammonium chloride.

3.4.2. Preparation of opsonized zymosan

The opsonization of zymosan particles was carried out as described earlier (Wiik et al., 1996) with some modifications. Briefly, zymosan (100 mg) was mixed in 5 mL phosphate buffer saline (PBS, pH 7.4) and 5 mL fresh pooled serum from healthy human volunteers. The mixture was incubated at 37 °C in a shaking water bath for 30 min, then centrifuged at 300*g* for 20 min and washed twice with PBS. The pellet was finally re-suspended in 5 mL of PBS. The mixture was kept at −20 °C until use and was brought to room temperature immediately before use.

3.4.3. Preparation of luminol, lucignin and PMA

Luminol (1.8 mg) was dissolved in 1 mL borate buffer and vortexed for 5−10 min. The solution was then further diluted up to 10 mL of $HBSS^{++}$ (with Ca and Mg), to give 180 μg luminol/mL. Lucignin (0.5 mM) solution was prepared by dissolving 1.276 mg lucigenin in 5 mL of $HBSS^{++}$. PMA was dissolved in DMSO, stored in small aliquots at −20 °C, and diluted in HBSS to a final concentration of 40 nM immediately before use.

3.4.4. Isolation of polymorphoneutrophils (PMNs)

Human whole blood was withdrawn from the antecubital veins of healthy volunteers into syringes containing 100 U/mL heparin, and placed in sterile tubes. The heparinized blood was diluted with of $HBSS^{--}$ (Ca and Mg Free) (pH 7.4), and then mixed with one third of dextran (3% in 0.9% NaCl) solution for differential sedimentation and removal of erythrocytes. After gentle mixing, it was kept at room temperature for 20 min undisturbed. The upper layer containing leucocytes was collected and gently layered over an equal volume of lymphocytes separation medium (LSM) and then subjected to centrifugation at 400*g* for 25 min at room temperature. After removal of the upper phase, neutrophils were collected and subjected to hypotonic lysis with sterile distilled water for one minute, and then washed twice with $HBSS^{--}$. The purity of isolated PMNL was >96% (as determined by trypan blue dye exclusion using light microscopy). Cells were re-suspended in Hank's balanced salt solution ($HBSS^{++}$) containing Ca and Mg to give 1×10^6.

3.4.5. Chemiluminescence assay

Luminol-enhanced chemiluminescence assay was performed as described by Helfand (Helfand et al., 1982). Compounds were serially diluted to final volume of 50 μL. To each well 50 μL of a PMN suspension (1×10^7 cells/mL) and 50 μL luminol (7×10^{-5} M) or lucigenin (400 μM) solutions were added. The neutrophils were challenged by adding 50 μL of opsonized zymosan A (OPZ; final concentration: 200 μg/mL) or PMA (final concentration 10 μM) and chemiluminescence was monitored every 1 min for 0.5 s during a 50 min period by using a Luminometer (Luminoskan RS Labsystem, Finland). Total integral levels were used to calculate the activity of test samples in relation to their corresponding controls (identical incubations without test sample). Experiments were performed in Hank's balanced salt solution (HBSS), buffered at pH 7.35 with NaHCO3 and supplemented with 0.1% (w/v) gelatin to avoid cell aggregation (HBSS-gel). PMA was dissolved in DMSO, stored at −20 °C, and diluted in HBSS to a final concentration of 40 mM immediately before use.

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References

Agrawal, P.K., 1989. ^{13}C NMR of Flavonoids. Elsevier, Amsterdam.
Agrawal, P.K., 1992. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. Phytochemistry 31, 3307–3330.
Allen, R., 1986. Phagocytic leukocytic oxygen activities and chemiluminescence: a kinetic approach to analysis. In: Deluca, M., McElory, D. (Eds.), Methods in Enzymology. Bioluminescence and Chemiluminescence, vol. 133 B. Academic Press, New York, pp. 449–493.
Baytop, T., 1999. Therapy with Medicinal Plants in Turkey. Istanbul University Press, Istanbul. pp. 66–167.
Beier, R.C., Mundy, B.P., Strober, G.A., 1980. Assignment of anomeric configuration and identification of carbohydrate residues by ^{13}C NMR. Can. J. Chem. 58, 2800–2804.
Bisset, N.G., 1994. Herbal Drugs and Phytopharmaceuticals. CRC Press, Stuttgart. pp. 273.
Bombardelli, E., Morazonni, P., 1994. Hypericum perforatum. Fitoterapia 66, 43–68.
Chaturvedula, V.S.P., Schilling, J.K., Kingston, D.G.I., 2002. New cytotoxic coumarins and prenylated benzophenone derivatives from the bark of *Ochrocarpos punctatus* from the Madagascar Rainforest. J. Nat. Prod. 65, 965–972.
Dahlgren, C., Brihein, G., 1985. Comparison between the luminol dependant chemiluminescence of polymorphnuclear leukocytes and of the myeloperoxidase –HOOH system influence of pH, cations and protein. Photobiology 41, 605–610.
Davis, P.H., 1988. Flora of Turkey and the East Aegean Islands, vol. 10. University Press, Edinburgh. pp. 96.
Erken, S., Malyer, H., Demirci, F., Demirci, B., Baser, K.H.C., 2001. Chemical investigations on some *Hypericum* species growing in Turkey-I. Chem. Nat. Compd. 37, 434–438.
Ferrari, J., Terraux, C., Sahpaz, S., Msonthi, J.D., Wolfender, J.-L., Hostettmann, K., 2000. Benzophenone glycosides from *Gnidia involucrata*. Phytochemistry 54, 883–889.
Gorin, P.A., Mazurek, M., 1975. Further studies on the assignment of signals in ^{13}C magnetic resonance spectra of aldoses and derived methyl glycosides. Can. J. Chem. 53, 1212.
Guner, A., Ozhatay, N., Ekim, T., Baser, K.H.C. (Eds.), 2000. Flora of Turkey and the East Aegean Islands. Edinburgh University Press, Edinburgh, p. 71. 11.
Gustafson, K.R., Blunt, J.W., Munro, M.H.G., Fuller, R.W., McKee, T.C., 1992. HIV inhibitory natural products.8. The guttiferones, HIV-inhibitory benzophenones from *Symphonia-Globulifera, Garcinia-Livingstonei Garcinia-Ovalifolia* and *Clusia-Rosea*. Tetrahedron 48, 10093–10102.
Hadjimitova, V., Traykov, T., Bakalova, R., Petrova, V., Lambev, I., Ohba, H., Ishikawa, M., Baba, Y., 2004. Chemiluminescent analysis of the antioxidant and immunomodulation effects of several psychotropic drugs on peritoneal macrophages. Luminescence 19, 319–321.
Helfand, S., Werkmeister, J., Roder, J., 1982. Chemiluminescence response of human natural killer cells. I. The relationship between target cell binding, chemiluminescence, and cytolysis. J. Exp. Med 156, 492–505.
Huang, Y-L., Chen, C-C., Chen, Y-J., Huang, R-L., Shieh, B-J., 2001. Three xanthones and a benzophenone from *Garcinia mangostana*. J. Nat. Prod. 64, 903–906.
Kitanov, G.M., Nedialkov, P.T., 2001. Benzophenone *O*-glycoside, a biogenic precursor of 1, 3, 7- trioxigenated xanthones in *Hypericum annulatum*. Phytochemistry 57, 1237–1243.
Markham, K.R., Ternai, B., Stanley, R., Geiger, H., Mabry, T.J., 1978. Carbon-13 NMR studies of flavonoids-III. Tetrahedron 34, 1389–1397.
Meneses, S.P., Marques, K.L., Pires, C.K., Santos, J.L.M., Fernandes, E., Lima, J.L.F.C., Zagatto, E.A.G., 2005. Evaluation of the total antioxidant capacity by using a multipumping flow system with chemiluminescent detection. Anal. Biochem. 345, 90–95.
Nedialkov, P.T., Kitanov, G.M., 2002. Two benzophenone *O*-arabinosides and a chromone from *Hypericum annulatum*. Phytochemistry 59, 867–871.
Ozen, T., Ayan, A.K., Cirak, C., Kevseroglu, K., 2005. Total phenol content of some *Hypericum* species growing in Turkey. Chem. Nat. Compd. 41, 232–233.
Pandey, R., Maurya, R., Singh, G., Sathimorty, B., Naik, S., 2005. Immunosupressive properties of flavonoids isolated from *Boerhaavia diffusa* Linn. Int. Immunopharmacol. 5, 541–553.
Rao, A.V.R., Venkatswamy, G., Pendse, A.D., 1980. Garcinol and cambogin. Tetrahedron Lett. 21, 1975–1978.
Wende, G., Fry, S.C., 1997. 2-*O*-*β*-D-xylopyranosyl-(5-O-feruloyl)-L-arabinose, a widespread component of grass cell walls. Phytochemistry 44, 1019–1030.
Wiik, P., Opstad, P.K., Boyum, A., 1996. Granulocyte chemiluminescence response to serum opsonized zymosan particlesex vivo during long-term strenuous exercise, energy and sleep deprivation in humans. Eur. J. Appl. Physiol. 73, 251–258.