Free Radicals and Antioxidants 3 (2013) S1–S10

Contents lists available at ScienceDirect

Free Radicals and Antioxidants

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

Original article

Isolation, identification and radical scavenging activity of phlorotannin derivatives from brown algae, *Ecklonia maxima*: An experimental and theoretical study

Henry M. Mwangi [a], Jan Van Der Westhuizen [b], Jeanine Marnewick [c], Wilfred T. Mabusela [a,d,**], Mwadham M. Kabanda [e,*], Eno E. Ebenso [e]

[a] Department of Chemistry, University of the Western Cape, Bellville 7535, South Africa
[b] Department of Chemistry, University of Free State, Bloemfontein 9301, South Africa
[c] Antioxidant and Oxidative Stress Unit, Cape Peninsula University, Bellville 7535, South Africa
[d] South African Herbal Science and Medicine Institute, University of the Western Cape, Bellville 7535, South Africa
[e] Department of Chemistry, North-West University (Mafikeng Campus), Mmabatho 2735, South Africa

ARTICLE INFO

Article history:
Received 17 June 2013
Accepted 23 October 2013
Available online 11 December 2013

Keywords:
Brown algae
Ecklonia maxima
Phlorotannins
Radical scavenging activity
Hydrogen atom transfer mechanism

ABSTRACT

Aim & background: Phlorotannins are a family of polyphloroglucinols with numerous biological activities including anticancer, antimalarial and antioxidant. They are highly sought for utilization in food ingredients, animal feeds, fertilizers and medicines. This work reports the isolation, from brown algae, of four phlorotannin derivatives namely phloroglucinol (**1**), eckol (**2**), 7-phloroeckol (**3**) and 2-phloroeckol (**4**). Their radical scavenging activity was assayed to elucidate their capacity to scavenge free radical species. Their structural and electronic features were then compared across structures to provide an explanation for the differences in their radical scavenging properties. Moreover, the two main radical scavenging mechanisms, namely hydrogen atom transfer (HAT) and electron transfer (ET), were checked to determine the preferred mode of radical scavenging.

Methods: Polyphenols were determined spectrophotometrically according to the Folin–Ciocalteu colorimetric methods and the antioxidant assays were determined by means of *ORAC assay* and the Trolox equivalent antioxidant capacity (TEAC) assay. Theoretical studies were performed by means of the Density Functional Theory (DFT) method.

Results: Theoretical predictions indicate that the radical scavenging activities follow the order **1** < **2** < **4** < **3**. Theoretical study also indicates that ET mechanism could be more significant than HAT mechanism because of the high BDE values.

Conclusion: Overall, the results suggest that the position of substitution of phloroglucinol unit on eckol (**2**) plays a significant role in determining the radical scavenging ability of the resulting eckol derivatives.

1. Introduction

Phloroglucinols are polyphenolic derivatives, many of which are found in nature in different environments including plants,[1] marine organisms,[2–6] and some bacteria species.[7] They exhibit various biological activities such as anticancer,[8–13] antiprotozoal,[1,14–16] antimicrobial[17–20] and antioxidant.[2–6,21] Because of their promising biological activities, they are increasingly investigated for their potential utilization in food, cosmetic and pharmaceutical applications for the design of dietary supplements and useful pharmacological drugs. Among phloroglucinol derivatives investigated for their dietary utilization are phlorotannins. Phlorotannins are found abundantly in marine brown algae species such as *Ecklonia cava*. Brown algae are found abundantly in East Asian countries where they are utilized as ingredients in food, animal feeds, fertilizers and medicines.[22] Recently, however, different marine brown algae species have been discovered worldwide and are increasingly investigated for their human beneficial bioactivity components, such as phlorotannins. One of the human beneficial effects of phlorotannins is that they are natural antioxidants – they

* Corresponding author. Tel.: +27 18 389 2352; fax: +27 18 389 2051.
** Corresponding author.
E-mail addresses: wmabusela@uwc.ac.za (W.T. Mabusela), mbyechura@gmail.com (M.M. Kabanda).

2231-2536/$ – see front matter
http://dx.doi.org/10.1016/j.fra.2013.10.006

have the ability protect the human body from cellular or molecular damage by reactive oxygen species including superoxide anion radical, hydroxyl radical (HO•) and alkyl radical (ROO•) free radicals. Free radical species have the potential to cause oxidative damage to almost all major groups of biological macromolecules (e.g., DNA), and have shown to lead to a number of degenerative diseases such as cardiovascular diseases, gastrointestinal degeneration diseases and cancer.[23,24] Therefore, the search for natural antioxidant poses an interesting area of study because of the potential beneficial effects on human health.[25–28]

The current study investigates the antioxidant radical scavenging activity of phlorotannin derivatives isolated from brown algae species, *Ecklonia maxima*, found on the Western Cape region of South Africa. *E. maxima* is considered a member of *Ecklonia* species and contains a variety of compounds, including carotenoids, fucoidans, and phloroglucinol derivatives, that play diverse biological and ecological roles. *Ecklonia* species are increasing utilized for therapeutic applications as they exhibit antioxidant and anti-inflammatory activities,[29] radical scavenging activity,[30,31] anticancer,[32] antibacterial activity[33] and HIV-1 reverse transcriptase activity.[34,35]

The objectives of the study include the isolation and characterisation of the different phloroglucinol derivatives from the *E. maxima* EtOAc fraction, assaying the antioxidant activity of the isolated phlorotannins ~~(using the Oxygen radical absorption capacity (ORAC) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS)))~~ and utilizing theoretical methods to explain the differences in the antioxidant radical scavenging properties of the isolated phloroglucinol derivatives. Although there have been various experimental studies on the antioxidant activities of marine organisms, such as fungi and brown algae, there is scarce theoretical studies detailing the structural and electronic properties of antioxidants from marine organisms.[36] Moreover, although some of the phlorotannins isolated from *E. maxima* (see Fig. 1) have previously been isolated from other species of brown algae,[4,37] the theoretical study reported in this work forms the first attempt to explain the effects of structural and electronic features on their antioxidant activity and provides a tentative explanation for their possible mechanism of action as radical scavengers.

The antioxidant radical scavenging activity is mainly governed by the hydrogen atom transfer (HAT) and electron transfer mechanism.[38–41] In the HAT mechanism, the antiradical property of phenol derivatives (ArOH) is related to their ability to transfer their phenolic H-atom to a free radical. The H-atom abstraction is described by the reaction:

$$R^{\bullet} + ArOH \rightarrow RH + ArO^{\bullet} \quad (1)$$

The termination of further chain reactions depends strongly on the stability of the ArO• radical species formed. This means that factors enhancing the stability of the ArO• radical increase the antiradical activity. The ability of phenolic antioxidants to donate a hydrogen atom is mainly governed by the homolytic O–H bond dissociation enthalpy (BDE):

$$BDE = H_r(ArO^{\bullet}) + H_h(H^{\bullet}) - H_p(ArOH) \quad (2)$$

where H_r is the enthalpy of the radical generated by H abstraction, H_h is the enthalpy of the H atom, and H_p is the enthalpy of parent compound. A low O–H BDE value, usually associated with greater ability to donate the H atom, corresponds to high radical scavenging ability by the phenolic compound.[42–45]

The ET mechanism is governed by the capacity of the studied compound to transfer an electron and is better described in terms of the ionization potential (IP).

$$R^{\bullet} + ArOH \rightarrow ArOH^{\bullet+} + R^{-} \quad (3)$$

The stability of the radical cation is better described by the IP value; the lower the IP, the easier is the electron abstraction.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, Folin Reagent, Sodium Carbonate, Gallic acid standard were purchased from Sigma–Aldrich Chemical Company, South Africa. Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), sodium bisulphite and formic acid were purchased from Fluka Chemicals (South Africa). Phosphate buffer, Fluorescein sodium salt, AAPH (2,2′-Azobis (2-methylpropionamidine) dihydrochloride), Sodium acetate buffer, TPTZ (2,4,6-tri[2-pyridyl]-s-triazine), L-Ascorbic acid were purchased from Sigma–Aldrich, South Africa.

2.2. Plant materials

The brown seaweeds were collected from the rocky reefs off the coast of the western part of the Western Province, South Africa (between January and May 2011). The collected materials were then freeze-dried, pulverized and deposited in the laboratory.

2.3. Preparation of methanolic/ethanolic extracts and fractions

The freeze-dried pulverized material of *E. maxima* (300 g) was extracted three times with 80% MeOH and then filtered. The filtrate

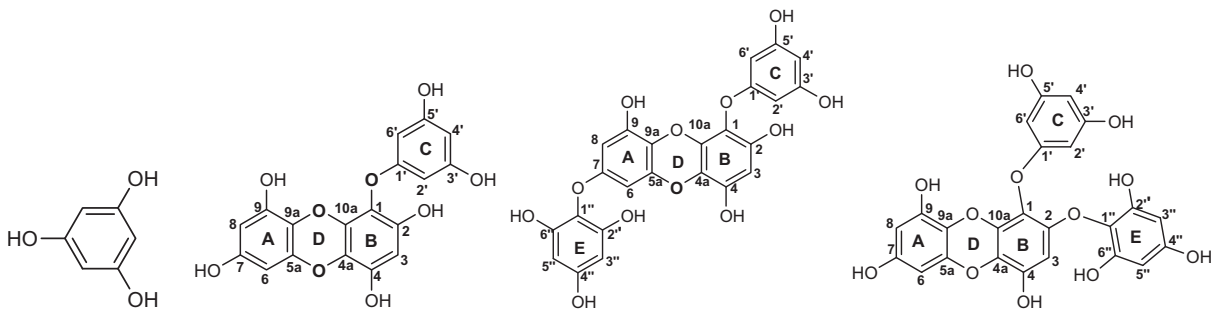

Fig. 1. Structures of the phloroglucinol derivatives isolated from the brown algae, *Ecklonia maxima*, and the atom numbering utilized throughout the study. The OH atoms are numbered with the same number as the C atom to which they are attached.

was evaporated at 40 °C to obtain the methanol extract. The extract was then suspended on distilled water, and partitioned with hexane, dichloromethane and ethyl acetate. The EtOAc fraction (5.0 g) that contained most of the active principles was subjected to chromatography on a silica gel column, with 500 ml volumes of EtOAc: methanol (10:1–5:5) as eluent, yielding 10 sub-fractions (E01–E10).

2.4. Quantification of total phenolic contents

Polyphenols were determined spectrophotometrically according to the Folin–Ciocalteu colorimetric method,[46] calibrating against Gallic acid standards and expressing the results as GAE with slight modifications. 25 μl of the extracts and isolates were taken for the experiment in triplicate. To this volume, 125 μl of diluted Folin–Ciocalteau reagent was added. After 5 min, 100 μl of 20% Na_2CO_3 was added and left for 2 h at room temperature before taking reading measured at 650 nm. A standard graph was plotted using Gallic acid. Data presented in Table 1, on the total phenolic content, are average of three measurements.

2.5. Isolation of individual phenolic compounds

The phlorotannins were isolated by partitioned extraction process and developed based on solvents of increasing polarity. The isolated phloroglucinol derivatives are shown in Fig. 1. Phloroglucinol (**1**) (25 mg) was purified from fraction E001 (50 mg) by high performance liquid chromatography (HPLC) using an Agilent HPLC system equipped with two pumps, degasser, auto-sampler, and a controller. The temperature of the column was set at 30 °C. The column consisted of a C18, (150 × 4.5 mm, 5 μm) with mobile phase A consisting of water/formic acid (100:0.1) and mobile phase B consisting of acetonitrile/formic acid (100:0.1). The chromatographic flow rate was set to 0.70 ml/min and maintained at 82:18 ratios respectively. Another HPLC of fraction E008 (200 mg), using identical solvent conditions, lead to the isolation of eckol (**2**) (20 mg), 7-phloroecckol (**3**) (20 mg) and 2-phloroeckol (**4**) (15 mg). These compounds were isolated from *E. maxima* for the first time.

2.6. Free radical scavenging activity

2.6.1. ORAC assay

The antioxidant capacity of the polyphenols based on hydrogen atom transfer reaction was assayed using 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) as peroxyl radicals source and fluorescein as a molecular probe. To the Trolox® standard wells, 12 μl of the standard were added per well in the designated 96-microwell black opaque plate. A similar amount of the Trolox® control phosphate buffer (12 μl) was added into the control wells. 12 μl of each of the nine samples supernatant was added in triplicate to wells. This was followed by adding fluorescein stock solution using a multichannel pipette into each well then a transfer of 50 μl of AAPH solution to each well. Finally, the 96-microwell plate was inserted into the fluorometer and the readings taken. Fluorescein consumption was evaluated from the decrease in the sample fluorescence intensity (excitation 485 nm, emission 530 nm) using a time base scan program. The fluorescence was then monitored kinetically with data taken every minute. The ORAC values were calculated using a regression equation ($Y = a + bX + cX^2$) between Trolox® concentration (Y) (μM) and the net area under the fluorescence decay curve (X). Data was expressed as micromoles of Trolox® equivalents (TE) per milligram of sample (μmole Trolox®/mg of sample). Trolox® in the experiment was used as a control sample.

Table 1
Total phenolic contents (*in mg GAE/g*) of some extracts and yields of compounds isolated from ethanolic fraction of *Ecklonia maxima*.

Sample	Total phenol content (%)
Hexane extract	0.124
DCM fraction	0.135
Ethyl acetate fraction	0.329
Methanol fraction	0.018
Phloroglucinol (**1**)	0.394
Eckol (**2**)	4.553
7-Phloroeckol (**3**)	26.570
2-Phloroeckol (**4**)	5.987

2.6.2. ABTS radical cation scavenging

The reduction of the radical cation of ABTS by antioxidants was measured utilizing the Trolox equivalent antioxidant capacity (TEAC) assay. To the Trolox standard wells, 25 μl of the standard per well was added to each of the designated wells in a clear 96-well plate. Then a control solution (Trolox in ethanol) was added to each of the control wells. Sample wells were filled with 25 μl of the sample and in triplicate to the wells. Diluted ABTS mix was then added to each of the wells using a multichannel pipette. The 96-well plate was kept for 30 min at room temperature before taking a reading. The TEAC readings were taken at 734 nm on the Multiscale spectrum plate reader. Inhibition of the sample was calculated by the following equation:

$$\text{Inhibition} = [(A_0 - A_1)/A_0]$$

A_0 expresses the absorbance of control; A_1 expresses the absorbance of the tested seaweed extract.

The ABTS radical anion scavenging assay was expressed as Trolox® equivalent antioxidant capacity (TEAC) and defined as μmole of Trolox® equivalents per 1 g of sample (μmole Trolox®/g of sample).

2.7. Statistical analysis

All the measurements were made in triplicate and all values were represented as the mean standard deviation (SD) values of three experiments. The means were statistically analysed using Student's *t*-test and processed with the SPSS program (Version 16). Values of $p < 0.05$ were considered statistically significant.

2.8. Computational methods

Fully relaxed geometry optimisations of the neutral and the radical species were performed utilizing DFT/B3LYP and DFT/UB3LYP methods respectively, where B3LYP is the Becke's Three Parameter Hybrid Functional using the Lee–Yang–Parr correlation functional.[47] Calculations were performed with the 6-31 + G* basis sets to take into consideration the effects of hydrogen bonding. The $<S^2>$ values, calculated for all radical species, has a 0.77–0.79 range, which is close to the value of 0.75 (corresponding to the pure doublet wave function). Therefore, the results of the calculations are less affected by spin contamination. Frequency calculations were performed on fully optimised conformers to determine the nature of the stationary points and to obtain thermochemical quantities necessary for the estimation of BDE and IP values.

The estimation of the O–H BDE values in the presence of a solvent was done by using the total free solvation energies of the neutral and the radical species, as suggested elsewhere.[45] Solvent effects on geometries and relative conformational stabilities have been taken into consideration using the polarizable continuum

model (PCM)[48] in the integral equation formalism (IEF) framework.[49] All calculations were performed with Gaussian03[50] and Spartan 10.V1.01 program.[51] The schematic representations were drawn using the ChemOffice package in the UltraChem 2010 version and conformers were drawn using Spartan 10.V1.01 program.

3. Results and discussion

3.1. Compounds identification

All the purified compounds were characterized by using their ^{1}H and ^{13}C NMR data and 2D experiments. Compound **1**: off-white powder; ^{1}H NMR (200 MHz, CD_3OD) δ5.66 (3H, s, H-2,4,6); ^{13}C NMR (200 MHz, CD_3OD,) δ95.5 (C-2,4,6), δ160.1 (C-1,3,5); ES-MS m/z 125.05 $[M+H]^+$. Compound **2**: light brown powder; ^{1}H NMR (600 MHz, CD_3OD) δ6.17 (1H, *s*, H-3), δ5.97 (1H, *d*, $J = 2.6$ Hz, H-6), δ5.96 (1H, *d*, $J = 2.6$ Hz, H-8), δ5.95 (1H, *t*, $J = 2.0$ Hz, H-4′), δ5.96 (2H, *d*, $J = 2.0$ Hz, H-2′,6′); ^{13}C NMR (600 MHz, CD_3OD); δ124.2 C-1; δ145.8 C-2; δ98.0 (*s*, C-3); δ141.9 C-4; δ123.1 C-4a; δ142.8 C-5a; δ94.4 (*d*, C-6); δ153.1 C-7; δ98.4 (*d*, C-8); δ145.7 C-9; δ123.4 C-9a; δ137.1 C-10a; δ160.4 C-1′; δ94.0 (*d*, C-2′); δ158.8 C-3′; δ96.3 (*d* C-4′); δ158.7 C-5′; δ94.0 (*d* C-6′); ES-MS, *m/z* 371.0397 $[M + H]^+$, (calcd, for $C_{18}H_{12}O_9$, 371.0403). elucidated as 1-(3′,5′-dihydroxyphenoxy) dibenzo[b,e] [1,4]dioxine-2,4,7,9-tetraol, an eckol. Compound **3**: light brown powder; ^{1}H NMR (600 MHz, CD_3OD) δ5.97 (1H, *s*, H-3), δ6.00 (1H, *d*, $J = 2.1$ Hz, H-6), δ6.02 (1H, *d*, $J = 2.1$ Hz, H-8), δ5.95 (2H, *s*, H-3″,5″), δ6.11 (2H, $J = 1.8$ Hz, H-2′,6′), δ6.01 (1H, *t*, $J = 1.8$ Hz, H-4′); ^{13}C NMR (600 MHz, CD_3OD); δ123.8 C-1; δ147.3 C-2; δ96.5 (*s*, C-3); δ141.6 C-4; δ123.1 C-4a; δ142.6 C-5a; δ94.6 (*d*, C-6); δ153.0 C-7; δ98.7 (*d*, C-8); δ145.6 C-9; δ123.4 C-9a; δ137.2 C-10a; δ160.5 C-1′; δ94.3 (*d*, C-2′); δ158.8 C-3′; δ96.7 (*d* C-4′); δ158.8 C-5′; δ94.3 (*d*, C-6′); δ125.4; δ150.7 C-2″; δ95.0 (*d*, C-3″); δ154.8 C-4″; δ95.0 (*d*, C-5″); δ150.7 C-6″; ES-MS *m/z* 495.0570 $[M-H]^+$ (calcd for $C_{24}H_{16}O_{12}$, 495.0564). It was completely elucidated as 1-(3′,5′-dihydroxyphenoxy)-7-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxine-2,4,9-triol, namely 7-phloroeckol. Compound **4**: light brown powder; ^{1}H NMR (CD_3OD, 600 MHz) δ5.97 (1H, *s*, H-3), δ6.00 (1H, *d*, $J = 2.1$ Hz, H-6), δ6.02 (1H, *d*, $J = 2.1$ Hz, H-8), δ5.95 (2H, *s*, H-3″,5″), δ6.11 (2H, $J = 1.8$ Hz, H-2′,6′), δ6.01 (1H, *t*, $J = 1.8$ Hz, H-4′); ^{13}C NMR (600 MHz, CD_3OD); δ123.9 C-1; δ145.7 C-2; δ96.8 (*s*, C-3); δ141.7 C-4; δ123.4 C-4a; δ142.7 C-5a; δ94.1 (*d*, C-6); δ155.0 C-7; δ98.6 (*d*, C-8); δ147.3 C-9; δ123.9 C-9a; δ137.3 C-10a; δ160.6 C-1′; δ96.5 (*d*, C-2′); δ159.0 C-3′; δ94.4 (*d*, C-4′); δ159.0 C-5′; δ94.4 (*d* C-6′); δ123.0 C-1″; δ150.8 C-2″; δ94.8 (*d*, C-3″); δ153.2 C-4″; δ94.8 (*d* C-5″); δ150.8 C-6″; ES-MS *m/z* 495.0561 $[M-H]^+$ (calcd for $C_{24}H_{16}O_{12}$, 495.0564). It was elucidated as 1-(3′,5′-dihydroxyphenoxy)-2-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxine-4,7,9-triol, namely 2-phloroeckol.

3.2. Antioxidant radical scavenging activity

The antioxidant capacity (AAPH and ABTS) is reported in Table 2 for all the isolated extracts. The ORAC results indicate that antioxidant activity can be attributed to micromoles that have the same antioxidant activity as Trolox® equivalent per gram of the tested solution. For the studied compounds the order of inhibition is such that **1** < **2** < **4** < **3**. These results suggest that the structural arrangement of the phloroglucinol units influences attack of free radical scavengers. The ABTS results follow similar trend as the ORAC results with the values of the isolates ranging from 0.342–83.153 μmol Trolox®E/g and 0.70–255.27 μmol TE/g respectively. Pure phlorotannins displayed a much higher total antioxidant capacity than the crude extracts; attributed to interferences from other substances within the crude samples. Interestingly enough, **3** and **4** have the same number of monomers and equal molecular masses but exhibit different radical scavenging capability. With both ORAC and ABTS, **3** have much higher radical scavenging activity than **4**.

Table 2
Antioxidant activity of phloroglucinol (**1**), Eckol (**2**), 7-Phloroeckol (**3**) and 2-Phloroeckol (**4**) in terms of ORAC and ABTS inhibition.

Compound	ORAC (μmol TE/g)	ABTS (μmol Trolox E/g)
Hexane extract	1.44	0.750
DCM extract	1.58	0.880
Ethyl acetate	4.01	1.412
Methanol	0.70	0.342
1	5.36	1.693
2	59.21	19.745
3	255.27	83.153
4	71.66	22.196

3.3. Conformation and radical stability

The B3LYP/6-31 + G (d) optimised geometries, corresponding to the lowest-energy conformer of each of the isolated phloroglucinol derivatives, are shown in Fig. 2. Studies on isolated phloroglucinol moiety have established that it has two conformations, one in which all the phenolic OH groups are oriented in the same way (uniform orientation, PG-1) and the other in which two of the phenolic OH groups are oriented towards each other (non-uniform orientation, PG-2). The conformation with uniform orientation of the phenolic OH has lower energy than the conformation with non-uniform orientation of the OH groups.[52] The lowest-energy conformers of structures **2**, **3** and **4** correspond to geometries in which the number of intramolecular hydrogen bonds (IHB) is maximized. Structure **2** can form a maximum of three O–H···O intramolecular hydrogen bonds (namely $H_9\cdots O_{10a}$, $H_2\cdots O_1$ and $H_4\cdots O_{4a}$). The H···O bond length is 2.20 Å for $H_9\cdots O_{10a}$ and $H_4\cdots O_{4a}$ and 2.21 Å for $H_2\cdots O_1$, the O···O distance is in the range of 2.72–2.75 Å and the $\hat{OHO}$ bond angle is ≈112°. These geometric parameters are indicative of weak IHB. In the lowest energy conformer, the phloroglucinol moiety linking to C_1 is oriented in such a way that the $C_{10a}-C_1-O_1-C_{1'}$ torsion angle is −73.8° (i.e., ring C is slightly inclined towards ring D). This orientation affords the formation of a weak $O_9-H_9\cdots\pi$ bond between the phenolic OH at C_9 and the π system of benzene ring C. H_9 is therefore engaged in bifurcated IHB, $H_9\cdots O_{10a}$ and $O_9-H_9\cdots\pi$.

The lowest energy conformer of structure **3** can form a maximum of five O–H···O IHB ($H_9\cdots O_{10a}$, $H_2\cdots O_1$, $H_4\cdots O_{4a}$, $H_{2''}\cdots O_{1''}$ and $H_{6''}\cdots O_{1''}$) and three unconventional IHB namely, $O_9-H_9\cdots\pi_C$, $C_{2'}-H_{2'}\cdots\pi_B$ and $C_6-H_6\cdots\pi_E$ (where π_B, π_C and π_E refers to the π systems of the aromatic ring B, C and E respectively). The $C_{10a}-C_1-O_1-C_{1'}$ torsion angle is −71.6° from the plane of ring B and the $C_6-C_7-O_7-C_{1''}$ torsion angle is 2.6° from the plane of ring A.

The lowest-energy conformer of structure **4** can form a maximum of four O–H···O IHB ($H_9\cdots O_{10a}$, $H_4\cdots O_{4a}$, $H_{2''}\cdots O_2$ and $H_{6''}\cdots O_2$) and three unconventional IHB namely $C_3-H_3\cdots\pi_E$, $C_{2'}-H_{2'}\cdots\pi_B$ and $O_{6''}-H_{6''}\cdots\pi_C$ (where π_B, π_C and π_E refers to the π systems of the aromatic ring B, C and E respectively) . The $C_{10a}-C_1-O_1-C_{1'}$ torsion angle is −84.8° from the plane of ring B and the $C_3-C_2-O_2-C_{1''}$ torsion angle is −107.6° from the plane of ring B, suggesting that ring C is inclined towards ring E. The geometric constrictions imposed by the different IHB accounts for the stability of **3** and **4**. For instance, it is noted that the unconventional intramolecular hydrogen bonds involving two aromatic rings (e.g., $C_3-H_3\cdots\pi_E$ and $C_{2'}-H_{2'}\cdots\pi_B$) impose a perpendicular arrangement of the benzene rings (e.g., the $C_7-O_7-C_{1''}-C_{2''}$ torsion angle in **3** is

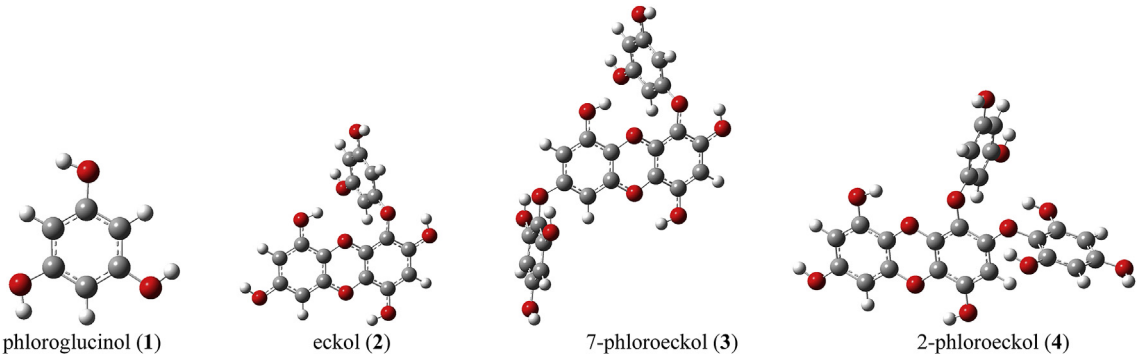

Fig. 2. Lowest-energy conformers of the optimised structures for the isolated phloroglucinol derivatives, B3LYP/6-31 + G(d) results *in vacuo*.

92.2°). The perpendicularity of benzene rings is known to stabilise molecular conformations.[53]

Structures **3** and **4** are structural isomers and a comparison of the lowest-energy conformer of **3** and **4** indicates that **3** is 2.365 kcal/mol lower than **4**. The preference of structure **3** (%population of 98.2) to structure **4** (%population of 1.8%) may be related to the fact that **3** can form an additional O—H···O IHB with respect to **4**, resulting in a five-member ring. IHB are known to determine conformational preferences and also to influence biological activities.[54–63] Moreover, both experimental and theoretical studies have established that the strength (kcal/mol) of the O—H···O IHB (resulting in a five member ring) in hydroxybenzenes is in the range of 0.71–2.22.[64,65] Therefore the energy difference between structure **3** and **4** is in the upper range of the O—H···O IHB in hydroxybenzenes.

The optimised neutral radical species, necessary to study reaction enthalpies related to the HAT mechanism, were generated from the lowest-energy conformer of each of the studied compounds and are shown in Fig. 3. The total spin density distribution for each neutral radical species is shown in Fig. 4 and the relative energies of the neutral radical species are reported in Table 3. The C_{3h} symmetry nature of the lowest-energy conformer of phloroglucinol suggests that only one radical species is generated by the H atom abstraction from each OH group. The H atom abstraction from each OH group present in **2** gives rise to six different radical species. The **2**-O_2, **2**-O_4 and **2**-O_9 radical species are formed by the removal of the IHB and are consequently less stable with respect to **2**-O_7 radical species, which is formed from free OH group (i.e., an OH group not engaged in IHB). However, **2**-O_2, **2**-O_4 and **2**-O_9 radical species are more stable than the **2**-$O_{3'}$ and **2**-$O_{5'}$ radical species mainly because the unpaired electron in **2**-O_2, **2**-O_4 and **2**-O_9 radical species is delocalized beyond the ring on which the H atom is removed while in **2**-$O_{3'}$ and **2**-$O_{5'}$ radical species the unpaired electron is distributed only on the ring with radicalized O atom. This means that the radical species of **2** are stabilised by the presence of IHB and the extent of delocalization of the unpaired electron; depending on the nature of the radical species formed, one of these factors might outweigh the others in contributing to the stabilisation of the radical species.

The abstraction of the H atom from each phenolic OH in **3** gives eight different radical species that are stabilised by a number of geometric and electronic factors including IHB, delocalization of the unpaired electron and steric effects. Radical species **3**-O_2, **3**-O_4, **3**-O_9, **3**-$O_{2''}$ and **3**-$O_{6''}$ are formed by the removal of the IHB while radical species **3**-$O_{3'}$, **3**-$O_{5'}$ and 3-$O_{4''}$ are generated from a free phenolic OH. For the results *in vacuo*, radical species **3**-O_4 has the lowest energy because, despite having fewer IHB than **3**-$O_{3'}$, **3**-$O_{5'}$ and **3**-$O_{4''}$, it distributes the unpaired electron across three rings (A, B and D), which does not happen in radical species **3**-$O_{3'}$, **3**-$O_{5'}$ and **3**-$O_{4''}$. Radical species **3**-$O_{2''}$ and **3**-O_2 are 0.331 kcal/mol and 0.343 kcal/mol respectively higher than **3**-O_4 as they combine different stabilisation factors; **3**-$O_{2''}$ is stabilised by the OH···O IHB and the unconventional O•···CH bond as a result of the proximity of the radicalized O atom to the CH group of ring C; **3**-O_2 radical species is stabilised more by the delocalization of the unpaired electron further than the ring on which the H atom is abstracted. **3**-$O_{6''}$, **3**-$O_{4''}$ and **3**-$O_{5'}$ radical species are nearly 2 kcal/mol higher than **3**-O_4 radical species; **3**-$O_{3'}$ is much higher in energy because of the inability to distribute the unpaired electron beyond the ring with radicalized O atom; **3**-O_9 radical species is the least stabilised probably because it is destabilised by the $O_9 \leftrightarrow O_{9a}$ and $O_9 \leftrightarrow \pi$ repulsions (with the aromatic ring D) as a result of the removal of the IHB.

The abstraction of the H atom from each phenolic OH in **4** also gives eight different radical species. The **4**-O_4, **4**-O_9, **4**-$O_{2''}$ and **4**-$O_{6''}$ radical species are formed by the removal of an IHB and the **4**-O_7, **4**-$O_{3'}$, **4**-$O_{5'}$ and **4**-$O_{4''}$ radical species are formed from a free phenolic OH. For the results *in vacuo*, the **4**-O_7 radical species has the lowest energy because it combines the highest number of IHB and a high tendency to distribute the spin density of the unpaired electron across three rings, A, B and D.

The relative energy trend varies in different media, which suggests that different radical species, for a given structure, are stabilised differently by the solvent; for structure **2**, the most stable radical species *in vacuo* and in chloroform is the **2**-O_7 and in water solution the most stable radical species is **2**-O_9; the lowest energy radical species for structure **3** is **3**-O_4 in all the media; the lowest energy radical species for structure **4** is 4-O_7 *in vacuo* and in chloroform and **4**-O_4 in water solution.

3.3.1. The ΔE_{iso} values

The stabilisation energy (ΔE_{iso}) is one of the parameters used to predict the ability of antioxidants to scavenge free radical species of phenolic derivatives and a high ΔE_{iso} is indicative of high radical scavenging activity.[66,67] A meaningful comparison, however, involves structures with similar central moiety which in this work corresponds to structure **2**, **3** and **4**. Table 4 reports the *in vacuo* ΔE_{iso} values among the studied eckol derivatives. The results show that **3** have the highest stability and **4** is slightly better stabilised than **2**. These results indicate that the addition of a phloroglucinol unit (ring E) on the eckol moiety (structure **2**) is preferable on ring A than on ring B. The positive ΔE_{iso} for **3** suggests increased scavenging activity with respect to **2**. Therefore both experimental findings and theoretical predictions confirm that structure **3** has the highest radical scavenging activities and that **4** is a slightly better radical scavenger than **2**, which is in agreement with the general trend that the stability of radical species, and hence the radical scavenging activity, depends mainly on the number of hydroxyl phenolic groups in the molecule.[36,42–44]

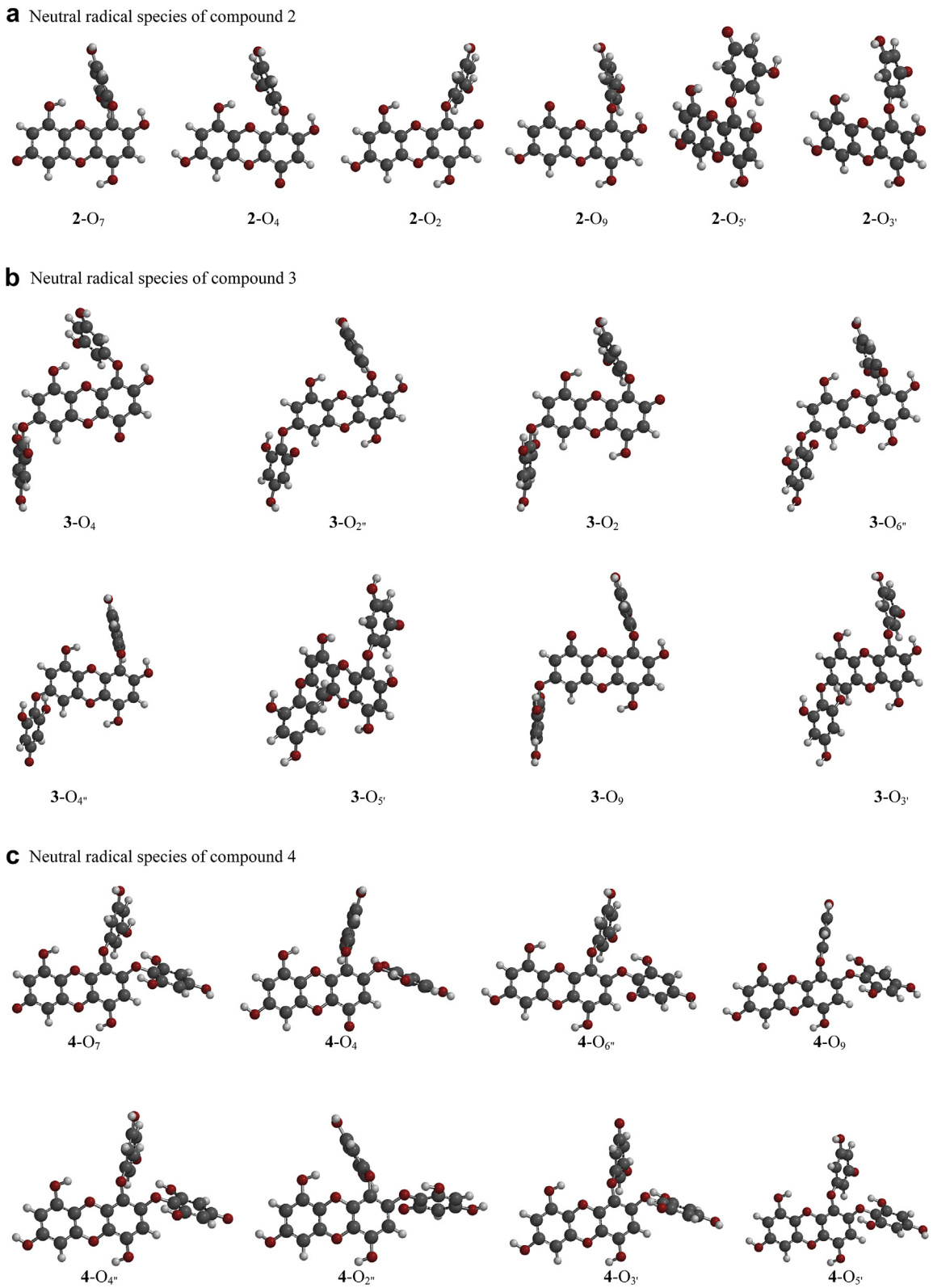

Fig. 3. *In vacuo* B3LYP/6-31 + G(d) optimised geometries for the neutral radical species of the isolated phloroglucinol derivatives. The conformers are arranged in order of increasing energy reported in Table 3.

3.4. The HAT mechanism and the BDE values

The role of the HAT mechanism in determining the free radical scavenging activity of the studied phloroglucinol derivatives is better understood by considering the O–H BDE values. The BDE values, calculated for each phenolic OH of each of the four compounds, are also reported in Table 3. A lower BDE indicates a greater tendency to donate H atom and therefore it corresponds to higher

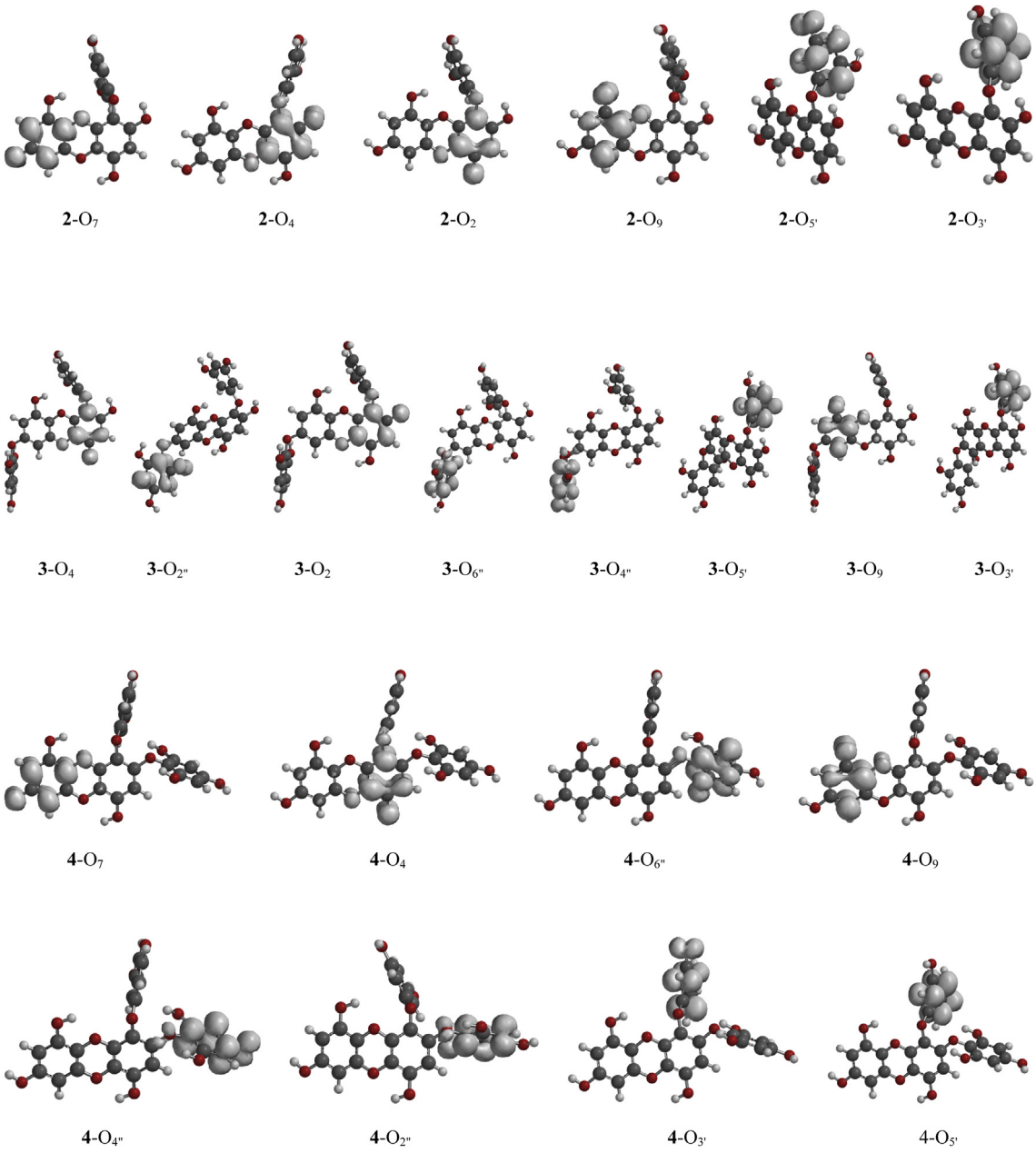

Fig. 4. Spin density distributions of investigated phlorotannin neutral radical species, B3LYP/6-31 + G(d) *in vacuo*.

reactivity.[35] The results indicate that the BDE value of phloroglucinol (89.0 kcal/mol) is slightly higher than that of a phenol calculated in this work (87.7 kcal/mol) and reported in experimental findings (87.3 ± 1 and 88.2 ± 0.5).[68,69] Since phenol is considered a reference compound for understanding the radical scavenging activity of polyphenolic derivatives (ArOH), the results suggest that the scavenging activity of phloroglucinol through the HAT mechanism is minimal.

The BDE values for **2** and **4** indicate that the 7-OH, with the lowest BDE value, is the most reactive site (i.e., the site with the greatest ability to donate H atom) and 3′-OH, with the highest BDE value, has the least ability to donate H atom; the BDE values for **3** indicate the importance of the 4-OH (in the lowest energy radical species) as H atom donor. A comparison of the BDE values across structures suggests that phlorotannins derivatives have better radical scavenging activity than phenol (i.e., they have lower BDE value than phenol) and that the ability to transfer the H atom from the phenolic OH increases in the order **2** > **4** > **3**. The results suggest that structures **2** and **4** preferably scavenge radical species through the HAT mechanism. Both **4** and **2** have lower BDE values than **3** because they have a free phenolic OH attached to ring A (i.e., O_7H_7), which on abstracting an H atom from it gives radical species whose unpaired electron is distributed throughout rings A, B and D (Fig. 4), indicating greater delocalization of the unpaired electron. The delocalization of the unpaired electron accounts for the stabilisation of the radical species. Structure **3**, which has a phloroglucinol moiety at C_7, does not have a free phenolic OH group from which an abstraction of H atom results in extended delocalization (i.e., beyond the ring on which the H atom is abstracted), and as a result it has higher BDE values than both **2** and **4**.

The BDE values of all the studied phloroglucinol derivatives are also lower than the BDE value of phenol (calculated in this work),

Table 3
Relative energies (kcal/mol) and BDE values (kcal/mol) for the calculated neutral radical species of the studied compounds, B3LYP/6-31 + G(d) results in different media

Structure	Relative energy (kcal/mol)[a]			Bond dissociation enthalpy, BDE (kcal/mol)		
	In vacuo	In chloroform	In water	*In vacuo*	In chloroform	In water
Phenol[b]	–	–	–	87.675	88.216	90.222
1-O_2	0.000	0.000	0.000	88.977	89.101	90.236
1-O_4	2.094	1.413	0.808	90.300	89.974	90.975
2-O_7	0.000	0.000	1.166	85.149	84.932	86.135
2-O_4	1.893	0.393	0.108	87.068	85.295	85.150
2-O_2	2.156	0.730	0.018	87.434	85.534	84.894
2-O_9	2.948	1.920	0.000	88.144	86.627	84.741
2-$O_{5'}$	4.308	4.762	6.042	89.487	89.642	90.918
2-$O_{3'}$	5.414	5.456	6.200	90.656	90.341	91.104
3-O_4	0.000	0.000	0.000	87.093	85.515	85.014
3-$O_{2''}$	0.247	1.321	2.566	87.325	86.387	86.983
3-O_2	0.271	0.451	0.036	87.434	85.954	85.002
3-$O_{6''}$	1.665	2.858	3.448	89.053	88.103	88.040
3-$O_{4''}$	1.903	3.333	5.774	88.813	88.970	90.809
3-$O_{5'}$	1.884	3.703	5.092	88.988	89.097	89.808
3-O_9	2.733	3.564	1.975	89.867	88.925	86.725
3-$O_{3'}$	3.351	4.936	5.811	90.516	90.350	90.550
4-O_7	0.000	0.000	0.691	85.164	86.573	85.347
4-O_4	1.770	0.762	0.000	86.985	85.737	85.929
4-$O_{6''}$	2.682	2.821	3.285	87.990	87.786	88.567
4-O_9	3.019	1.583	0.006	88.196	85.750	86.763
4-$O_{4''}$	3.188	3.554	4.947	88.372	90.865	88.936
4-$O_{2''}$	4.272	4.041	4.086	89.618	89.877	89.253
4-$O_{3'}$	4.677	5.522	6.817	89.972	92.572	90.861
4-$O_{5'}$	5.118	5.591	6.802	90.470	92.524	90.893

[a] Relative energy values are taken with respect to the lowest energy conformer in a given media
[b] All BDE values are shown relative to phenol calculated at the same level of theory.

indicating that in all the media, phlorotannins have the ability to scavenge radical species. The trend in the BDE values across structures is different in different media which emphasizes the role of the solvent in stabilising the radical species; the smallest BDE value, for each structure, often corresponds to the lowest-energy radical species.

The BDE values of each OH group of the four phlorotannins were compared with the BDE value of DPPH-**H** to provide an indication of the reactivity with stable DPPH radical species. The BDE value of DPPH-**H** species is calculated in this work to be 82.324 kcal/mol (experimental value = 80 kcal/mol).[69,70] The results therefore suggest that all phlorotannins have higher BDE values than DPPH-**H**, suggesting that reaction equation 1 is not expected to be thermodynamically favourable.

3.5. The ET mechanism and the IP values

The adiabatic IP values provide information for understanding possibility of the compounds to scavenge free radicals through the electron transfer mechanism. The IP values for the studied phlorotannins are reported in Table 5. The results indicate that all phloroglucinol derivatives have lower IP values than phenol suggesting that they have the ability to scavenge radical species through the ET mechanism. The scavenging of DPPH radical species by phlorotannins through the ET mechanism was also investigated by determining the IP value for the DPPH-**H** and comparing it with IP values of the studied phlorotannins. The *in vacuo* IP value for DPPH-**H** was calculated to be 168.465 kcal/mol, indicating that the IP values of phlorotannins (i.e., eckol (**2**), 7-phloroeckol (**3**) and 2-phloroeckol (**4**)) are smaller than that of DPPH-**H**; as a result, reaction equation 3 is expected to be thermodynamically favourable. This means that phlorotannins have high reactivity (i.e., through the ET mechanism) towards scavenging the stable DPPH radical species.

Table 4
Total energy (Hartree) of the lowest-energy conformer of the neutral and radical species and stabilisation energy (ΔE_{iso}, kcal/mol).[a]

Compound	$E_{radical}$ (kcal/mol)	$E_{neutral}$ (kcal/mol)	ΔE_{iso}
2	−1369.5122969	−1370.1456359	0.000
3	−1826.2265574	−1826.8634857	2.252
4	−1826.2257923	−1826.8597171	0.368

[a] The stabilisation energy is estimated as the energy difference between the lowest-energy conformer of the neutral species and neutral radical species for a given phlorotannin derivative and expressed as relative energy with respect to compound 2.

4. Conclusion

The experimental and quantum chemical studies on the antioxidant radical scavenging properties of phlorotannin derivatives performed provide an explanation for the differences in the radical scavenging activity of the isolated compounds. The isolates from the brown algae, *E. maxima* which were fully characterized are; phloroglucinol (**1**), eckol (**2**), 7-phloroeckol (**3**) and 2-phloroeckol (**4**). The antioxidant radical scavenging activity following the assays was in the order **1** < **2** < **4** < **3**. Compound **3** was found to exert significant inhibitory effect compared to the commercial antioxidant, ascorbic acid, indicating that **3** could be a good candidate for food, cosmetic and pharmaceutical applications.

Theoretical studies on the neutral and radical species of the studied compounds helped to identify the preferred geometries of the species and also the factors influencing the values of the BDE, which determines the hydrogen atom transfer mechanism, and the values of adiabatic IP, that determines the electron transfer mechanism. The stabilisation energy (ΔE_{iso}) was also investigated and it provided information on the ability of antioxidants to scavenge free radical species of phenolic derivatives.

The conformational studies indicate that the lowest-energy conformer of **2**, **3** and **4** are stabilised by intramolecular hydrogen bonds and electron delocalization throughout the rings. Compound **3** has high stability than its structural isomer **4**, mainly because it has an additional intramolecular hydrogen bond. The preferred neutral radical species for each eckol derivative is one in which the number of intramolecular hydrogen bonds is maximum and the delocalization of the single electron is highest.

The ΔE_{iso} for the eckol derivatives predicts that the radical scavenging activity follows the order **2** < **4** < **3**, which is in agreement with our experimental findings. These results agree with the general trend that the higher the number of phenolic OH, the greater is the radical scavenging activity.

The BDE values suggest that **2** and **4** may preferentially scavenge radical species through the HAT mechanism. The IP values suggest

Table 5
Calculated ionisation potential for the studied phenolic derivatives, B3LYP/6-31 + G(d) results in different media.

Structure	IP (kcal/mol)		
	In vacuo	In chloroform	In water
Phenol[a]	190.591	158.856	144.453
1	183.188	155.066	140.443
2	162.503	135.623	123.889
3	162.323	138.380	125.561
4	161.711	138.973	127.147

[a] All IP values are shown relative to phenol calculated at the same level of theory.

that all the compounds are capable of scavenging free radical species through the ET mechanism. Moreover, the ability of phlorotannins to scavenge free radical species (such as the stable DPPH radical investigated in this work), has shown that the scavenging activity is preferably through the ET than HAT mechanism.

Overall, the results suggest that the position of substitution of phloroglucinol unit on eckol (**2**) play a significant role in determining the radical scavenging ability of the resulting eckol derivatives. Compound **3** which has the phloroglucinol unit substituted on a different ring from the unit on ring B of eckol (**2**), has much higher radical scavenging activity than compound **4**, whose phloroglucinol unit is substituted on the same ring like the one on ring B of eckol (**2**).

Conflicts of interest

All authors have none to declare.

Acknowledgements

The authors gratefully acknowledge financial support, in a form of a research grant, from Lamicare group (South Africa). We also wish to express our gratitude to the UWC Council for additional financial support, Mr. Tyson Motsoabisane of the University of Free State (South Africa) for running the NMR experiments and Professor Liliana Mammino of the University of Venda for her technical assistance with some computations. M. M Kabanda thanks the North-West University (South Africa) for granting him a post-doctoral fellowship, allowing him to participate in this work. M. M Kabanda and Mwangi H. M are also grateful to the organizers of the ASSaf-2012 conference (South Africa), where their collaboration in this work was initiated.

Abbreviations

AAPH	2,2′-Azobis (2-methylpropionamidine) dihydrochloride
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
BDE	bond dissociation enthalpy
DFT	Density functional theory
HAT	hydrogen atom transfer
IHB	intramolecular hydrogen bond
ORAC	Oxygen radical absorption capacity
TEAC	Trolox equivalent antioxidant capacity
TE	Trolox® equivalents
TPTZ	2,4,6-tri[2-pyridyl]-s-triazine
PCM	Polarizable continuum model
IEF	integral equation formalism
B3LYP	Becke's Three Parameter Lee–Yang–Parr
TEAC	Trolox equivalent antioxidant capacity
GAE	Gallic acid equivalent
DPPH	2,2-diphenyl-1-picrylhydrazyl
PG	Phloroglucinol
IP	ionization potential

References

1. Verotta L. Are acylphloroglucinols lead structures for the treatment of degenerative diseases? *Phytochem Rev*. 2003;1:389–407.
2. Kang HS, Chung HY, Jung JH, Son BW, Choi JS. A new phlorotannin from the brown alga *Ecklonia stolonifera*. *Chem Pharm Bull*. 2003;51:1012–1014.
3. Kang HS, Chung HY, Kim JY, Son BW, Jung HA, Choi JS. Inhibitory phlorotannins from the edible brown alga *Ecklonia stolonifera* on total reactive oxygen species (ROS) generation. *Arch Pharm Res*. 2004;27:194–198.
4. Shibata T, Ishimaru K, Kawaguchi S, Yoshikawa H, Hama Y. Antioxidant activities of phlorotannins isolated from Japanese *Laminariaceae*. *J Appl Phycol*. 2008;20:705–711.
5. Kang KA, Lee KH, Chae S, Zhang R, Jung MS, Lee Y, Kim SY, Kim HS, Joo HG, Park JW, Ham YM, Lee NH, Hyun JW. Eckol isolated from *Ecklonia cava* attenuates oxidative stress induced cell damage in lung fibroblast cells. *FEBS Lett*. 2005;579:6295–6304.
6. Li Y-X, Wijesekara I, Li Y, Kim S- K. Phlorotannins as bioactive agents from brown algae. *Process Biochem*. 2011;46:2219–2224.
7. Yang F, Cao Y. Biosynthesis of phloroglucinol compounds in microorganisms – review. *Appl Microbiol Biotechnol*. 2012;93:487–495.
8. Ito H, Muranaka T, Mori K, Jin Z, Tokuda H, Nishino H, Yoshida T. Ichthyotoxic phloroglucinol derivatives from *Dryopteris fragrans* and their anti-tumor promoting activity. *Chem Pharm Bull*. 2000;48:1190–1195.
9. Fujimoto Y, Usui S, Makino M, Sumatra M. Phloroglucinols from *Baeckea frutescens*. *Phytochemistry*. 1996;41:923–925.
10. Cao S, Schilling JK, Randrianasolo A, Andriantsiferana R, Rasamison VE, Kingston DG. New cytotoxic alkyl phloroglucinols from *Protorhus thouvenotii*. *Planta Med*. 2004;70:683–685.
11. Hostanska K, Bommer S, Weber M, Krasniqi B, Saller R. Comparison of the growth-inhibitory effect of *Hypericum perforatum L*. extracts, differing in the concentration of phloroglucinols and flavonoids, on leukaemia cells. *J Pharm Pharmacol*. 2003;55:973–980.
12. Gartner M, Muller T, Simon JC, Giannis A, Sleeman JP. *Aristoforin*, a novel stable derivative of hyperforin, is a potent anticancer agent. *Chembiochem*. 2005;6:171–177.
13. Dona M, Dell'Aica I, Pezzato E, Sartor L, Calabrese F, Barbera MD, Donella-Deana A, Appendino G, Borsarini AA, Caniato R, Garbisa S. Hyperforin inhibits cancer invasion and metastasis. *Cancer Res*. 2004;64:6225–6232.
14. Tziveleka L, Vagias C, Roussis V. Natural products with anti-HIV activity from marine organisms. *Curr Top Med Chem*. 2003;3:1512–1535.
15. Wisespongpand P, Kuniyoshi M. Bioactive phloroglucinols from the brown alga *Zonaria diesingiana*. *J Appl Phycol*. 2003;15:225–228.
16. Singh IP, Bharate SB. Phloroglucinol compounds of natural origin. *Nat Prod Rep*. 2006;23:558–591.
17. Sieburth JM, Conover TJ. *Sargassum Tannin*. An antibiotic which regards fouling. *Nature*. 1965;208:52–53.
18. Nagayama K, Iwamura Y, Shibata T, Hirayama I, Nakamura T. Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*. *J Antimicrob Chemother*. 2002;50:889–893.
19. Eom SH, Heo SJ, Lee DS, Lee MS, Kim YM, Jung WK, Kim YM. Radical scavenging activity of *Poncirus trifoliata* extracts and their inhibitory effect against hydrogen peroxide induced cell damage. *J Korean Soc Appl Biol Chem*. 2011;54:479–487.
20. Braden KW, Blanton JR, Allen VG, Pond KR, Miller MF. *Ascophyllum nodosum* supplementation: a preharvest intervention for reducing *Escherichia coli* O157:H7 and *Salmonella spp*. in feedlot steers. *J Food Prot*. 2004;67:1824–1828.
21. Heo SJ, Park PJ, Park EJ, Kim SK, Jeon YJ. Antioxidant activity of enzymatic extracts from a brown seaweed *Ecklonia cava* by electron spin resonance spectrometry and comet assay. *Eur Food Res Technol*. 2005;221:41–47.
22. Tomas NV, Kim S-K. Potential pharmacological applications of polyphenolic derivatives from marine brown algae. *Environ Toxicol Pharm*. 2011;32:325–335.
23. Kang S-M, Lee S-H, Heo S-J, Kim K-N, Jeon Y- J. Evaluation of antioxidant properties of a new compound, pyrogallol-phloroglucinol-6,6′-bieckol isolated from brown algae, *Ecklonia cava*. *Nutr Res Pract*. 2011;5:495–502.
24. Artan M, Li Y, Karadeniz F, Lee S, Kim M, Kim S. Anti-HIV-1 activity of phloroglucinol derivative, 6,6′-bieckol, from *Ecklonia cava*. *Bioorg Med Chem*. 2008;16:7921–7926.
25. Khanavi M, Gheidarloo R, Sadati N, Ardekani MRS, Nabavi SMB, Tavajohi S, Ostad SN. Cytotoxicity of fucosterol containing fraction of marine algae against breast and colon carcinoma. *Pharmacogn Mag*. 2012;8:60–64.
26. Bhatia S, Sharma K, Namdeo AG, Chaugule BB, Kavale M, Nanda S. Broad-spectrum sun-protective action of Porphyra-334 derived from *Porphyra vietnamensis*. *Pharmacogn Res*. 2010;2:45–49.
27. Huda AWN, Munira MAS, Fitrya SD, Salmah M. Antioxidant activity of *Aquilaria malaccensis* (thymelaeaceae) leaves. *Pharmacogn Res*. 2009;1:270–273.
28. Zheleva-Dimitrova D, Zhelev I, Dimitrova-Dyulgerova I. Antioxidant activity of some *Carduus* species growing in Bulgaria. *Free Radicals and Antioxidants*. 2013;1:15–20.
29. Ragan MA, Glombitza KW. Phlorotannins, brown algal polyphenols. *Prog Phycol Res*. 1986;4:130–230.
30. Kim AR, Shin TS, Lee MS, Park JY, Park KE, Yoon NY, Kim JS, Choi JS, Jang BC, Byun DS, Park NK, Kim HR. Isolation and identification of phlorotannins from *Ecklonia stolonifera* with antioxidant and anti-inflammatory properties. *J Agric Food Chem*. 2009;57:3483–3489.
31. Li Y, Lee SH, Le QT, Kim MM, Kim SK. Anti-allergic effects of phlorotannins on histamine release via binding inhibition between IgE and Fc epsilonRI. *J Agric Food Chem*. 2008;56:12073–12080.
32. Li Y, Qian ZJ, Ryu B, Lee SH, Kim MM, Kim SK. Chemical components and its antioxidant properties in vitro: an edible marine brown alga, *Ecklonia cava*. *Bioorg Med Chem*. 2009;17:1963–1973.
33. Hashida W, Tanaka N, Kashiwada Y, Sekiya M, Ikeshiro Y, Takaishi Y, Tomoeones A- H. Cytotoxic phloroglucinol derivatives from *Hypericum ascyron*. *Phytochem*. 2008;69:2225–2230.
34. Nakayama Y, Takahashi M, Fukuyama Y, Kinzyo Z. An anti-plasmin inhibitor, eckol, isolated from the brown alga *Ecklonia kurome okamura*. *Agric Biol Chem*. 1989;53:3025–3030.

35. Asres K, Seyoum A, Veeresham C, Bucar F, Gibbons S. Naturally derived anti-HIV agents. *Phytother Res*. 2005;19:557–581.
36. Belcastro M, Marino T, Russo N, Toscano M. Structural and electronic characterization of antioxidants from marine organisms. *Theor Chem Acc*. 2006;115:361–369.
37. Zubia M, Fabre MS, Kerjean V, Lann KL, Stiger-Pouvreau V, Fauchon M. Antioxidant and antitumoural activities of some Phaeophyta from Brittany coasts. *Food Chem*. 2009;116:693–701.
38. Kozlowski D, Trouillas P, Calliste C, Marsal P, Lazzaroni R, Duroux JL. Density functional theory study of the conformational, electronic and antioxidant properties of natural chalcones. *J Phys Chem A*. 2007;111:1138–1145.
39. Trouillas P, Marsal P, Svobodová A, Vostálová J, Gažák R, Hrbác J, Sedmera P, Křen V, Lazzaroni R, Duroux JL, Walterová D. Mechanism of the antioxidant action of silybin and 2,3-dehydrosilybin flavonolignans: a joint experimental and theoretical study. *J Phys Chem A*. 2008;112:1054–1063.
40. Wright JS, Jonhson ER, DiLabio GA. Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. *J Am Chem Soc*. 2001;123:1173–1183.
41. Chiodo SG, Leopoldini M, Russo N, Toscano M. The inactivation of lipid peroxide radical by quercetin. A theoretical insight. *Phys Chem Chem Phys*. 2010;12:7662–7670.
42. Leopoldini M, Pitarch IP, Russo N, Toscano M. Structure, conformation, and electronic properties of apigenin, luteolin, and taxifolin antioxidants. A first principle theoretical study. *J Phys Chem A*. 2004;108:92–96.
43. Leopoldini M, Marino T, Russo N, Toscano M. Antioxidant properties of phenolic compounds. H-atom versus electron transfer mechanism. *J Phys Chem A*. 2004;108:4916–4922.
44. Leopoldini M, Marino T, Russo N, Toscano M. Density functional computations of the energetic and spectroscopic parameters of quercetin and its radicals in gas-phase and in solvent. *Theor Chem Acc*. 2004;111:210–216.
45. Kabanda MM. Antioxidant activity of rooperol investigated through Cu (I and II) chelation ability and the hydrogen transfer mechanism: a DFT study. *Chem Res Toxicol*. 2012;25:2153–2166.
46. Singleton VL, Rossi JA. Calorimetry of total phenolics with phosphomolibdic-phosphotungstic acid reagents. *Am J Enol Vitic*. 1965;16:144–158.
47. Becker AD. Density-functional thermochemistry. III. The role of exact exchange. *J Chem Phys*. 1993;98:5648–5652.
48. Tomasi J, Mennucci B, Cammi R. Quantum mechanical continuum solvation models. *Chem Rev*. 2005;105:2999–3093.
49. Cancès E, Mennucci B, Tomasi J. A new integral equation formalism for the polarizable continuum model: theoretical background and applications to isotropic and anisotropic dielectrics. *J Chem Phys*. 1997;107:3032–3041.
50. Frisch MJ, et al. *Gaussian 03, Revision D.01*. Gaussian, Inc.; 2003.
51. Shao Y, et al. *Phys Chem Chem Phys*. 2006;8:3172–3191.
52. Mammino L, Kabanda MM. A computational study of the interactions of the phloroglucinol molecule with water. *J Mol Struct (Theochem)*. 2008;852:36–45.
53. Kabanda MM, Mammino L. A comparative study of the dimers of selected hydroxybenzenes. *Int J Quantum Chem*. 2010;112:519–531.
54. Mammino L, Kabanda MM. A study of the intramolecular hydrogen bond in acylphloroglucinols. *J Mol Struct (Theochem)*. 2009;901:210–219.
55. Mammino L, Kabanda MMA. Computational study of the effects of different solvents on the characteristics of the intramolecular hydrogen bond in acylphloroglucinols. *J Phys Chem A*. 2009;113:15064–15077.
56. Mammino L, Kabanda MM. Adducts of acylphloroglucinols with explicit water molecules: similarities and differences across a sufficiently representative number of structures. *Int J Quant Chem*. 2010;110:2378–2390.
57. Mammino L, Kabanda MM. A computational study of the carboxylic acid of phloroglucinol *in vacuo* and in water solution. *Int J Quant Chem*. 2010;110:595–623.
58. Mammino L, Kabanda MM. A study of the interactions of the caespitate molecule with water. *Int J Quant Chem*. 2008;108:1772–1791.
59. Mammino L, Kabanda MM. Model structures for the study of acylated phloroglucinols and computational study of the caespitate molecule. *J Mol Struct (Theochem)*. 2007;805:39–52.
60. Kabanda MM, Ebenso EE. Structures, stabilization energies, and binding energies of quinoxaline$\cdots(H_2O)_n$, quinoxaline dimer, and quinoxaline$\cdots$Cu complexes: a theoretical study. *J Phys Chem A*. 2013;117:1583–1595.
61. Mammino L, Kabanda MM. The role of additional O–H$\cdots$O intramolecular hydrogen bonds for acylphloroglucinols' conformational preferences *in vacuo* and in solution. *Mol Sim*. 2013;39:1–13.
62. Kabanda MM, Mammino L, Murulana LC, Mwangi HM, Mabusela WT. Antioxidant radical scavenging properties of phenolic pent-4-en-1-yne derivatives isolated from *Hypoxis rooperi*. A DFT study in vacuo and in solution. Int J Food Prop. Accepted 13.07.13.
63. Kabanda MM, Ebenso EE. DFT study of the protonation and deprotonation enthalpies of benzoxazole, 1,2-benzisoxazole and 2,1-benzisoxazole and implications for the structures and energies of their adducts with explicit water molecules. J Theor Comput Chem. Accepted for publication 14.08.13.
64. Verevkin SP, Schick C. Vapor pressures, enthalpies of sublimation, and enthalpies of fusion of the benzenetriols. *Thermochim Acta*. 2004;415:35–42.
65. Mammino L, Kabanda MM. Interplay of intramolecular hydrogen bonds, OH orientations and symmetry factors in the stabilization of polyhydroxybenzenes. *Int J Quant Chem*. 2011;111:3701–3716.
66. Bentes ALA, Borges RS, Monteiro WR, de Macedo LGM, Alves CN. Structure of dihydrochalcones and related derivatives and their scavenging and antioxidant activity against oxygen and nitrogen radical species. *Molecules*. 2011;16:1749–1760.
67. Borges RS, Queiroz AN, Mendes APS, Araujo SC, Franca LCS, Franco ECS, Leal WG, da Silva ABF. Density Functional Theory (DFT) study of edaravone derivatives as antioxidants. *Int J Mol Sci*. 2012;13:7594–7606.
68. Denisov ET, Khudyakov IV. Mechanisms of action and reactivities of the free. Radicals of inhibitors. *Chem Rev*. 1987;87:1313–1357.
69. Wayner DDM, Lusztyk E, Pagé D, Ingold KU, Mulder P, Laarhoven LJJ. Effects of solvation on the enthalpies of reaction of tert-butoxyl radicals with phenol and on the calculated O–H bond strength in phenol. *J Am Chem Soc*. 1995;117:8737–8744.
70. Mahoney LR, Mendenhall GD, Ingold KU. Calorimetric and equilibrium studies on some stable nitroxide and iminoxy radicals. Approximate oxygen-hydrogen bond dissociation energies in hydroxylamines and oximes. *J Am Chem Soc*. 1973;95:8610–8614.