Antioxidant activity of (+)-bergenin—a phytoconstituent isolated from the bark of *Sacoglottis uchi* Huber (Humireaceae)†

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(+)-Bergenin (**1**) was isolated from *Sacoglottis uchi*, a species of vegetable found in the Amazon region and popularly used for the treatment of several hepatic problems. The structure of **1** was fully characterized using IR, GC–MS and NMR (1D and 2D) analyses. This phytoconstituent has been used as an oriental folk medicine for the treatment of many diseases and shows antihepatotoxic properties. Tests with *β*-carotene, DPPH and a heterogeneous Fenton system were carried out, confirming the antioxidant activity of **1**. Theoretical calculations were performed to investigate the formation of the radical derivatives of **1** using $^{\bullet}H$, $^{\bullet}OH$, $^{\bullet}CH_3$, and $^{\bullet}CCl_3$ as initiator radicals. DFT thermodynamic calculations showed that the methoxyl group (O-6–CH_3) is the most favorable site for radical attack. Frontier molecular orbital analysis showed that nucleophilic radical attack is favored on the aromatic ring of **1** where the LUMO is localized, with antibonding character with respect to the O-6–CH_3 bond. The possibilities of attack at other sites on **1** were investigated in detail in order to understand the regiospecificity of this reaction.

1. Introduction

Oxygenated metabolites are reactive species that can induce lipidic peroxidation, causing different toxicities, mainly tumorigenesis, mutagenesis, tissue necrosis, and hypersensitivity reactions. Oxidative stress in tissues and membranes has been related to artherosclerosis, diabetes, inflammation, Alzheimer's disease, and hepatitis.[1] Antioxidants can be used for the treatment of these diseases by removing oxidative stress. Hepatitis is directly associated with covalent bonds formed between reactive metabolites and the hepatic tissue. The metabolite is recognized as a strange body, stimulating antibody production and local hypersensitivity.[2,3] The hepatotoxic reactions promote the formation of oxygenated radicals having steric hindrance to interactions with detoxifying enzymes, such as epoxide hydrolase and glutathione S-transferase.[4]

Sacoglottis uchi Huber (Humiriaceae) is known in the Amazon region as *cumatê*, *paruru*, and *uchi.* This species is commonly found in firm land forests in the Purus and Solimões river basins.[5–8] The local population uses *uchi* bark tea for the treatment of many diseases, including leukemia and hepatic ills.[9] The fruits present high amounts of phytosterols, carboxylic acids (oleic, linoleic, and linolenic acids), eugenol, and fatty acid esters.[10,11]

We report, in this work, the first phytochemical study of the bark of *Sacoglottis uchi*, resulting in the isolation and structural characterization of (+)-bergenin (**1** in Fig. 1) using IR, GC–MS, and NMR (1D and 2D) analyses. This isocoumarin presenting a *C*-glycosidic skeleton and an aryl *δ*-lactone ring was also isolated from some species of vegetable,[12–15] showing anti-inflammatory, anticancer, anti-HIV, anticoagulant, antimalarial, hypolipidic, and other activities.[16–23] The antioxidant properties of **1**[24,25] can be demonstrated by its protective activity against the hepatotoxicity induced by carbon tetrachloride[26] and galactosamine,[27] and its protective effect against viral hepatitis in mice.[28] Some work shows that the hepatoprotective activity is increased in derivatives of **1**,[29] mainly in acetylbergenins.[30,31]

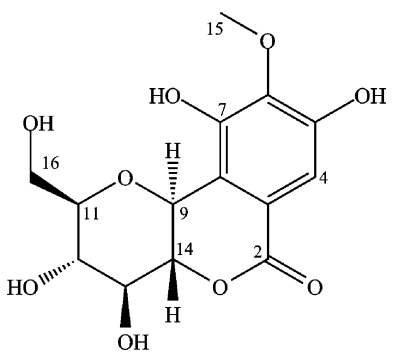

Fig. 1 Chemical structure of (+)-bergenin (**1**).

In this work, the antioxidant activity of **1** has also been investigated in the presence of the hydroxyl radical ($^{\bullet}OH$). Further, DFT calculations were performed aiming to investigate the regiospecificity in the formation of the radical derivatives. DFT methodology has already been successfully applied to studying the chemical properties of neutral, cationic, and anionic radicals.[32–39]

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† Electronic supplementary information (ESI) available: Total energies (E^{total}), zero-point energies (ZPE), and thermal contributions (G^{therm}) of **1** and its radical derivatives, and other species included in eqn (3) to (6); thermodynamic quantities (in kcal mol^{-1}) calculated for the reactions of **1** with $^{\bullet}H$, $^{\bullet}OH$, $^{\bullet}CH_3$ and $^{\bullet}CCl_3$. See DOI: 10.1039/b804385j

2. Experimental

2.1. General

The uncorrected melting point was determined using METTLER equipment, model FP62. The IR spectrum was taken on a Perkin-Elmer Spectrum 2000 FTIR spectrometer with the sample prepared in potassium bromide discs. Mass spectrum (EI, 70 eV) was taken on a Q-Mass 910 spectrometer coupled to a Perkin-Elmer Auto System gas chromatographer. The ^{1}H and ^{13}C NMR spectra, as well as the ^{1}H ^{1}H COSY ($^{1}J_{H,H}$ and $^{3}J_{H,H}$), ^{1}H ^{1}H NOESY ($J_{H,H}$ long distance), ^{1}H ^{13}C HMBC ($^{n}J_{H,C}$, n = 1, 2, 3, and 4), ^{1}H ^{13}C HMQC ($^{1}J_{H,C}$), and ^{1}H ^{13}C HSQC ($^{1}J_{H,C}$) experiments were taken on a Bruker DRX400 AVANCE spectrometer, using $CDCl_3$, DMSO-d_6, and CH_3OD as solvents. The chemical shifts were registered in parts *per* million (δ) relative to TMS used as internal standard. The coupling constants (J) were registered in Hertz. In the antioxidant activity tests, the absorbances were measured with a UV–vis 800M Analyser spectrophotometer.

2.2. Phytochemical procedures

Sacoglottis uchi was collected in February 2004 at the Reserva Adolpho Ducke, in Manaus-AM (Brazil). A voucher specimen of *Sacoglottis uchi* was deposited in the herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA-Manaus), under the code 82,627. The barks were dried, triturated and powdered. This material (3 000.0 g) was subjected to extraction using hexane and methanol as solvents, giving the extracts EH (11.83 g) and EM (212.48 g), respectively. The EM extract was recrystallized from ethanol : methanol (99 : 1), giving a white solid (3.00 g). (+)-Bergenin (**1**); m.p.: 232.0–234.0 °C; IR (KBr; cm^{-1}) ν 3450–3200, 2944, 2894, 2724, 1702, 1612, 1528, 1460, 1375, 1090, 1070, 859, 818, 587, and 541; ^{1}H NMR (400 MHz; DMSO-d_6) δ_H: 9.76 (s; HO–C-5), 8.45 (s; HO–C-7), 6.98 (s; H-4), 5.64 (d, J = 5.3 Hz; HO–C-13), 5.42 (d, J = 5.0 Hz; HO–C-12), 4.96 (d, J = 10.4 Hz; H-9), 4.91 (m; HO–C-16), 4.00 (dd, J = 10.4 and 9.5 Hz; H-14), 3.85 (dd, J = 10.9 and 3.2 Hz; H-16b), 3.78 (s; H-15), 3.65 (ddd, J = 9.5, 8.8, and 5.3 Hz; H-13), 3.58 (ddd, J = 7.6, 3.2, and 1.9 Hz; H-11), 3.44 (ddd, J = 10.9, 8.1, and 1.9 Hz; H-16a), and 3.20 (ddd, J = 8.8, 7.6, and 5.0 Hz; H-12); ^{13}C NMR (100 MHz; DMSO-d_6) δ_C: 163.4 (C-2), 150.9 (C-3), 148.1 (C-6), 140.6 (C-5), 118.1 (C-8), 115.9 (C-7), 109.4 (C-4), 81.7 (C-11), 79.8 (C-14), 73.7 (C-13), 72.1 (C-9), 70.7 (C-12), 61.1 (C-16), and 59.8 (C-15); MS (EI, 70 eV) m/z: 328 [M$^+$], 279, 208 (base peak), 195, 180, 152, and 61.

2.3. Antioxidant activity tests

The antioxidant activity of **1** was qualitatively analyzed by silica gel TLC using ethanol as the mobile phase and revealing with a methanolic solution of β-carotene (0.02%). Antioxidant compounds showed a persistent yellow coloration under solar irradiation. Another qualitative test was performed using the same TLC procedure but revealing with a methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH; 0.2%). After 30 min the antioxidant compounds were revealed by purple to yellow colorations.

The quantitative evaluation of the antioxidant activity of **1** was performed using the UV–vis absorbance band of a methylene blue solution in the presence of hydroxyl radicals (•OH). The decoloration of the methylene blue solution was used as an indicator of its oxidation. The radical •OH shows a high oxidation potential (ε = 2.80 V), being generated *in situ* by the reaction between hydrogen peroxide and magnetite (a heterogeneous Fenton system) according to eqn (1).[40,41]

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad (1)$$

To a 20 mL beaker was added 5.0 mL of the methanolic solution of methylene blue (0.16 mmol L^{-1}), magnetite (30.0 mg), H_2O_2 at 30% (0.2 mL), and methanol (0.5 mL). Three other mixtures were prepared, adding different quantities of **1** (7.0, 14.0 and 21.0 mg). The reaction was interrupted at 10 min intervals and the ferric derivatives were temporarily removed from the medium using a magnet. After this the UV–vis absorbances of the solution were measured at λ = 545 nm.

2.4. Theoretical methodology

Theoretical calculations were performed using the Gaussian 03 program package.[42] Gas phase optimization and evaluation of the harmonic frequencies at the PBE/6-311++G(d,p) level of theory[43] were performed. Geometries were characterized as true minima in the potential energy surface (PES) when all vibrational modes were real. The species •H, •OH, •CH_3, and •CCl_3 were considered as initiator radicals in the oxidation of **1**, resulting in the formation of the radical derivatives shown in Fig. 2. For the most important reactions the non-specific solvent effects were estimated using the united atoms Hartree–Fock/polarizable continuum model (UAHF/PCM). In the UAHF/PCM approach the solute is placed in a polarizable cavity formed by spheres centered on atomic groups. Inside the cavity the dielectric constant is the same as in a vacuum, while outside it takes the value of the solvent used (ε = 78.4 for water).[44] The DFT optimized geometries in the gaseous phase were used for all calculations of the UAHF radii obtained by single point calculations at the HF/6-31+G(d,p) level of theory. The solvation energies were estimated using the Gaussian 03 program package. It is important to mention that the combination of DFT and the continuum solvent model has already been successfully used for describing complex systems.[45–52] As it was pointed out elsewhere, for the open shell systems it is important to use the restricted open shell Hartree–Fock model (ROHF/PCM) in order to obtain reasonable solvation energy estimates.[46,47]

The antioxidant potential of **1** was investigated through thermodynamic calculations of the radical species formation according to eqn (2). It is worth considering the whole amount of reaction energy (ΔG^{total}) as the sum of two parts: electronic plus nuclear repulsion energy (ΔE^{ele}) and thermal correction to the Gibbs free energy (ΔG^{therm}). The thermal contribution is estimated using the ideal gas model and calculated harmonic vibrational frequencies to estimate the zero-point energy correction (ZPE) and the correction due to the thermal population of the vibrational levels.

$$\Delta G^{total} = \Delta E^{ele} + \Delta G^{therm} \quad (2)$$

The possible reaction mechanisms involving **1** are described by eqn (3) to (6). In eqn (3), radicals **O-5**, **O-7**, **O-12**, **O-13**, and **O-16** are formed from the hydroxyl groups of the rings A and C of **1**. By eqn (4) radical **O-6** and RCH_3 are formed at the same time. Radicals **C-2**, **C-2(OH)**, **C-2(CH_3)**, and **C-2(CCl_3)** are formed by

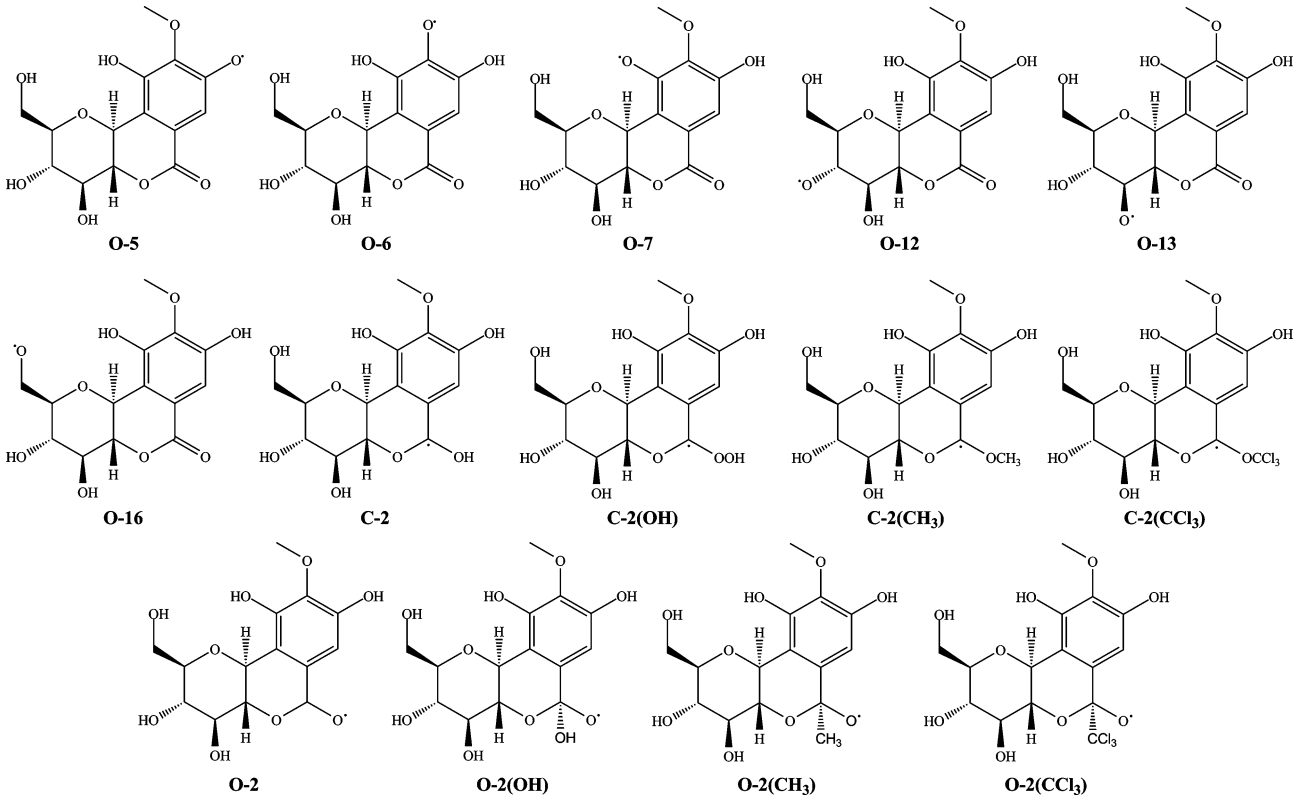
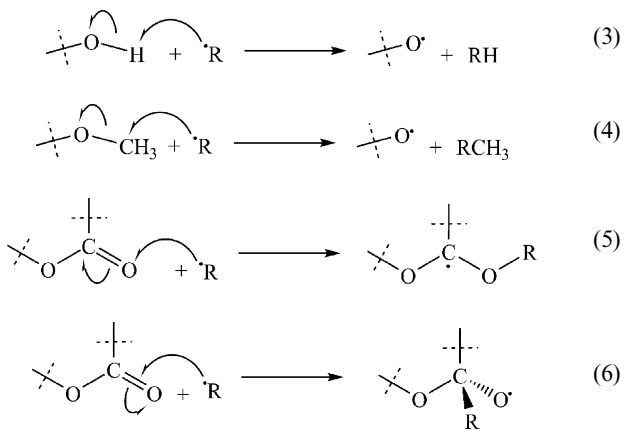


Fig. 2 Chemical structure of radical derivatives of (+)-bergenin.

the homolytic breakage of the π-bond of the ester group, and formation of a bond between the oxygen O–C-2 and the radicals $^{\bullet}$R [eqn (5)]. The formation of bonds between C-2 and the different radicals $^{\bullet}$R results in the species **O-2**, **O-2(OH)**, **O-2(CH$_3$)**, and **O-2(CCl$_3$)**, as shown in eqn (6). The stereochemistry of C-2 in these latter species was considered as having the alkoxyl radical in an equatorial position, *i.e.* α-position.



3. Results and discussion

3.1. Structural analysis

The IR spectrum of **1** shows absorptions between 3450 and 3200 cm^{-1} (ν_{O-H}), at 1702 cm^{-1} ($\nu_{C=O}$), 1612, 1528, 1460, and 1375 cm^{-1} (ν_{C-C} aromatic ring), and 1090 and 1070 cm^{-1} (ν_{C-O}). The mass spectrum shows the molecular ion peak [M$^+$] at *m/z* 328. The base peak at *m/z* 208 can be attributed to the coumarinic cation-radical. The ^{1}H NMR spectra of **1** in DMSO-d_6 and MeOD show broad signals at δ_H 9.76 (HO–C-5), 8.45 (HO–C-7), 5.64 (HO–C-13), 5.42 (HO–C-12), and 4.91 (HO–C-16). As these signals are not observed in the corresponding spectrum using $CDCl_3$ as the solvent, they can be attributed to acidic hydrogen atoms. ^{13}C NMR, DEPT 135°, ^{1}H ^{1}H COSY, ^{1}H ^{13}C HMQC, ^{1}H ^{13}C HSQC, and ^{1}H ^{13}C HMQC data of **1** correspond to (+)-bergenin. Fig. 3 shows the correlations observed in the ^{1}H ^{1}H NOESY contour map of **1**. These correlations correspond to (+)-bergenin. This structure shows the high stability of the *trans*-fused bicyclic system.[53]

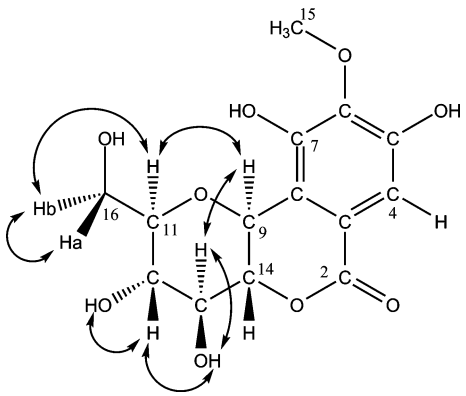


Fig. 3 Correlations observed in the ^{1}H ^{1}H NOESY contour map of **1** (400 MHz; DMSO-d_6).

3.2. Antioxidant activity analysis

The antioxidant activity tests of **1** with *β*-carotene and DPPH radical gave positive results. Fig. 4 shows the curves for the decoloration of the methylene blue solutions as a function of time. After 60 min the mixture without (+)-bergenin showed close to 25% decoloration. The mixtures containing 7.0, 14.0, and 21.0 mg of **1** showed less decoloration (close to 11%, 9%, and 7%, respectively). The $^{\bullet}$OH radical oxidizes **1** more quickly than it does methylene blue. Furthermore the methylene blue oxidation is not verified even in low concentrations of **1**, indicating its high antioxidant activity.

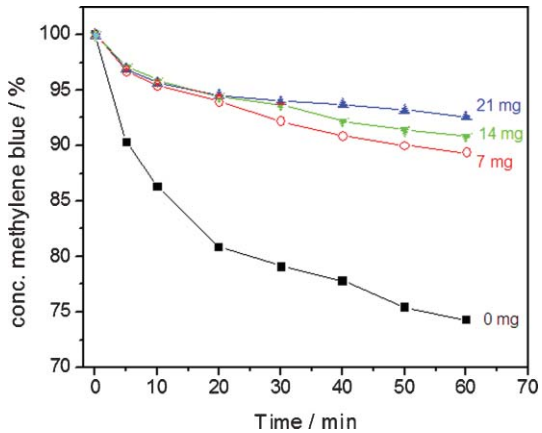


Fig. 4 Methylene blue oxidation in H_2O_2, Fe_3O_4, methanol, and 0.0, 7.0, 14.0 or 21.0 mg of (+)-bergenin as a function of time.

3.3. Theoretical results

All thermodynamic quantities of the species included in eqn (3) to (6), obtained using the PBE/6-311++G(d,p) level of theory, are shown in the ESI (Supplementary Table 1).† Table 1 shows the calculated thermodynamic quantities for reactions of **1** with the initiator radicals $^{\bullet}$H, $^{\bullet}$OH, $^{\bullet}CH_3$, and $^{\bullet}CCl_3$, respectively, to form the most stable radical **O-6**. The calculated values for the reaction of **1** with radical $^{\bullet}$H (Supplementary Table 2)† show that the formation of **O-6** ($\Delta G^{total} = -55.3$ kcal mol^{-1}) is 36.4 kcal mol^{-1} more favored than the generation of any other radical. Although all reactions of **1** with the radical $^{\bullet}$OH are thermodynamically favored (Supplementary Table 3)†, the formation of **O-6** is 8.3 and 8.9 kcal mol^{-1} more favored than the formation of **O-5** and **O-7**, respectively. Comparing the reaction energies for radicals **O-5** and **O-7** under attack by $^{\bullet}$H and $^{\bullet}$OH, the latter leads to relatively more stable products. Apparently, the secondary products (H_2 and H_2O, respectively) significantly contribute to the formation of the radical derivatives of **1**. For $^{\bullet}CH_3$ and $^{\bullet}CCl_3$ (Supplementary Tables 4 and 5)†, radical **O-6** is also the most favored and the other ones are, at least, 20.8 and 29.6 kcal mol^{-1} respectively, higher in energy.

The sites O-5 and O-7 are also favored for all the investigated radical reactions. These sites are similar and reaction at both leads to similar products. For the formation of **O-5**, **O-6**, and **O-7**, the solvation free energy (ΔG^{solv}) has been estimated. The calculated ΔG^{solv} values are similar for the three reactions and do not change the order of stability (**O-6** > **O-5** > **O-7**). In fact these radical derivatives of **1** are geometrically similar, therefore it is expected

Table 1 Thermodynamic quantities (in kcal mol^{-1}) calculated for the reaction of **1** with different radical species using the PBE/6-311++G(d,p) level of theory

Reaction	ΔE^{ele}	ΔG^{therm}	ΔG^{total}
(+)-Bergenin + $^{\bullet}$H → **O-6** + CH_4	−56.18	0.86	−55.3
(+)-Bergenin + $^{\bullet}$OH → **O-6** + CH_3OH	−45.56	2.45	−43.1
(+)-Bergenin + $^{\bullet}CH_3$ → **O-6** + CH_3–CH_3	−43.28	4.42	−38.9
(+)-Bergenin + $^{\bullet}CCl_3$ → **O-6** + CH_3–CCl_3	−34.32	−0.33	−34.7

that the solvation energy will not be important for determining the preferred site for radical reaction. From the thermodynamic analysis one can argue that the order of the secondary product stability is responsible for the regiospecificity of the antioxidant activity of **1** towards **O-6**. This hypothesis is reasonable because **O-5** and **O-7** (Supplementary Table 1)† have similar energy values ($\Delta E^{total} \sim 1.8$ kcal mol^{-1}).

The frontier orbitals of **1** can have an important role in driving the attack of highly reactive species such as the initiator radicals $^{\bullet}$H, $^{\bullet}$OH, $^{\bullet}CH_3$, and $^{\bullet}CCl_3$. The unpaired electrons of these radicals are in high energy orbitals and must be transferred to the lowest unoccupied molecular orbital (LUMO) of **1** (Fig. 5), *i.e.* a relatively nucleophilic radical has a higher energy singly occupied molecular orbital (SOMO) and will react faster with molecules having a low-energy LUMO. This orbital is mostly localized in the aromatic portion of the molecule. It also has an important contribution from the atomic orbitals of the methoxyl group. In fact, the LUMO has anti-bonding character with respect to the O-6–CH_3 bond. Therefore, it can suffer a nucleophilic radical attack, weakening the O–CH_3 bond and, consequently, favoring the formation of the radical **O-6**. However, according to the thermodynamic analysis, the **O-7**, **O-6** and **O-5** are similar (+)-bergenin radicals. The secondary products (H–R) formed from the radical attack on the sites O-7 and O-5 are the same, explaining why they have similar reaction energies (Supplementary Table 1)†. Therefore, we decided to calculate a model structure for the radicals **O-5** and **O-7** with the methyl group at the O-6 position replaced by hydrogen, permitting

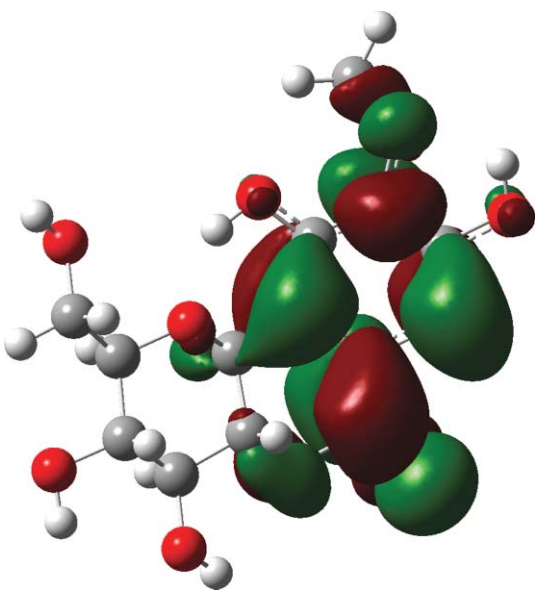


Fig. 5 LUMO of **1**, calculated using the PBE/6-311++G(d,p) level of theory.

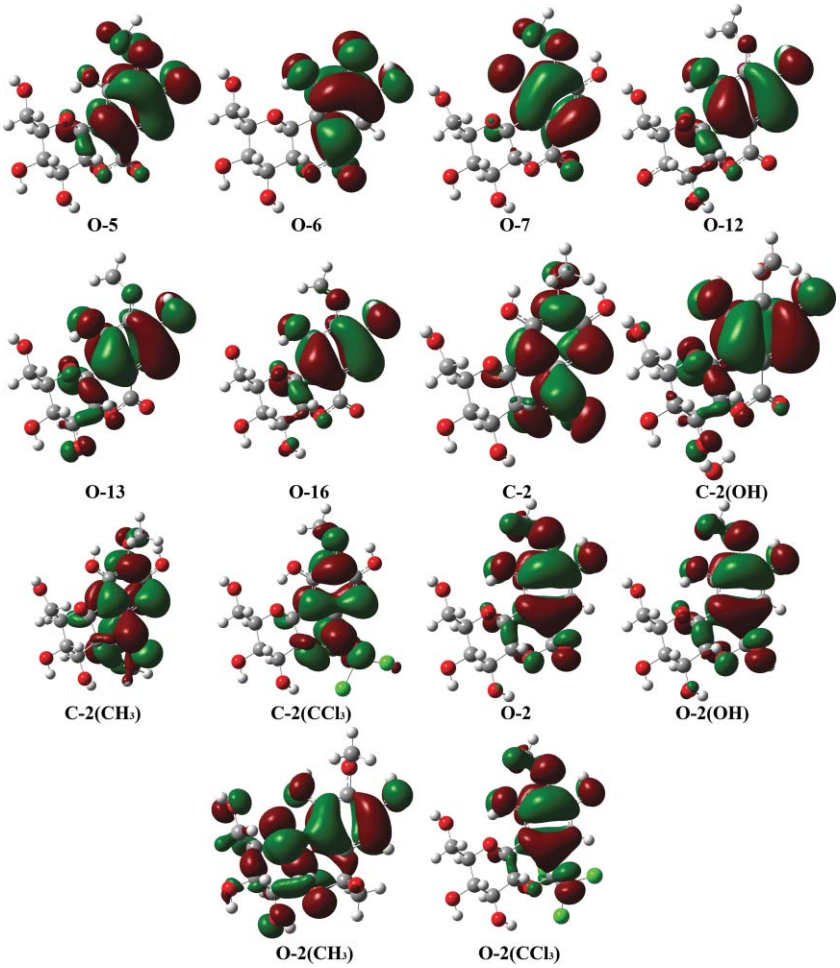


Fig. 6 SOMO of the radical derivatives of **1**.

the direct comparison of the stability of the three radicals and its effect on the regiospecificity. The most stable radical is **O-6**, and **O-5** and **O-7** are about 8.5 and 9.7 kcal mol^{-1} higher in energy, respectively. It is clear that the difference in the stability of **O-6** is predominantly due to the secondary product CH_3–R being more stable than the H–R one.

The SOMO of the radical derivatives of **1** are shown in Fig. 6. For all of them, the SOMO is mostly localized on the aromatic portion, showing that this region is responsible for the radical scavenging ability of (+)-bergenin. In fact, the SOMO is delocalized through the aromatic portion, stabilizing it and, consequently, favoring the radical formation.

Conclusions

The popular use of *Sacoglottis uchi* for the treatment of hepatic diseases can be attributed to the presence of (+)-bergenin as a phytoconstituent. (+)-Bergenin has been isolated and unambiguously characterized. Tests with β-carotene, DPPH and a heterogeneous Fenton system showed that (+)-bergenin is a good free radical scavenger and presents excellent antioxidant activity. The frontier orbital analysis and the calculated electronic and thermodynamic properties showed that the aromatic portion is responsible for the antioxidant activity of (+)-bergenin. The radical attack is favored at the aromatic portion due to the fact that the LUMO is completely localized on this region. Furthermore, the LUMO has anti-bonding character at O-6–CH_3, facilitating the breaking of the bond at the O-6 site. The singly occupied molecular orbital of the radical derivatives of (+)-bergenin are greatly stabilized due to their delocalization through the aromatic ring. The thermodynamic stability of the secondary product, CH_3–R, is also important in understanding the preference of radical attack towards the O-6 site when compared to the O-5 and O-7 sites. The presence of the aromatic ring in the structure of (+)-bergenin is of fundamental importance for its antioxidant activity and, modification of its structure can be envisaged to improve its radical scavenging properties.

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