

Bioactive pyrones and flavonoids from *Cryptocarya ashersoniana* seedlings

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Dedicated to Professor Otto Richard Gottlieb

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Abstract

The bioassay directed fractionation of the EtOH extract from leaves of *Cryptocarya ashersoniana* seedlings led to the isolation of two flavonol glucosides: *iso*-quercitrin and hyperin, which exhibited free radical scavenging activity towards DPPH (IC₅₀ 34.4 μM and 32.7 μM, respectively) and were compared to standard compounds rutin (IC₅₀ 27.0 μM) and catechin (IC₅₀ 41.4 μM). Investigation of extracts from the seedlings roots and stems afforded one antifungal styrylpyrone: goniothalamine, and two dihydropyrones: 6-propyl-5,6-dihydro-2-pyrone and the new 6-[(4'-ethyl-9'-oxabicyclo[3.3.1]non-6'-en-3'-yl)methyl]-5,6-dihydro-2H-pyran-2-one, which had its structure determined by detailed analysis of MS and NMR data, including 2D experiments.

Keywords: *Cryptocarya ashersoniana*, Lauraceae, pyrone, flavonoid, antioxidant, free radical scavenger, DPPH

Introduction

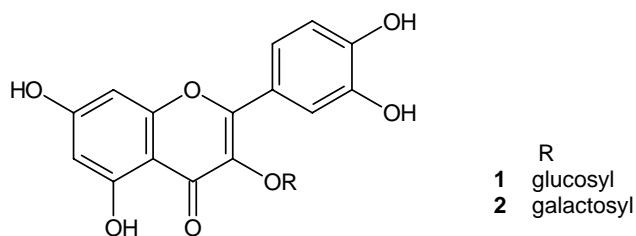
Cryptocarya genus belongs to Lauraceae family and comprises more than 350 species which occur in South America, Asia and Australia. Only three *Cryptocarya* species occurring in the Brazilian Atlantic Rain Forest have been chemically studied so far, and have shown to contain pyrones, flavonoids and alkaloids, in addition to sesquiterpenes in the essential oil.¹⁻⁴ Alkaloids and pyrones are widely distributed in this genus in contrast to the chemical profile of Lauraceae family, in which lignans and neolignans are considered chemotaxonomical markers.⁵

Cryptocarya species have been used as traditional medicines in South Africa for their antiinflammatory and other activities.^{6,7} Some of the pyrones and styrylpyrones showed larvicidal and antifertility activities, in addition to inhibition of breast cancer cell lines growth.⁸⁻

¹¹ In this work, the chemical composition of *C. ashersoniana* seedlings has been assessed, in the search for bioactive compounds. Additionally, as the chemical profile of seedlings may be quite diverse from adult specimens,¹² resulting from the developing enzymatic apparatus of the former, this work has also focused on chemical data to be used in chemical variability studies on *Cryptocarya* species currently performed in our lab.¹³ Seedlings of *C. ashersonian* were grown in our greenhouse and the extracts of their leaves, stems and roots were chemically investigated and led to the isolation of flavonol glucosides **1** and **2** from leaves, and pyrones **3-5** from stems and roots. Compounds **1-5** were tested against the phytopathogen fungi *Cladosporium cladosporioides* and *C. sphaerospermum* growth, and flavonol glucosides **1** and **2** were further evaluated for their free radical scavenging activities by spectrophotometric test using DPPH.

Results and Discussion

The EtOH extract from leaves of *C. ashersoniana* seedlings showed the presence of antioxidant compounds on the β -carotene test on TLC and afforded flavonol glucosides *iso*-quercitrin (**1**) and hyperin (**2**), which were identified by MS and NMR spectral analysis and comparison with literature data.¹⁴⁻¹⁶ They showed moderate free radical scavenging activity (IC_{50} 34.4 μ M and 32.7 μ M), respectively when compared to standard compound rutin (IC_{50} 27.0 μ M) and higher activity than catechin (IC_{50} 41.4 μ M) (Figure 1), which is probably due to the α,β -unsaturated carbonyl moiety conjugated to ring B and the catechol group. Compounds **1** and **2** showed no antifungal activity as they did not inhibit *Cladosporium cladosporioides* and *C. sphaerospermum* growth on TLC plates.



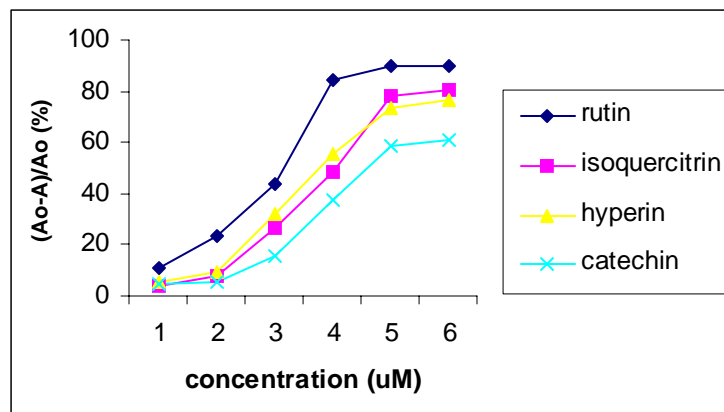


Figure 1. Free-radical scavenging activity of flavonol glucosides **1** and **2**, and reference compounds rutin and catechin.

From the EtOH extract of stems and roots of *C. ashersoniana* seedlings, pyrones **3-5** were isolated and had their structures determination based on MS and NMR analysis, as well as comparison with literature data.

The ^1H NMR spectrum of compound **3** showed four signals at δ 5.65, δ 5.71, δ 5.95 and δ 6.80 suggesting the presence of two disubstituted double bonds, including one α,β -unsaturated carbonyl moiety; and three signals at δ 3.58, δ 3.93 and δ 4.30, assigned to oxymethine hydrogens. The ^{13}C NMR spectrum of compound **3** showed five signals for sp^2 carbons, including one α,β -unsaturated carbonyl moiety and 11 signals for sp^3 carbons, including three oxymethine carbons at δ 70.7, δ 76.0 and δ 77.8. Combined with DEPT 135° data, which evidenced only five methylene carbons, and MS data, which showed the molecular ion peak at M^+ 262, the formula $\text{C}_{16}\text{H}_{22}\text{O}_3$ was established, suggesting the structure of a pyrone ring attached to an unsaturated bicyclic C_{11} aliphatic chain. One triplet at δ 0.86 (3H), correlating to a multiplet at δ 1.50 (2H) in the HOMOCSY spectrum and to signals at δ 11.9 (C-11') and δ 28.6 (C-10'), in the HMQC and HMBC spectra, respectively, evidenced an ethyl unit, which is linked to a methine carbon (δ_{C} 45.4, δ_{H} 1.75), assigned to C-4', as shown by HMBC cross-peaks between H-11' and C-4', as well as by HOMOCSY correlations of signals for H-4' and H-10'.

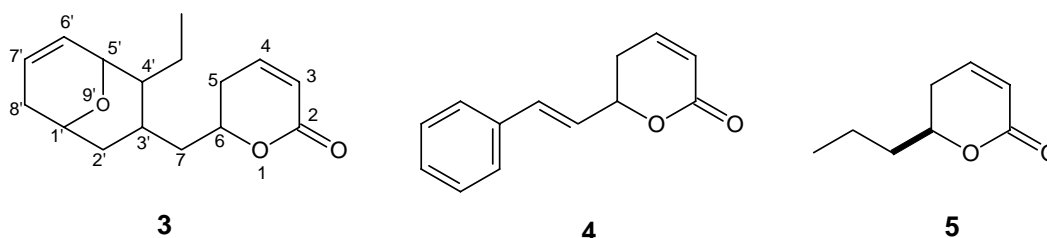


Table 1. – NMR data for compound **3**^a

Position	δ_C^b	δ_H^c	COSY	HMBC	TOCSY
2	164.0 s	–			–
3	121.5 d	5.95 dt (2.0;9.5)	H-4	H-5	H-4
4	144.8 d	6.80 dt (4.0;9.5)	H-3/H-5	H-5	H-3
5	29.9 t	2.25 m	H-4/H-6	H-7	H-6/H-7/H-3'
6	76.0 d	4.30 br tdd (3.5;7.8;8.6)	H-5/H-3'	H-7	H-5/H-7/H-3'
7	41.7 t	1.90 m	H-3'/H-4'/H-8'	H-7	H-2'/H-4'/H-5'
1'	70.7 d	3.93 br dq (3.0; 7.0)	H-5'/H-8'	H-5'	H-5'/H-8'
2'	36.9 t	2.30/1.50 m	H-3'/H-7/H-8'	H-7/H-3'	H-3/H-6/H-7/ H-2'/H-4'/H-8'
3'	37.5 d	1.60 m	H-7/H-2'/H-6	H-7	H-5/H-6/H-1'/ H-3'/H-8'
4'	45.4 d	1.75 m	H-7/H-10'	H-7/H-3' H- 5'/H-11'	H-7/H-10'/H11'
5'	77.8 d	3.58 t (7.5)	H-1'/H-8	–	H-1'/H-8'
6'	130.5 d	5.71 dt (2;11)	H-7'	–	H-7'
7'	128.4 d	5.65 ddd (2;3;11)	H-6'	–	H-6'
8'	33.9 t	3.14 br dt (2.0; 9.3)/ 1.90 m	H-2'/H-8'	–	H-2'/H-3'/H-5'/ H-8'
10'	28.6 t	1.50 m	H-4'/H-11'	H-11'	H-4'/H-11'
11'	11.9 q	0.86 t (7.5)	H-10'	H-10'	H-4'/H-10'

^a CDCl₃, 500 MHz and 125 MHz for ¹H and ¹³C NMR, respectively. Assignments were mostly based on HMQC and HOMOCOSY experiments.

^b multiplicities were obtained from DEPT 135° experiment.

^c δ , multiplicity, *J* (Hz).

Two signals at δ 2.25 (m, 2H) and δ 4.30 (br tdd, 1H) in the ¹H NMR spectrum were assigned to H-5 and H-6 of the pyrone ring, respectively, and showed correlations with signals at δ 29.9 and δ 76.0 in the HMQC spectrum, which were then assigned to the methylene C-5 and oxymethine C-6, respectively. HMBC correlations of C-5 and C-6 to methylene hydrogens at δ 1.90 (H-7) and, from the latter to the signals at δ 36.9 (C-2') and δ 37.5 (C-3'), established the linkage between the pyrone ring and the bicyclic moiety (Figure 2). One triplet at δ 3.58 and one broad double-quadruplet at δ 3.93 for the two remaining oxymethine hydrogens, showing correlations with signals at δ 77.8 (C-5') and δ 70.7 (C-1') in the HMQC spectrum, suggested an unsaturated six-membered ring with an ether linkage, after observation of a cross-peak between the oxymethine hydrogens H-1' (δ 3.93) and H-5' (δ 3.58) in the HOMOCOSY and TOCSY spectra (Figure 3) as well as the HMBC correlation of H-5' and C-1' (Figure 2). The TOCSY spectrum showed correlations of signals at δ 1.75 (H-4'), δ 1.60 (H-3') and δ 1.90 (H-7), which

suggested an additional cyclization between C-3' and C-4', leading to a bicyclic moiety. This explains further correlations, e.g. from H-7 (δ 1.90) and H-3' (δ 1.60) to C-4' (δ 45.4), observed in the HMBC spectrum; and a long distance correlation (J^A) between H-7 and H-4' in the HOMOCOSY spectrum. Additional HMBC and HOMOCOSY correlations confirmed the proposed structure for compound **3** (Table 1). The relative configuration of asymmetric centers C-1', C-3', C-4' and C-5' was established by analysis of coupling constants, e.g. $J_{\text{H-4'/H-5'}} = 7.5$ Hz, which evidenced the *cis* relationship of H-4' and H-5'; and additionally by NOESY experiments, which showed correlations between H-3'/H-4' and H-4'/H-5', and confirmed the *cis* orientation for these hydrogens. The *cis* relationship of H-1'/H-5' was assumed due to high steric hindrance of the (H-1'/H-5') *trans* isomer, as observed by molecular modeling and theoretical studies on steric energy minimization using the MM2 software and on heats of formation using the semiempirical PM3 and AM1 methods on SCF-MO-MOPAC-2000 software.¹⁷ A positive Cotton effect due to the carbonyl $n \rightarrow \pi^*$ transition of the α,β -unsaturated- δ -lactone moiety was observed at λ_{max} 255 nm ($\Delta\epsilon$ +2.84) in the CD spectrum of **3**. This observation indicated that the absolute configuration at C-6 was (S) on the basis of the rule first proposed by Sneath¹⁸ and later modified by Beecham,¹⁹ considering the conformation of the α -pyrone ring, which was inferred due to steric factors.²⁰ These observations led to the establishment of the new pyrone as 1'*R*',3'*S*',4'*R*',5'*S*',6*S*-[4'-ethyl-9'-oxabicyclo[3.3.1]non-6'-en-3'-yl)methyl]-5,6-dihydro-2H-pyran-2-one.

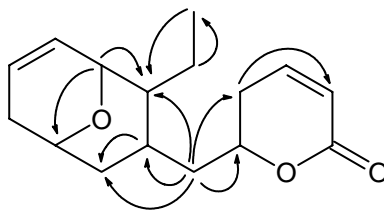


Figure 2. Selected HMBC correlations for compound **3**.

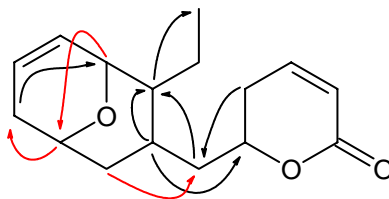


Figure 3. Selected HOMOCOSY (red arrows) and TOCSY (black arrows) correlations for compound **3**.

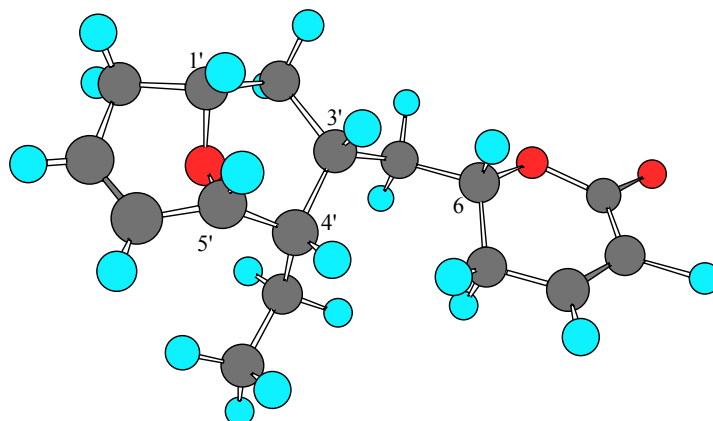
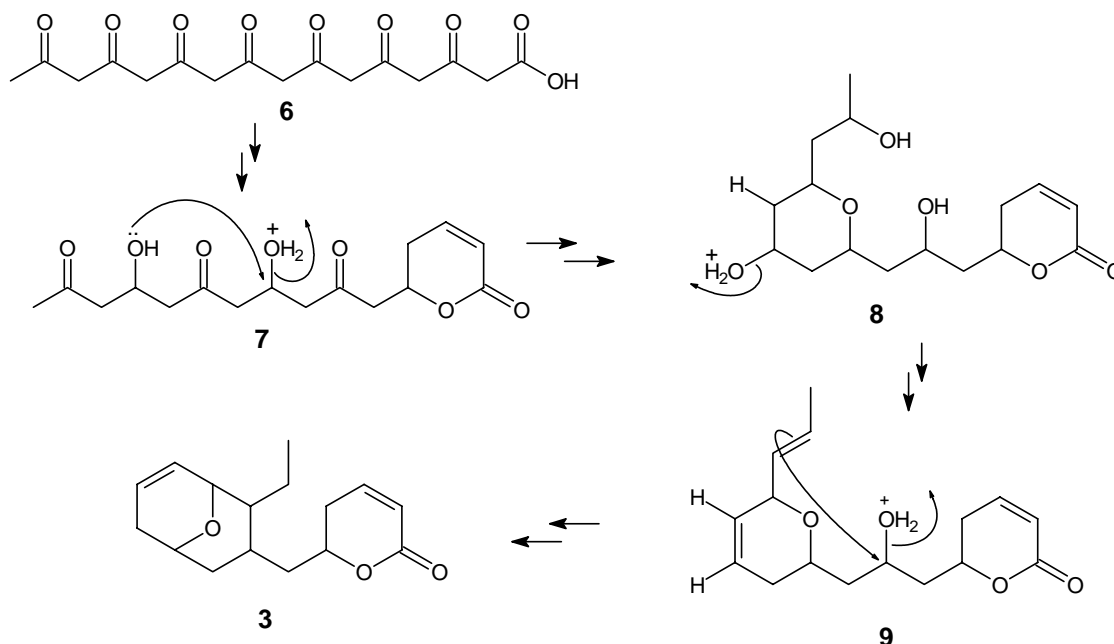
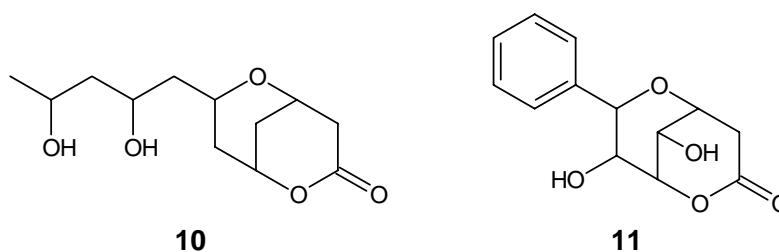


Figure 4. Structure of pyrone **3** evidencing the *cis* relationship of H-1', H-3', H-4' and H-5'.

The biogenetic pathway leading to this novel pyrone was proposed, in which an acetate derived C₁₆ chain has gone through cyclization reactions to originate the pyrone ring via nucleophilic attack followed by dehydration steps (Scheme 1). The subsequent attack of HO-5' onto C-1' with the ring closure via an ether linkage followed by dehydration and reduction steps might yield intermediate **8**. Two additional dehydration steps would yield intermediate **9**, which might go through a final cyclization, resulting from nucleophilic attack of C-4' onto C-3', and the subsequent formation of compound **3**. Bicyclic pyrones resulting from cyclization of the side-chain onto the α,β -unsaturated carbonyl ring as cryptocaryolone (**10**) and goniopyrone (**11**) have been reported solely from other *Cryptocarya* (Lauraceae) and *Goniothalamus* (Annonaceae) species, respectively^{21,22} and led to structures in which the pyrone C-C double bond is lost. Compound **3** differs from those as the driving force for both cyclization steps do not involve the α,β -unsaturated carbonyl moiety of the pyrone ring. Conversely, nucleophilic attacks, dehydration and reduction reactions involving hydroxy groups and double bonds in the side-chain led to a bicyclic structure linked to an intact pyrone moiety.



Scheme 1. Biogenetic proposal for compound 3.



Compound 4 was identified with goniotalamine, previously isolated from *Goniothalamus macrophyllus* (Annonaceae), from the bark of *Cryptocarya caloneura* and *C. moschata*^{23,24} and from leaves of *C. ashersoniana*.²⁵ It showed CNS activity and induced fetal abnormalities in rats.^{26,27} Compound 5 was identified with 5,6-dihydro-6-propyl-2H-pyran-2-one, which had been isolated previously from the fungus *Lasiodiplodia theobromae*, growing on fruits of *Palmae* species²⁸ and is now reported for the first time from a plant species. Compounds 3-5 were assayed for antifungal activity by bioautography with phytopathogen fungi *Cladosporium cladosporioides* and *C. sphaerospermum*.²⁹ Compound 5 showed moderate activity on the TLC assay and had its MIC further determined (8 μg) and compared to standard fungicidal compounds nystatin (MIC 0.5 μg) and myconazol (MIC 0.5 μg).

Experimental Section

General Procedures. Silica gel (Merck, 230-400 and 70-230 Mesh), Sephadex LH-20 (Pharmacia) and polyvinylpyrrolidone (PVPP) were used for column chromatography unless otherwise stated and solvents were redistilled prior to use. NMR spectra were recorded on Bruker AC-200 or Varian INOVA 500 spectrometers at 200 or 500 MHz, respectively, for ^1H NMR, and at 50 or 125 MHz, respectively, for ^{13}C NMR, with TMS as internal standard and $\text{DMSO-}d_6$ or CDCl_3 as solvent. ES-MS were recorded on a VG Platform II spectrometer and elemental analysis was performed on a Perkin-Elmer Analyser model 2400CHN. Theoretical study was done using the semiempirical PM3 and AM1 methods on SCF-MO-MOPAC-2000 software. CD curves were obtained using a ISA Jobin Yvon CD6 dichrograph and optical rotation was measured on a Perkin-Elmer 241 polarimeter using a quartz cuvette (length 1 cm).

Plant material. Seedlings of *C. ashersoniana* were grown in the greenhouse at Chemistry Institute, Sao Paulo State University in Araraquara, Brazil and were harvested when the seedlings were ca. 10 months old and 45 cm high.

Extraction and isolation. Leaves, stems and roots were dried at 40°C and ground separately. Each plant material was extracted with ethanol, which was then evaporated under vacuum and afforded the EtOH extracts of leaves (30.7 g), stems (3.7 g) and roots (16.7 g). The EtOH extract from leaves (10.0 g) was partitioned with organic solvents to afford the hexane (4.6 g), chloroform (1.1 g), EtOAc (3.3 g) and hydroalcoholic (0.3 g) fractions. The EtOAc fraction (3.0 g) was applied to a Sephadex LH-20 column (300.0 g), eluted with MeOH to give 10 fractions. Fraction 7 (0.234 g) was submitted to HPLC separation (RP-18; MeOH/ H_2O 72:28; flow 9 mL/min; UV detection at 280 nm) and afforded compounds **1** (12 mg) and **2** (7 mg). The EtOH extract (8.2 g) of roots and stems were combined after comparison by TLC analysis and submitted to MPLC (RP-18, H_2O /MeOH gradient), affording 11 pooled fractions (A-K). Fraction E (830 mg) was submitted to MPLC (silica gel, hexane/EtOAc gradient) and afforded 14 fractions (E1-E14). Fractions E4 (39 mg) and E9 (48 mg) were further purified by preparative TLC (CH_2Cl_2 /MeOH/*iso*-PrOH 97:2:1) and yielded compounds **4** (16 mg) and **5** (8 mg), respectively. Fraction G (480 mg) was also submitted to MPLC (silica gel, hexane/EtOAc gradient) and afforded 9 fractions (G1-G9). Fraction G6 (27 mg) was purified by HPLC (RP-18; MeOH/ H_2O 87:13; flow 9 mL/min; UV detection at 240 nm) and afforded compound **3** (6 mg).

Compound 3. $1'R^*,3'S^*,4'R^*,5'S^*,6S$ -6-[(4'-ethyl-9'-oxabicyclo[3.3.1]non-6'-en-3'-yl)methyl]-5,6-dihydro-2H-pyran-2-one. Brown oil, $\text{C}_{16}\text{H}_{22}\text{O}_3$ (calc. 73.25% C, 8.45% H; obs. 73.21% C; 8.48% H); $[\alpha]_D^{25} = +57.7^\circ$ (CHCl_3 , c 0.52); $\Delta\epsilon_{\text{Max}}$ (255nm) = 2.84 (MeOH, c 2.0); ESIMS, m/z (rel. int.) (40V, positive mode): 285 $[\text{M}+\text{Na}]^+$ (100), 263 $[\text{M}+\text{H}]^+$ (35), 262 $[\text{M}]^+$ (17). ^1H and ^{13}C NMR spectral data, see Table 1.

Bioautography with phytopathogen fungi on TLC plates.²⁵ Each sample is submitted to TLC analysis and, after dry, the plate is sprayed with *Cladosporium cladosporioides* or *C. sphaerospermum* spore solution. The plates are kept at 25°C in the dark for 2-3 days, when the

fungi grow all over the plates, except for spots where antifungal compounds are present, which remain white. The inhibition zones are measured and compared to standard compounds nystatin and myconazol.

Bleaching experiments on β -carotene.²⁶ Each extract (ca. 10 μ g) was assayed for the presence of antioxidants using a TLC test and two eluents ($\text{CHCl}_3/\text{MeOH}$ 7:3 and hexane/EtOAc 1:1). After developing and drying, TLC plates were sprayed with a 0.02% solution of β -carotene (Aldrich) in CH_2Cl_2 . Plates were placed under natural light until discoloration of background. The remaining yellow spots indicated the presence of antioxidant substances.

Spectrophotometric assay on the reduction of 2,2-diphenyl-1-picrylhydrazyl radical.²⁷ Reaction mixtures containing test samples (dissolved in MeOH) at several concentrations and 0.004% DPPH methanolic solutions were incubated at 25°C for 30 min. Absorbances of the resulting solutions were measured using a Milton Roy 20D spectrophotometer at 517 nm and the percent inhibition was determined by comparison with a MeOH treated control group.

Acknowledgments

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