Antioxidant and Cytotoxic Flavonols from *Calotropis procera*

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Phytochemical investigations of *Calotropis procera* leaves have led to the isolation of two new compounds: quercetagetin-6-methyl ether 3-*O*-*β*-D-$^4C_1$-galacturonopyranoside (**3**) and (*E*)-3-(4-methoxyphenyl-2-*O*-*β*-D-$^4C_1$-glucopyranoside)-methyl propenoate (**4**), along with eleven known metabolites: nine flavonol and two cinnamic acid derivatives. All metabolites were isolated for the first time from the genus *Calotropis*, except for **1** isolated previously from *Calotropis gigantea*. The structures were determined by spectroscopic methods (UV, ESI-MS, ^{1}H, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, and HMBC). The radical scavenging activity of the aqueous methanol extract and compounds **8**–**13** was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. Cytotoxic screening of the same compounds was carried out on brine shrimps as well.

Key words: Calotropis procera, Flavonols, Antioxidant

Introduction

Calotropis procera (Ait.) R. Br. is a xerophytic shrub belonging to the Asclepiadaceae family and commonly known as Arka in India. It is widely distributed in Asia, Africa, South America, and abundant in the northeast of Brazil. Asclepiadaceous plants are mainly either tropical or subtropical (South America and South Africa) with only a few species growing in temperate regions (Heywoo, 1978). *C. procera* is known to possess multifarious medicinal properties. It has been used for the treatment of leprosy, ulcers, tumours, piles, and diseases of spleen, liver and abdomen (Kirtikar and Basu, 1935). It also has insecticidal (Mours, 1997), larvicidal (Markouk *et al*., 2000), antibacterial, and antiparasitic activities (Larhsini *et al*., 1999). The root of *C. procera* is used as a carminative in the treatment of dyspepsia (Kumar and Arya, 2006). Further, the root bark and leaves of *C. procera* are used by various tribes of central India as a curative agent for jaundice (Samvatsar and Diwanji, 2000). The chloroform extract of the root also has been shown to provide protection against carbon tetrachloride-induced liver damage (Basu *et al*., 1992). An ethanolic extract of the flowers of *C. procera* is reported to have antimicrobial, anti-inflammatory, antipyretic, analgesic (Mascolo *et al*., 1988), anticancerogenic (Smit *et al*., 1995), and antimalarial (Sharma and Sharma, 1999, 2001) activities. The milky white latex of this plant has been reported to exhibit potent anti-inflammatory and analgesic, and weak antipyretic activities in various experimental models (Kumar and Arya, 2006; Kumar and Basu, 1994; Dewan *et al*., 2000a, b). The latex also inhibits the inflammatory response elicited by various inflammation mediators (Arya and Kumar, 2005). Besides, it has also been demonstrated to possess antioxidant, antihyperglycemic, and hepatoprotective properties (Roy *et al*., 2005; Padhy *et al*., 2007). Recently, the aqueous extract of the latex has been shown to inhibit cellular infiltration and afford protection against the development of neoplastic changes in the transgenic mouse model of hepatocellular carcinoma (Choedon *et al*., 2006), in addition to the application as an antidote for snake poisoning (Nandkarni, 1976).

The plant has been investigated phytochemically for cardenolides, anthocyanins, tannins, sterol, hydrocarbons, a steroid, and triterpenoids (Mascolo *et al*., 1988; Abdul Qasim and Abdul Malik, 1989). The wide and diverse biological activit-ies of *C. procera* stimulated us to isolate and characterize polyphenols from the aqueous methanol extract (AME) of *C. procera* leaves. The antioxidant capacity of the extract and the major isolated compounds **8**–**13** as well as their cytotoxic activity towards brine shrimps have been studied.

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Material and Methods

Equipment

The NMR spectra were recorded at 300, 400 (^{1}H) and 75, 100 (^{13}C) MHz, respectively, on a Varian Mercury 300 (Palo Alto, CA, USA) and JEOL GX-400 NMR spectrometer (Akishima, Japan), and δ values are reported in ppm relative to TMS in the convenient solvent. ESI-MS analyses were measured on a Finnigan LCQ deca LC/MS and double focusing sector field MAT 90 MS spectrometer (Finnigan, Bremen, Germany). UV spectra of pure samples were recorded, separately, in MeOH using different diagnostic UV shift reagents using a Shimadzu UV 240 spectrophotometer (Kyoto, Japan). For column chromatography (CC), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Darmstadt, Germany), polyamide 6S (Riedel de Haën AG, Seelze, Germany), and silica gel powder (G 60 E; Merck) were used. For paper chromatography Whatman No. 1 sheets (Maidstone, England) were used.

Plant material

Leaves of *Calotropis procera* were collected in spring from fields near Cairo (Desert), Egypt. The plant was authenticated by Wafaa M. Amer, Professor of Taxonomy, Department of Botany, Faculty of Science, Cairo University, Giza, Egypt. Voucher specimens (Reg. No. C–II) are kept in the herbarium, Medicinal Chemistry Department, Theodor Bilharz Research Institute, Giza, Egypt.

Brine shrimp lethality bioassay

Eggs of *Artemia salina* were allowed to hatch into their larvae (Fatope *et al.*, 1993). Compounds **8**–**13** were separately dissolved in distilled water to give four assay concentrations (1000, 500, 100, and 10 μg ml^{-1}); solubility was aided by Tween 80. Each dose was examined in triplicate. Potassium dichromate was used as a reference drug and dissolved in sea water, to obtain concentrations of 1000, 100, and 10 μg ml^{-1}. Assays were performed in test tubes with ten larvae each, and the final volumes were adjusted to 5 ml sea water immediately after adding the shrimps. After 24 h, the number of surviving shrimps was recorded for each dose. The LC_{50} values were calculated by the use of the Instate computer program.

Antioxidant activity

This activity was investigated *in vitro* for the aqueous methanol extract (AME) and compounds **8**–**13** using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, according to the method of Peiwu *et al.* (1999). In a disposable cuvette a methanolic solution of DPPH (2.95 ml, 100 μM) was added to a 50-μl sample of AME or the respective compounds (in MeOH) in different concentrations (2–100 mg ml^{-1} for quercetin, and 20–100 mg ml^{-1} for AME and compounds **9**–**13**). The absorbance was measured at 517 nm at regular intervals of 15 s for 5 min as described by Govindarajan *et al.* (2003). Ascorbic acid was used as standard (0.1 M). The percentage inhibition was calculated according to:

% inhibition (reactive reaction rate) =

$$\frac{\text{Abs.(DPPHsolution)} \cdot \text{Abs.(sample)}}{\text{Abs.(DPPHsolution)}} \cdot 100 \, .$$

Extraction and isolation

The air-dried powdered leaves of *C. procera* (750 g) were exhaustively extracted with 85% MeOH (5 x 1.5 l and 2 x 2.5 l, respectively), under reflux (70 °C). The AME was concentrated under reduced pressure and defatted with light petroleum ether (60–80 °C, 5 x 1 l) to give a dark brown viscous extract which was dissolved in water, and the water-insoluble residue was removed by filtration. The water-soluble portion was desalted by precipitation with excess MeOH to give a dry brown residue (60 g) that was suspended in H_2O and fractionated on a polyamide column (110 cm x 6 cm, 300 g) using a stepwise gradient from H_2O, H_2O/MeOH mixtures up to pure MeOH for elution. Based on comparative paper chromatography (Co-PC) with the use of UV light, 1% $FeCl_3$, or Naturstoff spray reagent for detection, the individual 75 fractions (each 1 l) were pooled into 5 collective fractions (A–E). Fraction A (H_2O, 14 g) was found to be a dark brown material with no phenolic character. Fraction B (10–40% MeOH, 11 g) was fractionated on cellulose C with 40% EtOH as an eluent, followed by a Sephadex LH-20 column using BIW (*n*-BuOH/2-propanol/H_2O, 4:1:5 v/v/v, organic layer) to afford pure **1** (47 mg) and **2** (42 mg). Fraction C (40–60% MeOH, 10 g) was subjected to repeated CC on cellulose and Sephadex LH-20 with 20–60% aqueous MeOH as an eluent, resulting in pure samples of **3** (32 mg)

and **4** (39 mg). Fraction D (70%, 11 g) was chromatographed on Sephadex with MeOH to give two major subfractions. With the use of UV light (365 nm) two major blue spots were detected in the first one which was applied to cellulose and eluted with 30–50% aqueous MeOH followed by Sephadex LH-20 chromatography with EtOH to give pure **5** (10 mg) and **6** (15 mg). Repeated CC of the second subfraction on Sephadex with EtOH, afforded a pure sample of **7** (19 mg). Fraction E (80–100% MeOH, 9 g), was fractionated on a silica gel column (70–230 mesh, Merck) and eluted with $CHCl_3$, followed by a gradual increase in EtOAc up to 100% (v/v), to afford three subfractions (E_1–E_3). Subfraction E_1 (0–50% EtOAc, 2.5 g) was separated on a Sephadex LH-20 column with BIW to afford two sub-subfractions, each of which was separately purified on Sephadex with MeOH to give compound **8** (120 mg) from the first sub-subfraction and compounds **9** (75 mg) and **10** (95 mg) from the second one. With the use of UV light (365 nm) two major dark purple spots were detected in subfraction E_2 (50–60% EtOAc, 3 g), which was separated on microcrystalline cellulose with BIW as eluent to afford two major sub-subfractions; each one was fractionated on Sephadex twice with MeOH to give compounds **11** (73 mg) and **12** (87 mg). The major constituent **13** (77 mg) was obtained in a chromatographically pure form by repeated fractionation of subfraction E_3 (60–100% EtOAc, 1 g) on Sephadex LH-20 using 70% aqueous EtOH as eluent. All separation processes were followed by 2D-PC and Co-C using Whatman No. 1 paper with *n*-BuOH/AcOH/H_2O (4:1:5, top layer) (S1) and 15% aqueous AcOH (S2) as solvent systems.

*Quercetagetin-6-methyl ether 3-O-β-*D*-$^4C_1$-galacturonopyranoside* (**3**): Yellow amorphous powder. – Rf = 0.53 (S1), 0.59 (S2). – UV/Vis λ_{max} (MeOH) = 261, 355; (NaOMe) 271, 345sh, 407; (NaOAc) 271, 333sh, 380; (NaOAc/H_3BO_3) 265, 380; ($AlCl_3$) 270, 310, 365, 403; ($AlCl_3$/HCl) 270, 300sh, 359, 392 nm. – ^{1}H NMR (400 MHz, DMSO-d_6): δ = 12.57 (1H, s, OH-5), 7.69 (1H, d, J = 1.5 Hz, H-2'), 7.65 (1H, dd, J = 8.4, 1.5 Hz, H-6'), 6.84 (1H, d, J = 8.4 Hz, H-5'), 6.47 (1H, s, H-8), 5.81 (1H, d, J = 7.2 Hz, H-1"), 3.60 (1H, br d, J = 12.3 Hz, H-6"a), 3.40–3.20 (4H, m, H-4", H-2", 3", 5", 6"b), 3.29 (3H, s, C6-Me). – ^{13}C NMR (100 MHz, DMSO-d_6): δ = 178.09 (C-4), 172.91 (C-6"), 157.91 (C-7), 157.02 (C-2), 152.72 (C-5), 149.02 (C-4'), 146.17 (C-3'), 134.45 (C-3), 132.18 (C-6), 121.43 (C-1'), 121.06 (C-5'), 118.65 (C-6'), 115.97 (C-2'), 104.14 (C-10), 103.72 (C- 1"), 94.32 (C-8), 77.09 (C-5"), 74.08 (C-3"), 74.61 (C-2"), 72.27 (C-2"), 60.34 (CH_3-6). – Negative ESI-MS: m/z = 507.07 [M – H]$^-$, 331.04 [M – H – galacturonic acid]$^-$, 317.02 [M – H – galacturonic acid – CH_3]$^-$, 301.03 [M – H – glacturonic acid – OCH_3]$^-$.

*(E)-3-(4-Methoxyphenyl-2-O-β-*D*-$^4C_1$-glucopyranoside)-methyl propenoate* (**4**): Off-white amorphous powder. – Rf = 0.71 (S1), 0.77 (S2). – Negative ESI-MS: m/z = 369.08 [M – H]$^-$, 207.21 [M – H – 162]$^-$, 193.18 [M – H – 162 – 14]$^-$, 179.15 [M – H – 162 – 2 x 14]$^-$. – ^{1}H NMR and ^{13}C NMR (300 and 75 MHz, DMSO-d_6): Table I.

Results

The defatted total aqueous methanol extract of *C. procera* leaves was fractionated by repeated column chromatographic separations to obtain compounds **1**–**13**. Based on chemical and physicochemical analyses, compounds **1**, **2** and **5**–**13** were identified as isorhamnetin 3-*O*-*β*-D-rutinoside (**1**), isorhamnetin 3-*O*-*β*-D-robinoside (**2**) (Agrawal, 1989), methyl caffeate (**5**), caffeic acid (**6**) (Lu and Foo, 2002), isoquercitrin (**7**), quercetin (**8**),

Table I. ^{1}H, ^{13}C NMR, and HMBC spectral data of **4** (300/75 MHz, DMSO-d_6).

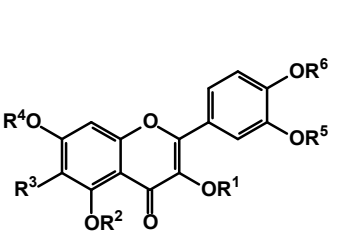
No.	δ_H	δ_C	HMBC
1		116.11	
2		156.99	
3	6.87 d (2.1)	101.11	C-1, 5
4		161.97	
5	6.72 dd (8.7, 2.1)	108.00	C-1, 3
6	7.61 d (8.7)	129.56	C-2, 4, 7
7	7.91 d (16)	138.52	C-2, 6, 8, 9
8	6.30 d (16)	117.24	C-1, 7
9		167.37	
OCH_3	3.78	52.00	C-9
OCH_3	3.76	55.29	C-4
1'	4.99 d (7.6)	100.10	C-2, 3'
2'	3.17*	73.33	C-4'
3'	3.39 t-like (10.8)	76.83	C-1', 5'
4'	3.20 t-like (10.5)	69.80	C-2', 6'
5'	3.29 m	77.29	C-3'
6'a	Hidden by water	60.44	C-4'
6'b	3.745*		

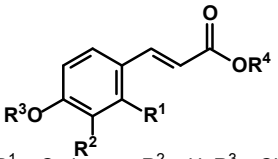
* Unresolved proton resonances.
δ in ppm and J values (Hz) are given in parentheses. All carbon and proton resonances were assigned on the basis of 2D (^{1}H-^{1}H COSY, HSQC and HMBC).

isorhamnetin (**9**), azaleatin (**10**), 3,3'-dimethoxy quercetin (**11**), 3,6,3',4'-tetramethoxy quercetin (**12**), and 3,6,7,3',4'-pentamethoxy quercetin (**13**) (Agrawal, 1989; Harborne and Mabry, 1982).

Compound **3** was expected to be a quercetin derivative based on its chromatographic properties [Rf values, fluorescence under UV light and colour change with NH_3, $FeCl_3$ and Naturstoff spray reagent (yellow, green and orange, respectively)]. On complete acid hydrolysis of **3**, galacturonic acid was detected in the aqueous phase and quercetagetin-6-methyl ether in the organic phase (Co-PC with authentic samples). The negative ESI-mass spectrum of **3** showed a molecular ion peak at *m/z* 507.07 $[M - H]^-$. The fragment ion peak at *m/z* 331.04 indicated the loss of galacturonic acid. The fragmentation of the molecular ion peak at *m/z* 331.04 revealed a daughter ion peak at *m/z* 317.02 ($[M - H -$ galacturonic acid $- CH_3]^-$), while the losses of an OCH_3 group afforded the ion peak at *m/z* 301.03, proving the presence of a methoxy group. The fragment ion peak at *m/z* 182.02 was due to the $C_8H_6O_5$ ion, resulting from the retro-Diels-Alder fragmentation of **3**, suggesting the location of the methoxy group at the A-ring of a flavonol. The ^{1}H NMR spectrum of **3** clearly indicated a singlet for one OH group at δ 12.57 ppm, due to hydrogen bonding to the C-4 carbonyl carbon atom. Additionally the presence of a disubstituted B-ring was confirmed by an ABX-spin coupling system at δ 7.69 (d, J = 1.5 Hz), 7.65 (dd, J = 8.4, 1.5 Hz), and 6.84 ppm (d, J = 8.4 Hz) for H-2', H-6', and H-5', respectively. The singlet signal at δ 6.47 ppm for H-8 together with the singlet at δ 3.29 ppm (3H) for an OMe group were confirmative for a tri-substituted A-ring with the methoxy group most probably at C-6. Typically, 16 carbon signals were similar to those of quercetagetin-6-methyl ether (Fabio *et al*., 1999), assigned in the ^{13}C NMR spectrum of aglycone **3**. Etherification at the C-6 position was further deduced from the downfield location of C-6 (~ + 2 ppm) relative to that of quercetagetin (Agrawal, 1989; Fabio *et al*., 1999). This evidence was supported by an intrinsic downfield shift of the methoxy signal at δ 60.34 ppm (Hussein *et al*., 2003), accordingly, the aglycone identity was confirmed to be quercetagetin-6-methyl ether. The presence of the 3-*O*-galacturonopyranosyl group in **3** was deduced from its anomeric proton signal at δ 5.81 ppm (d, J = 7.2 Hz). This evidence was further substantiated by the slight upfield shift of C-3 and a characteristic downfield shift of C-2 (~ – Δ 10 ppm) (Harborne, 1984). The identity of the sugar moiety in **3** was further confirmed as galacturonopyranoside, due to the resonance of C-6'' at δ 172.91 ppm. The stereostructure of the sugar moiety was established as β-$^4C_1$-pyranose based on the typical J and δ values in both of its ^{1}H and ^{13}CNMR signals. The remaining carbon resonances of **3** were completely assigned by comparison with previously published corresponding data of structurally related compounds (Fabio *et al*., 1999; Harborne, 1984). Therefore, **3** was finally identified as quercetagetin-6-methyl ether 3-*O*-β-D-$^4C_1$-galacturonopyranoside (Fig. 1).

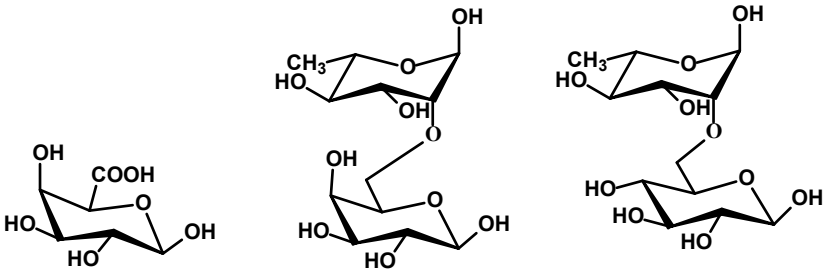
Compound **4** showed chromatographic properties, colour changes in UV light, and UV absorption maxima (237, 302 nm) of cinnamic acid derivatives (Cheel *et al*., 2005). The negative ESI-mass spectrum of **4** exhibited a molecular ion peak at *m/z* 369.08 $[M - H]^-$. The fragment ion peak at *m/z* 207.21 $[M - H - 162]^-$ suggested a hexosyl residue in the molecule. The two fragment ion peaks at *m/z* 193.18 $[M - H - 162 - 14]^-$ and 179.15 $[M - H - 162 - 2 \times 14]^-$ indicated the sequential loss of two methyl groups. On complete acid hydrolysis of **4**, glucose was detected in the aqueous phase (Co-PC with authentic samples). In the aromatic region of its ^{1}H NMR spectrum, an AX-spin coupling system of two *E*-olefinic protons was observed at δ 7.91 (H-7) and 6.30 ppm (H-8) (each, d, J = 16 Hz). In addition the characteristic signals of the aglycone moiety (1,2,4-tri-substituted phenyl ring) were assigned in the form of an AMX-spin coupling system at δ 7.61 ppm (d, J = 8.7 Hz), 6.87 (d, J = 2.1 Hz), and 6.72 (dd, J = 8.7, 2.1 Hz) of H-6, H-3, and H-5, respectively. Additionally, two signals in the aliphatic region were observed at δ 3.78 and 3.76 ppm (each singlet and integrated to three protons) for two methoxy groups. The relative upfield anomeric proton assigned at δ 4.99 ppm (d, J = 7.6 Hz) through its one-bond correlation in HSQC with its own downfield anomeric carbon signal at δ 100.10 ppm was an evidence for the presence of a glucoside in the phenyl ring rather than an ester (Marzouk *et al*., 2007). The attachment of a glucose moiety to C-2 was assigned on the basis of the three-bond correlation peak between H-1' at δ 4.99 ppm and C-2 at δ 156.99 ppm in the HMBC spectrum. The glucose moiety was deduced to have the β-$^4C_1$-pyranose stereostructure based on the J value of the anomeric proton and δ values of its ^{1}H and





4: R^1 = *O*-glucose, R^2 = H, R^3 = CH_3, R^4 = CH_3
5: R^1 = H, R^2 = OH, R^3 = H, R^4 = CH_3
6: R^1 = H, R^2 = OH, R^3 = H, R^4 = H

1: R^1 = -D-rutinoside, R^2 = H, R^3 = H, R^4 = H, R^5 = CH_3, R^6 = H
2: R^1 = -D-robinoside, R^2 = H, R^3 = H, R^4 = H, R^5 = CH_3, R^6 = H
3: R^1 = -D-glacturonopyranoside, R^2 = H, R^3 = OCH_3, R^4 = H, R^5 = H, R^6 = H
7: R^1 = -D-glucopyranoside, R^2 = H, R^3 = H, R^4 = H, R^5 = H, R^6 = H
8: R^1 = H, R^2 = H, R^3 = H, R^4 = H, R^5 = H, R^6 = H
9: R^1 = H, R^2 = H, R^3 = H, R^4 = H, R^5 = CH_3, R^6 = H
10: R^1 = H, R^2 = CH_3, R^3 = H, R^4 = H, R^5 = H, R_6 = H
11: R^1 = CH_3, R^2 = H, R^3 = H, R^4 = H, R^5 = CH_3, R^6 = H
12: R^1 = CH_3, R^2 = H, R^3 = H, R^4 = H, R^5 = CH_3, R^6 = CH_3
13: R^1 = CH_3, R^2 = H, R^3 = H, R^4 = CH_3, R^5 = CH_3, R^6 = CH_3



β-D-Galacturonopyranoside *β*-D-Robinoside *β*-D-Rutinoside

Fig. 1. Chemical structures of compounds isolated from the leaves of *Calotropis procera.*

^{13}C resonances (Table I). The ^{13}C NMR spectral data of compound **4** showed the presence of seventeen carbon signals, six signals were assigned to a glucose moiety, and the remaining signals were assigned to aromatic, olefinic, and dimethoxy carbon atoms. The estrification of **4** at C-9 was deduced from the characteristic signal of one of the two methoxy groups at δ 52.00 ppm. This was further deduced from the observation of the three-bond correlation beak between one of the methoxy proton signals at δ 3.78 ppm and the C-9 signal at δ 167.37 ppm in the HMBC spectrum (Fig. 2). Similarly, the connectivity of the other methoxy group to C-4 of the aglycone was proven by the correlation peak of OCH_3 at δ 3.76 ppm and C-4 at δ 161.97 ppm. All other ^1H and ^{13}C resonances were also confirmed by the ^1H-^1H-COSY, HSQC, and HMBC spectra and by comparison with previously reported data for structurally related compounds (Chen *et al.*, 2007). Thus, the structure of **4** was established as shown in Fig. 1

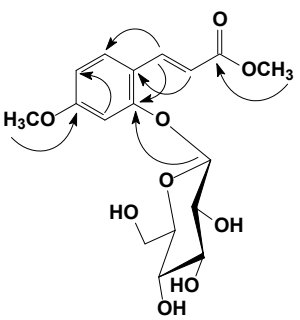


Fig. 2. Selected HMBC correlations for **4**. Arrows point from H to C atoms.

and **4** was named (*E*)-3-(4-methoxyphenyl-2-*O*-*β*-D-$^4C_1$-glucopyranoside)-methyl propenoate.

Among many recent advances in cancer chemotherapy, phytochemicals play an important role as cancer chemotherapeutic drugs. The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity and can be employed for this purpose as it appears to be a convenient, rapid, and inexpensive assay for the determination of cytotoxicity. In the brine shrimp lethality bioassay study of compounds **8** – **13**, only **8**, **9,** and **10** exhibited activity on brine shrimp larvae of *Artemia salina* with LC_{50} values of 10, 22, and 21 mg ml^{-1}, respectively. Potassium dichromate served as the positive control in this assay and gave an LC_{50} value of 2.28 mg ml^{-1}. The structure-activity relationship conducted in this study provided some new information on quercetin which was the most cytotoxic compound among the substituted flavonols. It seems that, on one hand, a methyl substitution of the A- or B-ring, respectively, with a free 3-OH group caused a 50% decrease in the activity of quercetin, *i.e.* isorhamnetin (**9**) and azaleatin (**10**), whereas full methylation of the quercetin hydroxy groups totally abolished it. Therefore, the presence of free hydroxy groups on the A- or B-ring seems to be necessary for the cytotoxic activity of quercetin. On the other hand, 3-etherification completely abolished the activity. This result confirms the importance of the 3-OH substitution of quercetin as a corner stone in the activity.

The aqueous methanol extract (AME) and compounds **8**–**10** exhibited a significant scavenging activity against DPPH radicals *in vitro*. The maximum effect was observed for AME and compounds **8**–**10** after 5 min (Table II, Fig. 3).

Classes of flavonoid compounds have graded antioxidative activities (Peng *et al.*, 2003). The strongest antioxidant activity of flavonols, among other flavonoidal subclasses, was attributed to their main structural features (the presence of a C-ring composed of a 4-keto group, 2,3-double bond and 3-hydroxy group, which form a large π-bond conjugated system with both the A- and B-ring). This conjugation in the structures of our flavonol metabolites is the responsible factor for the stabilization of the aryloxyl radical after hydrogen abstraction in the free radical scavenging process. The quantitative assay revealed varying degrees of antioxidant activity of compounds **8**–**13**. Quercetin (**8**) with free phenolic OH groups (without any methoxy substitution) was the most active compound. Compounds **9**–**13** are quercetin derivatives with different methoxylation patterns. Of the five aglycone derivatives **9**–**13**, compounds **9** and **10** with 3'-OMe and 5-OMe, respectively, were the most active ones. Compounds **9** and **10** have the same oxygenation pattern as the remaining aglycone derivatives but with only C-3'- or 5-methoxylation, respectively. The presence of these free OH groups could be linked to higher antioxidant activity of the two compounds com-

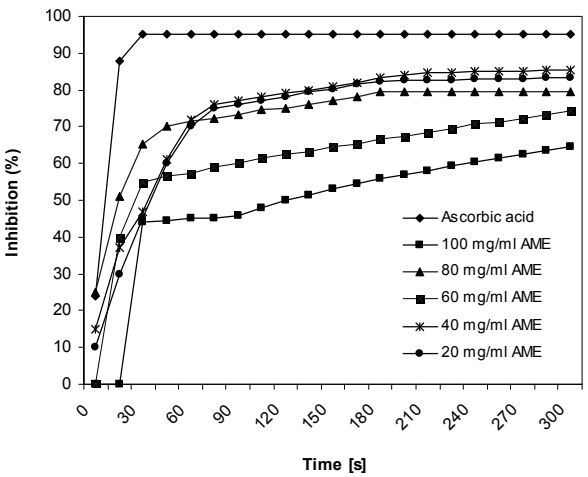


Fig. 3. Antioxidant activity of AME of *C. procera* (20, 40, 60, 80, 100 mg ml^{-1}) and ascorbic acid (0.1 M) *in vitro*, using the DPPH radical scavenging activity method.

Table II. Antioxidant activity after 5 min of AME and compounds **8**–**10** at different concentrations using the spectrophotometric DPPH assay.

Investigated material	Inhibition of DPPH free radical scavenging activity after 5 min at different concentration [mg ml^{-1}]								
	2	4	8	10	20	40	60	80	100
AME	-	-	-	-	83.2	85.5	74.3	79.5	64.7
8	82.1	84.5	85.3	86.1	87.2	88.7	89.3	89.7	86.3
9	-	-	-	-	78.1	79.5	80.3	81.5	79.3
10	-	-	-	-	79.3	80.5	81.9	82.7	80.3

pared to other aglycone derivatives with more methoxylated groups. Methylation of free OH groups in flavonoids substantially reduces the antioxidant activity of the compounds (Op de Beck *et al*., 2003). In line with this observation, compounds **11**, **12**, and **13** with a 3-OMe beside a 3'-OMe group in case of **11**, 6,3',4'-OMe group in case of **12**, and 6,7,3',4'-OMe group in case of **13** did not inhibit 50% of DPPH scavenging capacity at concentrations up to 100 mg ml^{-1}. The weak activity exhibited by these compounds could be linked to the methylation of some of their phenolic OH groups (Op de Beck *et al*., 2003). Methylation of the 3-OH group of compounds **11**–**13** reduced the activity significantly when compared with compounds **9** and **10** with a free 3-OH group. Compound **8** has the same oxygenation pattern as its five derivatives **9**–**13**, but all its OH groups are free and this significantly enhances its antioxidant activity. This observation agrees with an earlier structure-activity study (Op de Beck *et al*., 2003). Our results indicate that only compounds **9** and **10** had strong antioxidant activity but they are still less active than compound **8** (quercetin).

In conclusion, we have clearly shown that *Calotropis procera* displays antioxidant activity *in vitro* and cytotoxic activity in the brine shrimp lethality bioassay, due to the presence of quercetin derivatives. Further studies are needed to confirm these properties of *Calotropis procera in vivo*.

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