ORIGINAL ARTICLES

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Phenolic profiling of an extract from *Eugenia jambos* L. (Alston) - the structure of three flavonoid glycosides – antioxidant and cytotoxic activities

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Phenolic metabolite profiling and identification using high performance liquid chromatography (HPLC) coupled to high resolution accurate mass spectrometry (HR-ESI-MS) with detection of negative ions was used for assaying the complex mixture of phenolics of an aqueous ethanol leaf extract of *Eugenia jambos* L. (Myrtaceae). Eight known polyphenolics were tentatively identified, and, in addition, three hitherto unknown flavonol-*O*-glycosides were detected in the extract. These unknowns were taken as the targets and isolated by means of consecutive polyamide S6, MCI gel and repeated Sephadex LH-20 column fractionation. The isolation and purification were monitored by HPLC/ESI-MS. The isolates were subsequently identified as quercetin 3-*O*-xylosyl-(1"' → 2")-*O*-xyloside, myricetin 7-methylether 3-O-xylosyl-(1"' → 2")-rhamnoside and myricetin 3',5'-dimethyl ether 3-*O*-xylosyl-(1"' → 2")-*O*-rhamnoside. All known metabolites were also separated by applying the same chromatographic techniques. ESI-MS, ^{1}H and ^{13}C NMR spectra were then recorded, completely interpreted and confirmed by HR-ESI-MS and 2D NMR spectroscopy. In order to get information about biological activities of *E. jambos* the extract was tested for radical scavenging activity by DPPH and ORAC assay. In addition, its cytotoxicity was assessed by the neutral red assay against non-tumorigenic HaCaT keratinocytes and the human bladder carcinoma cell line 5637.

1. Introduction

Numerous investigations show that the biological activities of some plant phenolics, especially flavonoids are most probably due to their strong antioxidant properties (Croft et al. 1998). Several plant families are known to be rich in flavonoids. Among these families, the Myrtaceae include species which provide extracts rich in flavonoids, ellagitannins and gallotannins. Despite the valuable discoveries made through studies of constitutive phenolics of several myrtaceous species (Hussein et al. 2007, 2004; Seligmann et al. 1981) many of these plants remain virtually unexplored from a chemical and biological point of view. Thus, we are initiating here a study of one of these plants, namely, *Eugenia jambos* L. (Synonym: *Syzygium jambos* (L.) Alston) as potential sources of new bioactive phenolics. The plant is native to Southeast Asia but it has also been introduced widely on every continent except Antarctica, and has become established and invasive in several regions. *E. jambos* is a large shrub or small-to-medium-sized tree, typically three to 15 metres high, with a tendency to low branching. Its leaves and twigs are glabrous and the bark, though dark brown, is fairly smooth too, with little relief or texture. The leaves are lanceolate, 2 cm to 4 cm broad, 10 cm to 20 cm long, pointed, base cuneate with hardly any petiole, lively red when growing, but dark, glossy green on attaining full size. The flowers are in small terminal clusters, white or greenish white, the long, numerous stamens giving them a diameter of 5 – 8 cm (Chittenden 1951). Literature reviews on *E. jambos* fruits, flowers, seeds, leaves, bark and root preparations either in a form of decoctions or extracts stated that they have been used in different ailments such as central nervous system disorders, diuretic, gastrointestinal, diabetes, rheumatism, microbial infections, lung diseases and disorders (Mohanty and Cock 2010; Djipa et al. 2000). The present study has been undertaken to investigate in detail the phenolic metabolite profile of *E. jambos* aqueous ethanol leaf extract in association with its antioxidant capability and cytotoxicity. During the course of this work, we were able to apply the efficient screening technique of HPLC/HR-ESI-MS (Nawwar et al. 1997) to determine the profile of the phenolics existing in that extract and discover the compounds which might possess new structural features to be taken as targets for comprehensive phytochemical analysis. The HPLC/ESI-MS technique was used to overcome the significant complexity (shown by paper chromatographic analysis) of the *E. jambos* leaf extract. According to the received HPLC/ESI-MS data, there are probably about 160 to 200 compounds contained in the extract investigated, some are very minor, some are minor, few are of more abundance, and several are obviously of isomeric structure (Fig. 1a/b). Consequently, we isolated and determined the structure of eleven metabolites, among which three have not been reported previously to occur as natural products, namely, quercetin 3-O-xylosyl-(1"' → 2")-O-xyloside,

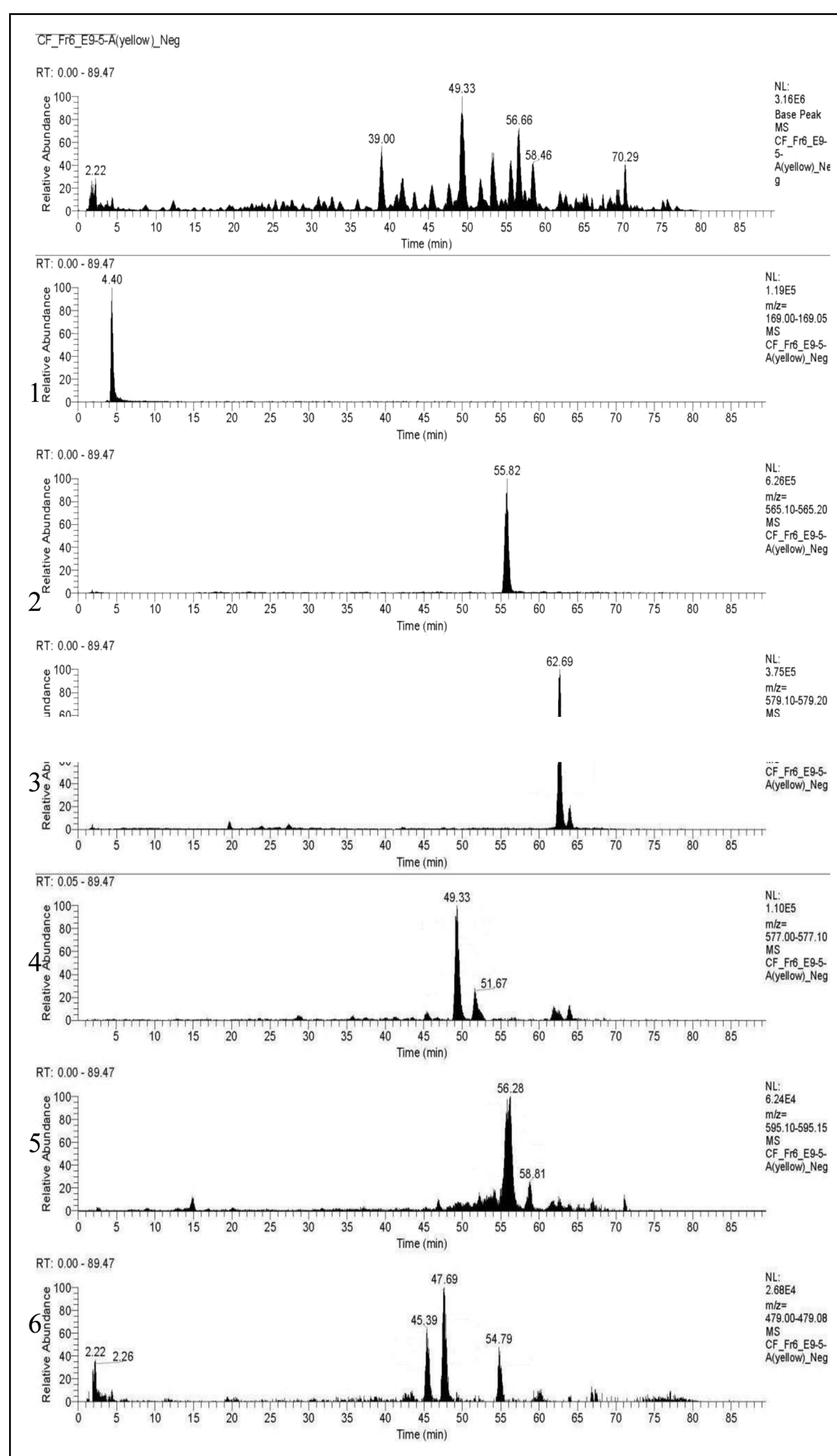

Fig. 1: a HPLC/Accurate ESIMS (Negative mode) of *E. jambos* extract and the major phenolics ions. Top trace: basepeak chromatogram, showing all components in the mixture, following traces. Components according to Table 1 (first half) with accurate masses in a 0.1 mass window to exclude structure with the same nominal masses. Fig. 1**b:** HPLC/Accurate ESIMS (Negative mode) of *E. jambos* extract and the major phenolics ions. Top trace: basepeak chromatogram, showing all components in the mixture, following traces. Components according to Table 1 (second half) with accurate masses in a 0.1 (or less) mass window to exclude structure with the same nominal masses.

myricetin 7-methyl ether 3-O-xylosyl-(1"' → 2")-rhamnoside and myricetin 3',5'-dimethyl ether 3-O-xylosyl-(1"' → 2")-rhamnoside. Besides, we evaluated the antioxidant capacities using the DPPH and the ORAC methods and assessed the cytotoxicity against the keratinocyte cell line HaCaT and the human bladder carcinoma cell line 5637 using the neutral red assay (NRU) for that extract and a major isolate the known phenolic ellagic acid. It was separated pure as column fraction X (2DPC, CoPCand UV, EI-MS, ^{1}H, 13C NMR) (Nawwar et al. 1994). [It should be noted, however, that the known phenolic ellagic acid was separated pure as column fraction X (2DPC, CoPCand UV, EI-MS, ^{1}H, ^{13}C NMR (Nawwar et al. 1994)].

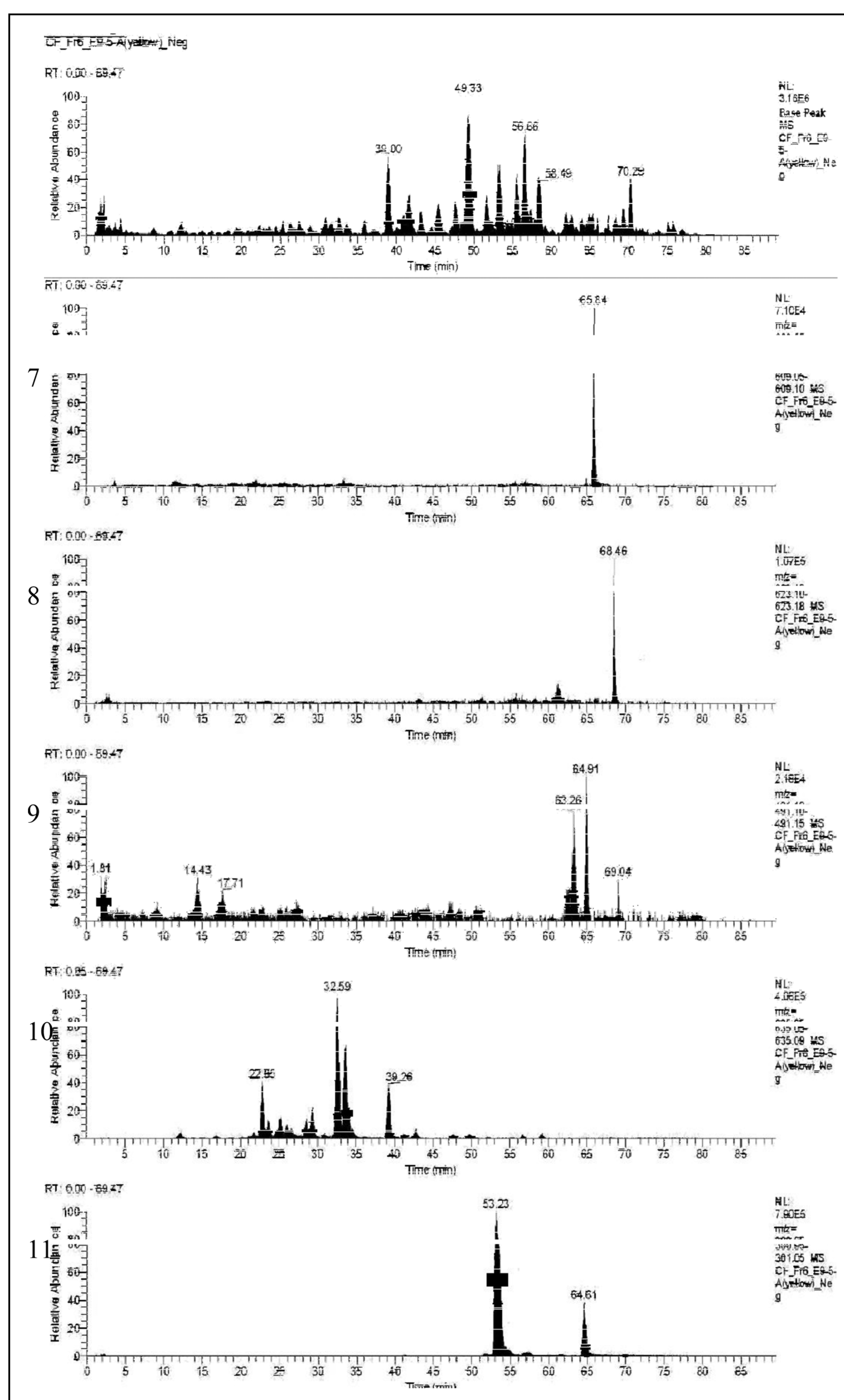

Fig. 1: *(Continued).*

2. Investigations, results and discussion

The aqueous ethanol whole plant extract of *E. jambos* contains a complicated mixture of phenolics, which includes mainly flavonoid-*O*-glycosides as previously detected by 2D paper chromatographic screening. The search for new, potentially biologically active compounds becomes much more efficient after sorting out all the known structures in that mixture. Therefore, the application of reversed-phase HPLC combined with ESI-MS metabolite profiling is the most promising technique aiming at phenolic compounds, the determination of negative ions will yield the most interesting signals. The same technique can then also be used to follow the isolation steps until the pure target phenolic compounds are obtained. Optimum HPLC/MS conditions were established under the premises that (a) the separation of several classes of compounds should be possible in general, (b) the phenolic compounds should be separated and (c) the system must be robust and yield reproducible results (retention times, intensity distributions) over an extended time period to allow tentative identification based on the limited information from ESIMs (generally $[M - H]^-$ ions and fragmentation by CID, when necessary), Fig. 1a/b. During the analysis of the

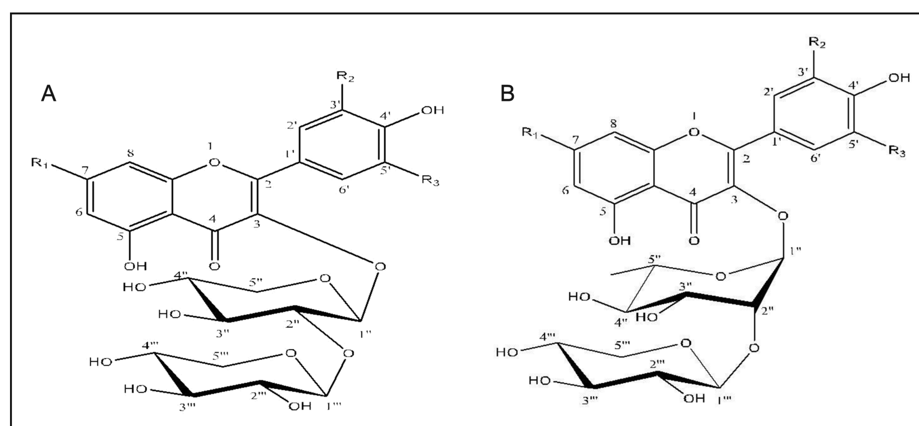

Fig. 2: a Compound 2: R1 R2 = OH, R3 = H. b: Compound 7: R1 = OMe, R2 & R3 = OH Compound 8: R1 = OH, R2 & R3 = OMe.

Table 1: Molecular mass, molecular formula and retention time (R_t) of isolated compounds

N_r	R_t (min)	Compd.	State	Molecular Formula	Molecular Mass
1	4.4	Gallic acid	Known	$C_7H_6O_5$	170.0215
2	55.82	Quercetin 3-*O*-xylosyl- (1→2) Xyloside	Unknown	$C_{25}H_{26}O_{15}$	566.1272
3	62.69	Quercetin 3-*O*-xylosyl-(1→2)rhamnoside	Known	$C_{26}H_{28}O_{15}$	580.1428
4	49.33	Quercetin 3-*O*-glucuronide	Known	$C_{21}H_{18}O_{13}$	478.0747
5	56.28	Myricetin 3-*O*-xylosyl-(1→2) Rhamnoside	Known	$C_{26}H_{28}O_{16}$	596.1377
6	47.69	Myricetin 3-*O*-glucoside	Known	$C_{21}H_{20}O_{13}$	480.0904
7	56.84	Myricetin 7-methylether 3-*O*-xylosyl(1→2)rhamnoside	Unknown	$C_{27}H_{30}O_{16}$	610.1534
8	68.46	Myricetin 3',5'-dimethyl ether 3-*O*-xylosyl(1→2)rhamnoside	Unknown	$C_{28}H_{32}O_{16}$	624.1690
9	64.91	Myricetin 3',5'-dimethyl ether 3-*O*-rhamnoside	known	$C_{23}H_{24}O_{12}$	492.1268
10	32.59	1,2,3-Tri-*O*-galloyl-β-glucose	Known	$C_{27}H_{24}O_{18}$	636.0963
11	53.23	Ellagic acid	Known	$C_{14}H_6O_8$	302.0063

extracts, $[M - H]^-$ ions were detected as base peaks often without any further fragment for all metabolites. However, dimeric ions $[2M - H]^-$ and doubly charged $[M - 2H]^{-2}$ ions were also observed in some instances. Such observations were taken as evidence that this class of compound generally forms $[M - H]^-$ type ions as expected. The chromatograms revealed the presence of 160 polar constituents some of which are of phenolic nature (Number of double bonds and oxygen functions exhibited by the molecular formula and molecular mass). The majority of these phenolics are shown to be of known structures on the basis of their $[M - H]^-$ ions, molecular formula and their ESI mass spectra. The results of all of the tentatively determined phenolics are given in Table 1. After tentative identification of the new compounds, they were isolated.

2.1. Structure elucidation

Compound 2 was isolated as an amorphous yellow powder, which appeared to be a quercetin derivative with a substituted 3-hydroxyl group from the results of acid hydrolysis which gave quercetin and xylose (CoPC) and from its UV spectral analysis with diagnostic reagents which showed bathochromic shift with NaOAC for band II, bathochromic shift for band I with NaOAc + H3BO3, stable shift of band I with NaOMe and a 17 nm hypsochromic shift of band I with AlCl3/AlCl3 + HCl (Mabry et al. 1970; Markham and Mabry 1975). In the negative ion ESI mass spectra a molecular ion was observed at m/z = 565 $[M – H]^-$. However, fragment ions at m/z 301 due to quercetin $[M – 1]^-$ ion was found, thus identifying the 3-substituent as a dixyloside. The molecular formula of **2** was concluded to be $C_{25}H_{26}O_{15}$ from its negative HRESI mass spectrum which showed an $[M-H]^-$ ion at *m*/z = 565.1272 (calcd. for C25H25O15, 565.4570). Structural elucidation of **2** was achieved by ^1H and ^{13}C NMR spectroscopy (DMSO-d_6), including HMQC and HMBC spectroscopy, which allowed the full assignment of all carbon and proton resonances. The ^1H and ^{13}C NMR spectra (Table 1) unambiguously identified **2** as a quercetin derivative with a 3-*O*-substituent (Merkham et al. 1978). The number and characteristic shifts of the glycosidic carbon signals indicated the presence of two pentose systems in the pyranose form. The assignment of their ^1H and ^{13}C signals followed directly from the HMQC spectra. In all cases the ^{13}C shifts of their carbon number 2 signals indicated that one of these carbons, (C - 2“') bears a free OH group while the OH of the second carbon, (C-2”) is substituted. Both sugars had β-glycosidic linkages from the magnitude of the vicinal proton couplings of the anomeric protons (J = 7.1 Hz) in the ^1H spectrum. That the intermediate sugar linked to the hydroxyl group at C-3 of the aglycone was readily followed from the low field shift of its anomeric proton (H-l“, 5.35 ppm) and unambiguously confirmed by a long-range correlation between this proton and the quercetin C-3 (δ 135.3 ppm) in the HMBC spectrum. This latter spectrum also allowed the interconnectivity of the two sugar units to be unambiguously determined. The anomeric proton H-I”' (δ 4.28 ppm) showed a correlation with the intermediate xyloside carbon, C-2“ (δ 83.53 ppm). The shift of C-2”' was compatible with the terminal sugar being xylose (δ 73.66 ppm). This was indirectly confirmed as the 13C shifts of the terminal sugar were only compatible with those of β-xylose moiety. Hence, the structure of **2** was determined as quercetin 3-*O*-β-xylopyranosyl(1→2)-*O*-β-xylopyranoside, which is not detected previously in natural sources.

Compound 7 gave on acid hydrolysis myricetin 7-O-methyl ether (europetin), rhamnose and xylose (CoPC). UV spectral analysis of **7** in methanol and in the presence of diagnostic reagents (Mabry et al. 1970; Markham and Mabry 1975), suggested that the sugar moieties are bonded to the aglycone moiety at positions 3 (no shift with NaOAc, large shift with AlCl3 intense stable shift with NaOMe).

Therefore**,** compound **7** is europetin 3-xylosylrhamnoside. The suggested structure of **7** was confirmed by NMR spectroscopy. From the ^{13}C NMR spectra**,** the presence of a rhamnose moiety followed from signals in the methyl region. The position of this signal at δ 18.1 ppm indicated that the rhamnose moiety was attached directly to the europetin hydroxyls, because attachment to sugar hydroxyls would shift the signals downfield to about 21 ppm (Nawwar et al. 1984b). That the rhamnoside moiety must be attached to position 3 of europetin followed from the upfield shift of this C–3 carbon signal and the corresponding *ortho-* and *para*-carbon signals were shifted downfield (see Experimental). Similar shifts are well known from the work of Markham et al. (1987). The β-configuration of the xylosyl moiety was derived from its anomeric carbon chemical shift at δ 105.42 (Nawwar et al. 1984b). The α-configuration of the rhamnose moiety followed from its anomeric carbon chemical shift values at δ 99.218. Attachment of the xylosyl moiety to C-2 of rhamnose was indicated by the shift of the rhamnose C-l signal to δ 99.2 pm (γ-upfield shift caused by C - l of xylose) and of the rhamnose C-2 signal to δ 80.4 (β-downfield shift caused by C-l of xylose). The chemical shift values of all the sugar carbons confirmed the pyranose form of the two sugar. The rhamnose anomeric proton gave rise to signal, the position of which (δ 5.27) indicated the attachment of the anomeric carbon to the europetin hydroxyl, and the half-width of which (ca 4 Hz) proved the α-cofiguration at the anomeric carbon. The anomeric proton of the xylose moiety at δ 4.45 ppm (d, J = 7 Hz) proved the β-configuration. The conformation of the two sugar moieties is 1C4 for the rhamnose moiety and 4C1 for the xylose moiety. This follows from the α and β-configurations discussed above. From the above given analytical data, compound **7** is confirmed to be europetin 3-*O*-β-xylopyranosylsyl-(1 2)-*O*-α-rhamnopyranoside, which represents a new natural product as far as the available current literature is concerned.

Compound 8 yielded on acid hydrolysis, glucose and rhamnose as the sugar residues, which were confirmed by CoPC against authentic samples. The UV and EI-MS of the released aglycone suggested that it is syringetin (3',5-dimethoxy myricetin). This was confirmed by the ^{1}H NMR spectrum which exhibited two meta-coupled doublets (J = 2 Hz) at δ 6.42 (H-8) and 6.25 (H-6), a proton singlet at δ 7.35 assigned to H-2' and H-6' and another singlet at δ 3.79 integrated to 6 methyl protons of two equivalent methoxyl groups, a set of chemical shifts which were closely similar to the corresponding signals in the spectrum of free syringetin (Lawrenceet al. 2005). The UV spectrum of **8** in methanol and its changes after addition of shift reagents suggested that the compound is a flavonol glycoside with free hydroxyl groups at positions C-5 and C-4', a feature confirmed by the ^{1}H NMR peaks at 12.60 (OH-5) and 9.20 (OH-4); both disappeared upon addition of D_2O. The 1H spectrum also exhibited two anomeric proton signals downfield at δ 4.28 (J = 7.1 Hz) and 5.40 (J = 1.2 Hz), assignable to a β-xylosyl anomeric proton and to α-rhamnoside anomeric proton, respectively. These chemical shifts prove that the xyloxyl moiety is attached to an alcoholic sugar OH group and that the rhamnoside moiety is directly connected to the phenolic aglycone OH group number 3. In the negative mode ESI mass spectra a molecular ion was observed at m/z = 623 [M – H]$^-$. However, fragment ions at m/z 345 due to [M – 1]$^-$ ion attributed to the aglycone, syringetin was found, thus identifying the 3-O-substituent as a 3-O-xylosyl rhamnoside. The molecular formula of **8** was concluded to be C27H30O16 from its negative HRESI mass spectrum which showed an [M-H]$^-$ ion at *m*/z = 623.1690, (calcd. for $C_{28}H_{31}O_{16}$, 623.5361). Changes of diagnostic values were observed in the ^{13}C NMR spectrum of compound **8** in comparison with free syringetin. The C-3 was relatively upfield at δ 134.8, compared to those of the syringetin 136.5 (Lawrenceet al. 2005), obviously, due to glycosylation at this carbon. This was further confirmed by HMBC spectrum, which showed correlations between the anomeric proton of rhamnose (δ 5.40) and the C-3 (δ134.8), and a correlation of the xylose anomeric proton (δ 4.28) and C-2" of the rhamnoside moiety (δ 80.4). The comparatively upfield location of the anomeric rhamnoside carbon, C – 1" (δ 98.82) is due to the vicinal substitution with the xylosyl moiety. From the foregoing spectral evidence, compound **8** was identified as syringetin 3-*O*-β-xylopyranosyl-(1→2)-*O*-α-rhamnopyranoside, a new natural product which is reported here to occur in nature for the first time.

2.2. Known compounds

Quercetin 3-O-xylosy-(l"'→2")-rhamnosid (Slowing et al. 1994), myricetin 3-oxylosy-(1"'→2")-rhamnoside (Soike et al. 1990), myricetin 3',5'-dimethyl ether 3-orhamnoside (Guo et al. 1998), quercetin 3-O-glucuronide (Nawwar et al. 1984a), myricetin 3-O-glucoside (Lawrence et al. 2005), 1,2,3-tri-O-galloyl-β-glucose (Nawwar et al. 1994) and ellagic acid (Nawwar et al. 1994).

2.3. Biological activities

In order to get information about biological activities of *E. jambos* the extract was tested for radical scavenging activity by DPPH assay and by ORAC assay, for cytotoxicity on HaCaT keratinocytes and for those on the human bladder carcinoma cell line 5637 by neutral red assay. Because of minimal amounts of isolated compounds the only compound which could be tested as pure substance was ellagic acid. The EC_{50} of the extract for radical scavenging activity in DPPH assay was 13.52±0.69 μg/mL and in ORAC assay 34.35±12.45 μg/mL. Ellagic acid considerably contributed to this effect with an EC_{50} value of 8.64±1.24 μg/mL in the ORAC assay. This value is lower than that of the positive control Trolox which had an EC_{50} of 28.0±14.3 μg/mL. The IC_{50} values for cytotoxicity of the extract were 106.74 ± 10.89 μg/mL against the non-tumorigenic HaCaT keratinocytes and 55.24 ± 2.67 μg/mL against the bladder carcinoma cells. The IC_{50} values for ellagic acid were 65.77 ± 2.57 μg/mL against HaCaT cells and 75.34 ± 4.56 μg/mL against 5637 cells. Etoposide, the positive control for cytotoxicity, reduced cell viability of HaCat cells with an IC_{50} of 1.47 μg/mL and that of 5637 cells with an IC_{50} of 2.27 ± 0.30 μg/mL. The vehicle in which the test samples were dissolved had no influence on measured parameter.

3. Experimental

3.1. General

NMR spectra were acquired in DMSO-d_6 on a Jeol ECA 500 MHz NMR spectrometer, at 500 MHz. Standard pulse sequence and parameters were used to obtain one-dimensional ^{1}H and ^{13}C, and two dimensional COSY, HSQC and HMBC spectra. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts to DMSO-d_6 and were converted to TMS scale by adding 39.5. The extracts were analysed using a nanoLC system (Agilent 1100 series, Germany) equipped with a Zorbax 300SB-C18 (150 mm × 75 μm, Agilent) analytical column and a Zorbax 300SB-C18 (5 × 0.3 mm; Agilent) trap column coupled to the mass spectrometer. The analysis was performed at a flow of 0.35 μL min – 1 using a

mobile phase of 5% ACN and 0.1% formic acid (v/v) (Solvent A) and 99.9% ACN and 0.1% formic acid (v/v) (Solvent B). High resolution ESI mass spectra were measured using a Finnigan LTQ FT Ultra mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Nanomate ESI interface (Advion Biosystems, USA). An electrospray voltage of 1.7 kV (+/−) and a transfer capillary temperature of 200 °C were applied. Collision induced dissociation (CID) was performed in the ion trap using a normalized collision energy of 35 %, activation time of 30 ms, 0.25 activation Q and a precursor ion isolation width of 2 amu. High resolution product ions were detected in the Fourier transform ion cyclotron resonance (FTICR) cell of the mass spectrometer. UV recording was made on a Shimadzu UV–Visible-1601 spectrophotometer. Chromatographic analysis (PC) was carried out on Whatman No. 1 paper, using solvent systems: (1) H_2O; (2) 6% HOAc; (3) BAW (n-BuOH–HOAc– H_2O, 4:1:5, upper layer).

3.2. *Plant materials*

Leaves of *Eugenia jambos* were collected from the Zoo garden at Cairo, on April, 2013 and identified by Prof. Salwa Kawashty at the Department of Phytochemistry and Plant Systematic, National Research Centre (NRC), Cairo, Egypt. A voucher specimen (E 1732) has been deposited at the herbarium of the NRC.

3.3. *Preparation of extract*

Fresh leaf material (2 kg) was extracted with hot EtOH/H_2O (3:1, 3 times, each with 3 l, for 8 h, under reflux). The solvent was evaporated under vacuum. The resulting dry sticky material thus left (150 g), dissolved in 200 ml H_2O, was applied to a Polyamide 6S chromatographic column (Riedel-de Haen, Seelze, Hannover, Germany) and eluted with H_2O, followed by H_2O/MeOH mixtures of decreasing polarity to yield ten major fractions (I – X). Following removal of the solvents ten fractions (I – X, fraction I eluted with H_2O; II with 10 %, III with 20 %, IV with 30 %, V with 40 %, VI with 50 %, VII with 60 %, VIII with 70 %, IX with 80 % and X with 90 %) were individually collected and subjected to two dimensional paper chromatography (2DPC).

3.4. *Isolation and identification of phenolics*

Compound 1 (187 mg) was purely isolated from fraction II (1.8 g) by repeated column fractionation over Sephadex LH-20 using MeOH/H2O mixture (20 %) for elution. Compound **2** (108 mg) and **3** were individually isolated from fraction III (9.21 g) by MCI gel column fractionation, using H_2O as solvent, followed by preparative paper chromatography (Prep. PC), using BAW as solvent. Compound **4** (99 mg) was purely isolated from 1.1 g of fraction IV by applying repeated Sephadex LH-20 column fractionation and elution with H_2O–EtOH (30:70) mixture. Compound **5** (87 mg) was separated from fraction V (3.35 g) through fractionation over polyamide column and elution with a mixture of MeOH–C_6H_6–H_2O (60:38:2). Compound **7** (121 mg) was isolated pure from fraction VI (2.80 g) by applying the same technique used for the separation of **6**. Compound **7** was obtained pure (78 mg) from fraction VII (1.68 g) by applying repeated Sephadex LH-20 column fractionation and elution with H_2O–EtOH (70:30) mixture. Compounds **8** (64 mg) and **9** (70 mg) were individually obtained pure from fraction VIII by applying polyamide column fractionation, using a mixture of MeOH–C_6H_6–H_2O (60:38:2) as solvent. Extraction of fraction IX (1.7 g) with ether (150 ml, 3 times, each for h, under reflux) followed by filtration gave 242 mg of dried ether insoluble material which was dissolved in dry acetone (50 ml). Treatment of the obtained solution with dry ether led to the precipitation of an off-white precipitate. The precipitation process was repeated thrice and the formed precipitate was collected and examined chromatographically to prove that it is a pure sample of compound **10** (84 mg). Crystallization of the material of fraction X (740 mg) from pyridine afforded dull yellow pure crystalline material of compound **11**.

3.4.1. *Quercetin 3-O-β-xylosylpyranosyl-(1→2)-O-β-xylopyranoside (2)*

UV: λ_{max} (MeOH): 268, 350; (+NaOAc) 272, 305, 388; (+NaOAc + H_3BO_3) 262,365; (+AlCl3) 252 sh, 270, 388 sh, 279, 415; (+AlCl3 + HCl) 277, 398 nm, (NaOME) 262, 383. ESI (negative mode): *m/ z* = 565 [M-H]-. Negative HRESIMS: negative mass spectrum:[M-H]- ion at *m/z* = 565.1272 (calcd. for $C_{25}H_{25}O_{15}$, 565.4570). EI-MS: *m/z* (%):302 (100 %), 153 (10 %), 60 (15 %). 1H NMR (DMSO-d6) of **2:** δ (ppm): 7.33 (1 H, d, J = 2 Hz, H-2'), 7.36 (1 H, d,d, J = 2 HZ and J = 8 Hz, H-6'), 6.91(1 H, d, J = 8 Hz, H-5'), 6.40 (1 H, d, J = 2 Hz, H-8), 6.19 (1 H, d, J = 2 Hz, H-6), 5.24 (1 H, d, J = 7.1 Hz, H-1''), 4.28 (1 H, d, J = 7.1 Hz, H – 1'''), 3.2 -4.1 (m, sugar protons overlapped with sugar OH and water signals). ^{13}C NMR: Table 2.

Table 2: ^{13} C NMR assignments of compounds 2, 7 and 8 in DMSO-d$_6$

Carbon number	Compound **2**	Compound **7**	Compound **8**
Aglycone			
2	156.8	156.9	156.4
3	135.3	135.0	134.8
4	178.6	178.5	177.5
5	16.1	161.8	161.3
6	99.3	98.4	99.0
7	164.8	165.7	164.1
8	93.4	92.7	94.1
9	156.8.1	158.5	156.4
10	104.1	105.6	104.0
1'	122.1	120.0	120.2
2'	116.1	108.5	108.1
3'	147.9	146.3	147.5
4'	146.8	137,1	139.3
5'	117.2	146.3	147.5
6'	1202	108.5	108.1
OMe		56.7	56.6
Rhamnose			
1¨		99.1	98.8
2¨		80.5	80.4
3¨		70.5	70.4
4¨		71.8	72.1
5¨		70.03	70.02
Me		18.1	18.1
Xylose			
1¨	98.76	106.4	105.5
2¨	83.53	73.6	73.7
3¨	73.25	75.9	76.1
4¨	68.12	70.5	70.4
5¨	65.45	65.0	65.1
Xylose			
1ÿ	105.77		
2ÿ	73.66		
3ÿ	76.29		
4ÿ	69.12		
5ÿ	65.45		

3.4.2. *Myricetin 7-methyl ether 3-O-xylopyranosylsyl-(1→2)-α-rhamnopyranoside (7)*

UV: λ_{max} (MeOH), (nm): 256, 356; (+NaOAc) 257, 358; (+NaOAc + H_3BO_3) 268, 390; (+AlCl3) 269, 395; (+AlCl3 + HCl) 271, 303, 401; 253, 404 nm. ESI MS (negative mode): *m/ z* = 609 $[M-H]^-$. Negative HRESIMS: negative mass spectrum: $[M-H]^-$ ion at *m/z* = 609.1534, (calc. for $C_{27}H_{29}O_{16}$, 609.5096). EI-MS: *m/z* (%): 332 (100 %) $[M]^+$, 317 (7 %). 153 (10 %) $[B_2^+]$, 60 (15 %). ^{1}H NMR (DMSO-d$_6$) of **7:** δ (ppm): 6.97 (2H, s, H-2'& H – 6'), 6.33 (1 H, d, J = 2 HZ H-6), 6.54 (1 H, d, J = 2 Hz, H-8), 5.27 (1 H, broad, $\Delta\nu 1/2$ = 4 Hz, H-1''), 4.45 (1 H, d, J = 7 Hz, H – 1'''), 3.87 (7-OMe), 3.2 - 4.1 (m, sugar protons overlapped with sugar OH and water signals), 18.1 (3 H, s, Me rhamnose). 13_C NMR: Table 2.

3.4.3. *Syringetin 3-O-β-xylopyranosyl-(1→2)-O-α-rhamnopyranoside (8)*

UV: λ_{max} (MeOH), (nm): 265, 352; (+NaOAc) 275, 356; (+NaOAc + H_3BO_3) 268, 355; (+AlCl3) 266, 308 sh, 405; (+AlCl3 + HCl) 266, 310, 362, 404; (+NaOME) 265, 422 nm. ESI-MS (negative mode): *m/ z* = 623 $[M^-H]^-$. Negative HRESIMS: negative mass spectrum: $[M^-H]^-$ ion at *m/z* = 624.1690, (calc. for $C_{27}H_{30}O_{16}$, 623.5361). EI-MS: *m/z* (%):346 (100 %), 332 (7 %), 60 (15 %). ^{1}H NMR (DMSO-d6) of **8:** δ (ppm): δ 6.42 (1 H, d, J = 2 Hz, H-8) and 6.25 (1 H, d, J = 2 Hz, H-6), δ 7.35 (2 H, s, H-2' and H-6'), δ 3.79 (6 H, s, Me-3' & Me-5'), 5.4 (1 H, d, J = 2 Hz, H-1''), 4.28 (1 H, d, J = 7.1 Hz, H – 1'''), 3.2 -4.1 (m, sugar protons overlapped with sugar OH and water signals), 18.1 (Me rhamnose). ^{13}C NMR: Table 2.

3.5. Biological assays

3.5.1. Determination of radical scavenging activity (DPPH assay)

In order to measure antioxidant activity, DPPH (2,2-Diphenyl-1-pycrylhydrazyl), free radical scavenging assay was used. This assay measures the free radical scavenging capacity of the investigated samples. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant which can donate an electron to DPPH, the purple colour which is typical for free DPPH radical decays and the change in absorbency at 517 nm is followed specrophotometrically. The radical scavenging activity of extract and ellagic acid was determined according to the method of Brand-Williams and Cuvelier (1995) as described in Nawwar et al. (2014). All experiments were carried out in triplicate. Ascorbic acid was used as positive control.

3.5.2. Oxygen radical absorbance capacity (ORAC assay)

Reactive oxygen species, ROS, are generated by the thermal degradation of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and quench the signal of the fluorescent probe fluorescein. The subsequent addition of antioxidants reduces the quenching by preventing the oxidation of the fluorochrome (Lucas-Abellán et al. 2008). The test was done in quadruplicate according to Nawwar et al. (2014). The vitamin E derivate 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox) was used as positive control.

3.5.3. Cell cultivation and Neutral red assay

The cytotoxicity was measured by the neutral red uptake assay (Lindl et al. 1989) using two human cell lines: non tumorigenic HaCaT keratinocytes and urinary bladder cancer cells (5637). Principle of this method is that only living cells are able to manage the active uptake of neutral red. HaCaT cells (CLS Cell Lines Service, Eppelheim, Germany) and 5637 cells (ATCC HTB-9) were cultured in RPMI 1640 medium (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 8 % fetal bovine serum (Sigma Aldrich, Taufkirchen, Germany) and antibiotics (100 U/ml penicillin/100 μg/ml streptomycin; Sigma Aldrich, Taufkirchen, Germany) at 95% humidity, 5% CO_2 and 37 °C. The cells were sub-cultured twice a week and regularly tested for mycoplasma contamination. The test was done as described in Nawwar et al. (2014). All samples were tested in triplicate with six technical replicates and etoposide (Alexis Biochemicals, ≥ 98% purity) served as positive control.

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