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Constitutive Phenolics and Hepatoprotective Activity of *Eugenia supra-axillaris* Leaves

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BIOLOGICAL investigation of the aqueous methanolic leaf extract of *Eugenia supra-axillaris* (Myrtaceae) was performed to prove potent hepatoprotective activitiy. Phytochemical investigation led to the characterization of ten phenolic compounds for the first time from this plant. They include the flavonoid glycosides; myricetin 3-*O*-β-xylopyranosyl (1→2)-α-rhamnopyranoside, myricetin 3-*O*-α-rhamnopyranoside and quercetin-3-*O*- α-rhamnopyranoside, the flavonoid aglycones; pinocembrin, myricetin and quercetin, together with phenolics; gallic acid, ellagic acid, 5-*O*-monogalloylquinic acid and nilocitin. Three phenolics were isolated for the first time from the plants of the genus *Eugenia*, namely, 5-*O*-monogalloyl quinic acid, nilocitin and pinocembrin.

Keywords: Plant phenolics, *Eugenia supra-axillaris,* Myrtaceae, Flavonoids, Gallotannins and Hepatoprotective activity.

Plants of the genus *Eugenia* (Myrtaceae) are known to synthesize and accumulate significant amount of phenolics [(1-5).]including flavonoids and tannins. The genus *Eugenia* was reported to provide extracts used as antimicrobial[(6)], anti-inflammatory, analgesic, antipyretic[(2)], antidiabetic[(7)], antiviral, antitumor[(8)], hypotensive, vasodilator[(9)] and antioxidant [(10)]. There was nothing reported in the available current literature about the biological activities nor the phytochemistry of *Eugenia supra-axillaris.* On the other hand, ten phenolic compounds were isolated from *Eugenia supra-axillaris* among them; three were isolated for the first time from the plants of the genus *Eugenia,* namely, 5-*O*-monogalloyl quinic acid, nilocitin and pinocembrin.

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The aim of the present work is to evaluate the hepatoprotective activitiy and to study the chemical composition of the aqueous methanolic extract of *Eugenia supra-axillaris* leaves.

Materials and Methods

Drugs and chemicals

Silymarin was kindly provided by The British Dispensary (L.P.) Co. Ltd.; diagnostic kits were purchased from Boehringer Mannheim GmbH Diagnostica, E. Merck, Postfach 4119, D-6100, and Darmstadt, Germany. All other chemicals were of highest quality available and were obtained from commercial sources.

Animals

Sprague Dawley rats of both sexes weighing 100 - 120 gm were used throughout the experiments. The animals were divided into 8 equal groups (six rats each), housed under standard environmental conditions (23 ± 1◦C, 55 ± 5 % humidity and a 12-h light: 12-h dark cycle) and maintained on a standard laboratory diet *ad-libitum* with free access to water.

Plant material

Fresh leaves of *Eugenia supra-axillaris* (Myrtaceae) were collected from Giza Zoo, Cairo, Egypt, on July 2006 and identified by Dr. M. El-Gibali, former researcher in the National Research Center. A voucher specimen is deposited at the NRC herbarium.

Instruments and materials for phytochemical investigation

^{1}H-NMR spectra were measured by Jeol EX-270 NMR spectrometer, 270 MHz. ^{1}H chemical shifts were measured relative to TMS and ^{13}C-NMR chemical shifts to DMSO-d_6 and converted to TMS scale by adding 39.5. Conditions: spectral width = 4 KHz for ^{1}H and 19 KHz for ^{13}C, 32 k data points and flip angle of 45°. ESI-MS spectra were measured on SSQ Finnigan MAT 4600 quadrupole mass spectrometer (Institute für chemie, Humboldt universität, Berlin).Chromatographic analysis 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).

Extraction and isolation

The air dried plant leaves were powdered and extracted with MeOH: H_2O (3:1). The resulting extract was filtered and dried under reduced pressure to give a dark brown amorphous extract amounting (38.95%) of the dried plant material. The received extract was then subjected to qualitative tests specific for phenolics which proved the presence of appreciable amount of phenolics (dark green colour with $FeCl_3$) including flavonoids (red colour with Mg/HCl reduction test) then applied to polyamide (6S Riedel De Häen, AG-Seelze- Hannover) column and eluted with H_2O, followed by H_2O-MeOH mixtures of decreasing polarities, whereby five fractions (I-V) were desorbed.

Compounds 1 and 2 were isolated from 20% MeOH fraction (I) by polyamide column, using H_2O/MeOH mixtures with decreasing polarity as eluent, followed by preparative paper chromatography (PPC) on Whatman No. 3MM paper using BAW as eluent. Compound 3 was isolated from 40% MeOH fraction (II) by repeated precipitation from a concentrated MeOH solution by addition of diethyl ether. Compounds 4 and 5 were isolated from 60% MeOH fraction (III) by polyamide column, using MeOH: Benzene: H_2O, 60:38:2 as eluent, followed by PPC using BAW. Compounds 6 and 7 were isolated from 80% MeOH fraction (IV) by polyamide column, using H_2O/MeOH mixtures with decreasing polarity, followed by PPC using BAW and 6% HOAc to afford compounds 6 and 7, respectively. Compounds 8, 9 and 10 were isolated from 100% MeOH fraction (V) by polyamide column, using 60:38:2, followed by PPC using BAW.

3,4,5-trihydroxybenzoic acid, gallic acid (1)

R_f-values: 44(H_2O), 55 (6% HOAc), 72 (BAW), UV λ_{max}: MeOH (272); ESI-MS: $[M-H]^-$ at m/z 169.0 (molecular mass= 170); NMR data: δ ppm 6.98 (s, H-2 & H-6), ^{13}C-NMR: δ ppm 120.6 (C-1), 108.8 (C-2 & C-6), 145.5 (C-3 & C-5), 138.1 (C-4), 167.7 (C=O).

5-O-monogalloyl quinic acid (2)

R_f-values: 65 (H_2O), 55 (6% HOAc), 27 (BAW); UV λ_{max}: MeOH (275); ESI-MS: $[M-H]^-$ at m/z 343.2 (molecular mass= 344); NMR data: δ ppm 6.95 (s, H-2 & H-6); quinic acid moiety: δ ppm 1.7-2.05 (m, H-2' & H-6'), 5.55-5.67 (m, H-3'), 3.57 (dd, J=3.37 &12.5 Hz, H-4'), 3.92-4.0 (m, H-5'); ^{13}C-NMR: δ ppm 120.1(C-1), 108.9 (C-2 & C-6), 145.6 (C-3 & C-5), 138.4 (C-4), 165.6 (C=O); quinic acid moiety: δ ppm 74.0 (C-1'), 37.4 (C-2'), 71.3 (C-3'), 71.1 (C-4'), 68.9(C-5'),36.9 (C-6'), 176.0 (C=O).

Myricetin -3-O- β-xylopyranosyl (1'''→2'') α-rhamnopyranoside (3)

It was obtained as yellow amorphous powder, R_f – values: 43 (H_2O), 67 (6% HOAc), 48 (BAW); UV λ_{max}: MeOH (257, 359), MeOH / NaOAc (266, 368), MeOH / NaOAc / H_3BO_3 (255, 303sh, 395), MeOH /$AlCl_3$ (272, 310sh, 405), MeOH /$AlCl_3$ / HCl (271, 360, 400) and MeOH / MeONa (266, 395); ESI-MS: $[M-H]^-$ at m/z 595.5 (molecular mass= 596); NMR data: δ ppm 6.21(d, J=2.5 Hz, H-6), 6.35 (d, J=2.5 Hz, H-8), 6.86 (s, H-2' & H-6'); rhamnose moiety 5.23 (broad s, $\Delta \nu_{1/2}$ = 4 Hz, H-1''), 4.1 (dd, J=3.5& 1.5 Hz, H-2''), 3.63 (dd, J=9& 3.5 Hz, H-3''), 3.78 (dd, J=9& 4 Hz, H-4''), 3.45 (m, H-5''), 0.92 (d, J=6 Hz, CH_3-rhamnose); xylose moiety: δ ppm 4.16(d, J=7.1 Hz, H-1'''), 3.16-3.45 (m,H-2''' to H-4'''); ^{13}C-NMR: δ ppm 156.5 (C-2), 134.4 (C-3), 177.9 (C-4), 161.32 (C-5), 93.8 (C-6), 165.6 (C-7), 99.1 (C-8), 156.9(C-9), 103.5 (C-10), 119.3 (C-1'), 107.7 (C-2' &C-6'), 146.0 (C-3'& C-5'), 137.0 (C-4'); rhamnose moiety: δ ppm 101.1 (C-1''), 80.8 (C-2''), 70.0 (C-3''), 71.9 (C-4''), 69.4 (C-5''), 17.6 (CH_3-rhamnose); xylose moiety: δ ppm 106.6 (C-1'''), 73.9 (C-2'''), 76.3 (C-3'''), 70.3 (C-4'''), 65.7 (C-5''').

Myricetin -3-O- α-rhamnopyranoside (4)

It was obtained as yellow amorphous powder; R_f – values: 21(H_2O), 43(6% HOAc), 66(BAW); UV λ_{max}: MeOH (259, 366), MeOH / NaOAc (267, 342), MeOH / NaOAc / H_3BO_3 (257, 393), MeOH /$AlCl_3$ (268, 312, 408), MeOH / $AlCl_3$ / HCl (269, 400) and MeOH / MeONa (267, 395); ESI-MS: $[M-H]^-$ at m/z 463.32 (molecular mass= 464); NMR data: δ ppm 6.16(d, J=2.5 Hz, H-6), 6.36(d, J=2.5 Hz, H-8), 6.89 (s, H-2' & H-6'); rhamnose moiety 5.2 (broad s, $\Delta \nu_{1/2}$ = 4 Hz, H-1''), 3.95-4.03 (m, H-2''), 3.6 (dd, J=9& 3.5 Hz, H-4''), 3.1-3.2 (m, H-5''), 0.85 (d, J=6 Hz, CH_3-rhamnose); ^{13}C-NMR: δ ppm 157.3(C-2), 134.3(C-3), 177.7(C-4), 161.3 (C-5), 93.9 (C-6), 165.9 (C-7), 99.2 (C-8), 156.6 (C-9), 103.6 (C-10), 119.7 (C-1'), 108.0 (C-2' &C-6'), 146.0 (C-3'& C-5'), 136.7 (C-4'); rhamnose moiety: δ ppm 102.0 (C-1''), 70.5 (C-2''), 71.4 (C-3''), 70.7 (C-4''), 70.2 (C-5''), 18.5 (CH_3-rhamnose);

Quercetin -3-O- α-rhamnopyranoside (5)

It was obtained as yellow amorphous powder; R_f – values: 22(H_2O), 48(6% HOAc), 68(BAW); UV λ_{max}: MeOH (259, 348), MeOH / NaOAc (276, 372), MeOH / NaOAc / H_3BO_3 (272, 383), MeOH /$AlCl_3$ (268, 352, 408), MeOH / $AlCl_3$ / HCl (268, 400) and MeOH / MeONa (270, 355, 405); ESI-MS: $[M-H]^-$ at m/z 447 (molecular mass= 448); NMR data: δ ppm 6.25 (d, J=2.5 Hz, H-6), 6.46(d, J=2.5 Hz, H-8), 7.49 (d, J=2.5 Hz, H-2'), 6.98 (d, J= 8 Hz, H-5'), 7.4 (dd, J= 8 & 2.5 Hz, H-6'); rhamnose moiety: δ ppm 5.3 (broad s, $\Delta \nu_{1/2}$ = 4 Hz, H-1''), other rhamnose protons are hidden by water, 0.82(d, J=6 Hz, CH3-rhamnose).

2,3-di-O-galloyl(α/β)-D-glucopyranose, nilocitin (6)

R_f – values: 60 (H_2O), 65(6% HOAc), 50 (BAW); UV λ_{max}: MeOH (276); ESI-MS: $[M-H]^-$ at m/z 482.9 (molecular mass= 484); NMR data: galloyl moieties: δ ppm 7.05(s), 7.07(s), 7.1(s) in ratio of 1:1:2, respectively, α- glucose moiety: δ ppm5.47 (d, J=3.3, H-1), 4.92 (dd, J= 8 and 3.3, H-2), 5.78 (t, J=8, H-3), 3.6-4.0 (m, H-4, H-5, H-6, H-6'), β- glucose moiety: δ ppm 4.98 (d, J= 7.5, H-1), 5.08 (t, J=7.5 Hz, H-2), 5.41 (t, J= 7.5, H-3), 3.6-4.0 (m, H-4, H-5, H-6, H-6'); ^{13}C-NMR: δ ppm 121.6, 121.3 (C-1) of both galloyl moieties of both anomers), 110.0 (C-2 & C-6 in all galloyl moieties), 138.7, 138.9 (C-4 in all galloyl moieties), 164.8, 165.2, 165.4 and 165.5 (C=O in all galloyl moieties), α-glucose moiety: δ ppm 98.3 (C-1), 72.2 (C-2, C-3& C-5), 68.3 (C-4), 60.6 (C-6); β- glucose moiety: δ ppm 94.5 (C-1), 73.1 (C-2), 75.5 (C-3), 68.3 (C-4), 76.7 (C-5), 60.6 (C-6).

5,7-dihydroxy flavanone, pinocembrin (7)

It was obtained as white amorphous powder; R_f – values: 0(H_2O), 1(6% HOAc), 92 (BAW); UV λ_{max}: MeOH (289, 325sh), MeOH / NaOAc (253, 323), MeOH / NaOAc / H_3BO_3 (291, 326sh), MeOH /$AlCl_3$ (311, 375), MeOH /$AlCl_3$ / HCl (309, 373) and MeOH / MeONa (245, 324); NMR data: galloyl moieties: δ ppm 5.55(dd, J=4 & 12.5 Hz, H-2), 3.15(dd, J=14 & 16.5 Hz, H-3_{ax}), 2.8 (dd, J= 16.5 & 4 Hz, H-3_{eq}), 6.0 (s, H-6 & H-8), 7.35-7.55(m,C_6H_5); ^{13}C-NMR: δ ppm 78.0 (C-2), 42.0(C-3), 196.5 (C-4), 161.0 (C-5), 95.0 (C-6), 164.7 (C-7), 95.0 (C-

8), 160.0 (C-9), 102.0 (C-10), 139.5 (C-1'), 126.5 (C-2' &C-6'), 128.5 (C-3'& C-5'), 128.0 (C-4').

Ellagic acid (8)

R_f – values: 0(H_2O), 9(6% HOAc), 48 (BAW); UV λ_{max}: MeOH (255, 262); NMR data: δ ppm 7.5 (s, H-4 & H- 9); ^{13}C-NMR data: δ ppm 136.4 (C-4a & C-6a), 140.2 (C-2 & C-7), 148.0 (C-3 & C-8), 111.4 (C-4 & C-9), 107.7 (C-4a & C-9a), 112.3 (C-4b & C-9b), 159.2 (C-5 & C-10).

Myricetin (9)

It was obtained as yellow amorphous powder; R_f – values: 0(H_2O), 9 (6% HOAc), 54 (BAW); UV λ_{max}: MeOH (256, 376), MeOH / NaOAc (269, 342), MeOH / NaOAc / H_3BO_3 (258, 392), MeOH /$AlCl_3$ (271,435), MeOH /$AlCl_3$ / HCl (266, 428) and MeOH / MeONa (262, 322, 423); NMR data: δ ppm 6.18(d, J=2.5 Hz, H-6), 6.34(d, J=2.5 Hz, H-8), 7.24 (s, H-2' & H-6'); ^{13}C-NMR: δ ppm 147.2 (C-2), 136.7 (C-3), 177.9 (C-4), 161.4 (C-5), 94.6 (C-6), 165.7 (C-7), 99.0 (C-8), 156.6 (C-9), 104.3 (C-10), 119.8 (C-1'), 108.0 (C-2' &C-6'), 145.9 (C-3'& C-5'), 137.5 (C-4').

Quercetin (10)

It was obtained as a yellow amorphous powder; R_f-values: 0 (H_2O), 7 (6% HOAc), 75 (BAW); UV λ_{max}: MeOH (255, 366sh, 370), MeOH/NaOAc (276, 375), MeOH/NaOAc/H_3BO_3 (272, 388), MeOH/$AlCl_3$ (270, 360sh, 440), MeOH/$AlCl_3$/HCl (258, 400) MeOH/MeONa (252, 330,430); NMR data: δ ppm 6.2 (d, J=2.5 Hz, H-6), 6.4 (d, J=2.5 Hz, H-8), 7.65 (m, H-2'), 6.88 (d, J= 8 Hz, H-5'), 7.65 (m, H-6'), ^{13}C-NMR: δ ppm 146.9 (C-2), 135.5 (C-3), 175.8 (C-4), 160.7 (C-5), 98.2 (C-6), 163.9 (C-7), 93.3 (C-8), 156.2 (C-9), 103.1 (C-10), 122.1 (C-1'), 115.3 (C-2'), 145.0 (C-3'), 147.6 (C-4'), 115.6 (C-5'), 120.0 (C-6').

Experimental

Acute toxicity study

The different oral doses of *Eugenia supra-axillaris* were given to several groups of rats. Animals were kept under observation to record any toxic symptoms or mortalities during the first 72 hr to 7 days post-treatment.

Hepatoprotective study

Six groups of normal rats (12 rats each) were used; first five groups were given aqueous methanol extracts of *Eugenia supra-axillaris* orally (100 mg and 200 mg / kg b. wt.), standard drug silymarin [11], (100 mg / kg b.wt., while, the control group was given distilled water (10 ml /kg b.wt.). After 15 days of daily

treatment, 6 rats from each group were given 50 % CCl_4 in liquid paraffin (1.5 ml / kg b.w., orally) to induce hepatic injury according to the method of Yadav and Dixit [12]. After 48 hr of the induction of hepatic damage, all animals were sacrificed and blood samples were collected from retro-orbital venus plexus in plain test tubes. Serum was prepared for biochemical analysis of γ-glutamyl transferase (GGT) according to Rosalki *et al.*[13], aspartate and alanine aminotransferase (AST and ALT) activities according to the method of Thefeld *et al.* [14], cholesterol [15], using BioMerieu kits. Another heparinized tube, blood samples were collected for determination of reduced glutathione (GSH) in whole blood and it mixed gently and kept on ice until analysis for GSH.

Statistical analysis

The obtained results were analyzed by ANOVA single factor using Excel 2003 Microsoft Corp (11.5612.5606), Redmond, WA software package.

Results

Acute toxicity study

Eugenia supra-axillaris extract did not show any toxic symptoms or mortalities among rats treated with oral doses for each extract during the observation period (24 hr 7 days post-treatment). Doses were starting from 0.1 gm up to 4 gm / kg b. wt.

Hepatoprotective study

Eugenia supra-axillaris extracts (100 and 200 mg/ kg) or silymarin did not change the levels of GGT and ALT, while, the values of AST and cholesterol were decreased significantly than normal control group (Table1). Whereas, the higher dose of *Eugenia supra-axillaris* (200 mg/kg) and silymarin caused significant elevation in GSH values, otherwise, the lower dose of *Eugenia supra-axillaris* non-significantly elevated GSH level as shown in Table 1.

Pre-treatment with *Eugenia supra-axilaries* extracts 100 and 200 mg/ kg) in CCl_4-hepatic-damaged rats had significant reduction in the studied serum biochemical assays (GGT, ALT, AST and cholesterol levels) when compared to the CCl_4-treated rats. These values declined to be near the normal values; specifically cholesterol was back to its normal values as prior CCl_4-treatment in all treatments (lower and higher doses of *Eugenia supra-axillaris* or silymarin).

TABLE 1. Hepatoprotective effect of (Es), (100 mg and 200 mg / kg.b.wt. / p.o.), silymarin (100 mg/ kg.b.wt./p.o.)in normal and CCl_4- hepatic damaged rats.

Groups	Normal rats				CCL_4- hepatic damaged rats			
	Control	Silymarin	Dose I	Dose II	CCL4	Silymarin	Dose I	Dose II
GGT	A 1.27± 0.09	A 0.97± 0.02	A 1.13± 0.04	A 1.00± 0.03	B 6.35± 0.22	C 2.70± 0.19	D 3.90± 0.23	C 2.98± 0.09
ALT	A 30.17± 0.70	A 29.17± 1.05	A 27.00± 1.24	A 26.33± 1.12	B 56.67± 2.50	CD 38.00± 1.61	C 41.00± 1.15	D 34.17± 0.91
AST	A 43.33± 2.42	B 32.50± 0.85	B 38.05± 1.73	B 33.22± 1.37	C 81.17± 2.36	D 53.00± 1.18	E 60.33± 2.63	DE 55.00± 3.14
Cholesterol	A 71.83± 1.14	B 49.33± 1.23	CE 55.33± 1.33	BE 50.33± 0.71	D 82.00± 1.51	BE 50.83± 1.47	CE 57.50± 0.92	E 54.33± 1.80
GSH	A 33.17± 0.48	B 41.17± 1.17	AD 35.67± 0.56	B 40.83± 1.01	C 18.00± 0.58	A 31.00± 0.86	A 32.00± 0.97	D 36.50± 0.96

ANOVA one way, the means within the vertical columns with different alphabetical superscripts are significantly different at $P< 0.05$.

Discussion

Extract of *Eugenia supra-axillaris* does not produce any gross behavioral changes or mortality even at a dose of 4 g /kg, p.o. in rats as also reported in the literature [16].

Administration of CCl_4 led to increase the serum enzymes level by 2–3-folds as compared to control group. Treatment of rats with *Eugenia supra-axillaris* p.o. markedly prevented CCl_4 induced elevation of serum GGT, AST and ALT (Table 1). CCl_4 induces fatty liver and cell necrosis [17] and plays a significant role in inducing triacylglycerol accumulation, depletion of GSH, increased lipid peroxidation, membrane damage, depression of protein synthesis and loss of enzymes activity [18]. Being cytoplasmic in location, the damage marker enzymes GGT, AST, ALT are released in serum [19]. It has been shown that protective agents exert their action against CCl_4 induced liver injury by impairment of CCl_4-mediated lipid peroxidation, either through decreased production of free radical derivatives[11, 20] or due to the antioxidant activity of the protective agent itself[21].

The extract of *Eugenia supra-axillaris* used in the study preserved the structural integrity of the hepatocellular membrane in a dose dependent manner as evident from the protection provided as compared to the enzyme levels in CCl_4 treated rats. Furthermore, protective mechanism not specific to CCl_4 may be responsible for hepatoprotective activity of the aqueous methanolic extract *Eugenia supra-axillaris*.

Conclusion

Ten phenolic compounds were isolated for the first time from *Eugenia supra-axillaris* leaves including the flavonoid glycosides; myricetin 3-*O*- β-xylopyranosyl (1→2) α-rhamnopyranoside, myricetin 3-*O*- α-rhamnopyranoside and quercetin 3-*O*- α-rhamnopyranoside, the flavonoid aglycones; pinocembrin, myricetin and quercetin, together with phenolics; gallic acid, ellagic acid, 5-*O*-monogalloylquinic acid and nilocitin.

Acute toxicological study of the aqueous alcoholic extract of *Eugenia supra-axillaris* had no toxic effect and no mortalities were observed in rats orally administrated different doses of extract up to 4 gm/kg b.wt.

Hepatic damaged rats showed significant elevation in the liver function tests: GGT, ALT, AST cholesterol and triglycerides. Moreover, GSH level was decreased significantly than normal control value.

Rats pre-treated with aqueous alcoholic extract of *Eugenia supra-axillaris* significantly reduced the elevated values of GGT, ALT, AST cholesterol and triglycerides and increased the level of GSH in a dose dependent manner. These results were obvious especially in the higher dose (200 mg / kg. b.wt.).

The activity of the higher dose of *Eugenia supra-axillaris* extract (200 mg / kg. b.wt.) was similar to that obtained by the standard natural hepatoprotective silymarin.

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المكونـات الفينوليـه والنشـاط الـواقى للكبـد لأوراق نبـات الكبابـه الصينى

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لمعرفة نشاطها الواقى للكبد وقد أجريت دراسة فيتوكيميائيه على النبات أمكن من خلالها التعرف على عشرة مركبات فينوليه لأول مرة فى هذا النبات وهى جليكوزيدات فلافونويديه وأجليكونات فلافونويديه الى جانب حمض أحادى جاللويل كينيك والنيلوسيتين والبينوسمبرين.