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Phytochemical and Biological Investigation of *Thunbergia grandiflora*

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Abstract
Investigation of 70% aqueous methanol of *Thunbergia grandiflora* Roxb leaves extract (Family Acanthaceae) led to isolation of ten compounds; Caffeic acid (1), Quercetin-3-*O*-rutinoside-7-*O*-α-L-rhamnopyranoside (2), Kaempferol-3-*O*-α-L-rhamnopyranosyl-7-*O*-β-D-$^4C_1$-arabinopyranoside- 4'-methyl ether (3), Kaempferol-6-C-sophoroside (4) Kaempferide 3-*O*- α-L-arabinofuranoside (5), Kaempferol-3-*O*-α-L-rhamnopyranoside (6), isoquercetrin (7), Quercetrin (8), Quercetin (9) and Kaempferol (10) for the first time. The isolated compounds were identified by using spectroscopic techniques as UV,-ESI/MS, ^{1}H-NMR and ^{13}C-NMR. Aqueous methanol extract of *Thunbergia grandiflora* Roxb leaves exhibited marked hypoglycemic activity comparable to metformin as reference drug and antioxidant activity that was evaluated by glutathione and DPPH radical scavenging activity methods in comparison with vitamin E, as well as hepatoprotective effect to liver damaged rats using CCl_4 and compared with silymarin as reference drug.

Keywords: *Thunbergia grandiflora*, flavonoids, antioxidant, hypoglycemic, hepatoprotective

1. Introduction
Acanthaceae is a taxon of dicotyledonous flowering plants, containing approximately 250 genera and nearly 4000 species among them genus *Thunbergia*, with more than 100 species. *Thunbergia grandiflora* Roxb is a long-lived (perennial), vigorous, climbing plant [1]. Although genus *Thunbergia* generally was reported to contain variety of compounds e.g. flavonoids, iridoid glycosides [2-5] but few phytochemical studies have been reported on *Thunbergia grandiflora* Roxb leaves. The aim of this study is investigation of phenolic constituents, evaluation of hypoglycemic, antioxidant and hepatoprotective activities of *Thunbergia grandiflora* Roxb.

2. Material and methods
2.1 *Plant material*
Leaves of *T. grandiflora* Roxb were collected from plants cultivated in El Kanater Gardens, Qaliubiya, Egypt. The identity of the plant was established by Prof. Dr. Wafaa M. Amer, Department of Botany, Faculty of Science, Cairo University. Voucher specimens (T-5) are kept in the Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University. The leaves were air-dried and reduced to No.36 powder and kept in tightly closed container until extraction process.

2.2 *General equipments and chemicals*
The ^{1}H NMR, 1D and 2D (300 and 400 MHz) and ^{13}C NMR (75 or 100 MHz) spectra (DMSO-d_6) were recorded on a Varian Mercury 300 (Varian Instruments, Palo Alto, CA, USA) at a proton frequency of 300, JEOL- and 400 GX spectrometers (Tokyo, Japan) at 400 MHz using TMS as internal standard. The δ-values are reported as ppm relative to TMS and *J*-values in Hz. UV analyses of pure isolates were recorded as methanol solutions (Merck) and with different diagnostic UV shift reagents in case of flavonoids [6], on Shimadzu UV 240 (P/N 204-58000) (Columbia, OH, USA) spectrophotometer. HPLC-grade methanol (Merck) was used for sample preparation. All other solvents used for extraction and separation processes were obtained from El-Nasr Chemicals Co., Abou Zaabal, Egypt. Aglycones and sugars obtained by acid hydrolysis were identified by co-TLC and PC with authentic samples, using S_1, and S_2 then visualized by specific spray reagents (e.g., vanillin HCl and aniline hydrogen phthalate).

2.3 Quantitative estimation of phenolic and flavonoid contents
Total phenolic content of *T. grandiflora* Roxb leaves was determined by the Folin-Ciocalteus

reagent method [7], it was expressed as GAE (Gallic Acid Equivalents) while, their total flavonoid content was determined by the aluminium chloride colorimetric methods, it was expressed as quercetin equivalents [8].

2.4 Extraction and isolation

Air dried powdered of *T. grandiflora* Roxb leaves (1Kg) were subjected to extraction with 70% methanol under reflux (5L, then 4 x 3L, 1.5h each, 70 °C). The combined methanol extract was evaporated under reduced pressure at 50 °C. The sticky brown residue from total extract (130 g) was subjected to successive extraction with increasing polarity by reflux with petroleum ether (4 x 1L, 50 °C, 1h), chloroform (3 x 1L, 40 °C, 1h) then MeOH (6 x 1L, 50 °C, 1h). The solvent-free yellowish-brown residue (85 g dry methanol-soluble portion) obtained which was suspended in 10 % CH_3OH: H_2O and applied on a polyamide S (Fluka, St. Louis, MO, USA) column (200 g, 100 x 8 cm) using a step gradient from 10% CH_3OH in H_2O to 100 % CH_3OH for elution to yield 48 fractions of 1L each. Similar fractions were collected together to yield eleven collective fractions (I-XI). Fraction II (eluted with 10%-20% methanol-water) was extracted with *n*-butanol saturated with H_2O then the extract was applied on sephadex LH-20 CC and eluted with ethanol to give 10 fractions (20 ml each). The eluted fractions were monitored by TLC to be collected into two sub-fractions. TLC of the first sub-fraction showed minor compounds which are difficult to be isolated. The second sub-fraction was further successively applied on sephadex LH-20 for isolation of compound 1. Fractions III and IV (30% aqueous methanol) were subjected to successive CC on sephadex LH-20 using different solvent systems to isolate pure compound 2. Fractions V and VI were eluted with 40% aqueous methanol and subjected to successive CC on sephadex LH-20 using different solvent systems for isolation of compound 3 and compound 4. Fraction VII and VIII (50% aqueous methanol fractions) were subjected to successive CC on sephadex LH-20 using different solvent systems to isolate pure compounds 5 and 6. TDPC of fraction IX using solvent system S_1 and S_2 [S_1: *n*-butanol-acetic acid-water (4:1:5, top layer) S_2: 15% aqueous acetic acid and] respectively, revealed the presence of one major spot of flavonoid nature. The fraction (2gm) was subjected to repeated column chromatography on sephadex LH-20 employing different solvent systems; the organic layer of *n*-butanol-isopropanol-water (BIW), 4: 1: 5 as eluent and absolute ethanol to give pure compounds 7 and 8. Fraction X and XI (70% -80% aqueous methanol fractions) were subjected to successive CC on sephadex LH-20 to isolate compound 9 and compound 10. The fractions were monitored by TLC (Merck F_{254} plates 20 x 20 cm using S_2, 2D-PC and Comp-PC using Whatmann No. 1 paper (Whatmann, Maidstone, UK systems S_1 and S_2). The compounds were visualized by spraying with Naturstoff (NP/PE). (diphenyl borinic acid ethanol amine complex 1% in methanol), followed by ethylene glycol 400 (5% in ethanol v/v) for color as flavonoids revealing reagents.

3. Experimental data of isolated compounds

Compound 1: White powder (35 mg) m.p 210°, R_f values: 0.48 (S_1) and (0.77) S_2 on PC, gave blue color with $FeCl_3$ reagent. UV spectral data λ_{max} (nm): (MeOH): 262, 292sh, 326; (+ NaOMe): 264, 371. Negative ESI/MS showed signals at 179[M -H]$^-$, 151[M- H- CO]. ^{1}H-NMR spectral data (400 MHz, DMSO-d_6) δ_{ppm} 12 (s- OH), 9.1 (s- COOH), 7.39 (1H, d, J = 16 Hz, H-7), 7.02 (1H, d, J = 2.5 Hz, H-2), 6.95 (1H, dd, J = 8, 2 Hz, H-6), 6.75 (1H, d, J = 8 Hz, H-5), 6.15 (1H, d, J = 16 Hz, H-8). ^{13}C-NMR spectral data (100 MHz, DMSO-d_6) δ_{ppm} 168.48(C=O), 148.58(C-4) 146 (C-3), 145 (C-7), 126.14(C-1), 121.66(C-6), 116.19(C-8), 115.64 (C-5), 114.98(C-2).

Compound 2: Yellow amorphous powder (40 mg); gave positive Molisch's test. It appears as a dark purple fluorescence under the UV light and changed yellow with ammonia vapors; yellow fluorescence UV (360 nm) with NP/PE and green color with $FeCl_3$ spray reagents and its R_f value in both S_1 and S_2 is 0.19 and 0.68 respectively. UV spectral data λ_{max} nm: MeOH: 258, 269 (sh), 357; NaOMe: 250, 270 (sh), 397; NaOAc: 260,290 (sh), 370; NaOAc/H_3BO_3: 261, 291 (sh), 382; $AlCl_3$: 279, 301 (sh), 352 (sh), 445; $AlCl_3$/HCl: 274, 300 (sh), 355 (sh), 405. Negative ESI-MS: m/z 755 [M^+- H]$^-$. ^{1}H-NMR spectral data (400 MHz, DMSO-d_6): δ_{ppm} 12.64 (^{1}H, s, H-bond OH-5), 7.54(1H, d, *J*=2 HZ, H-2'), 7.5 (1H, dd, *J*=8.4, 2 Hz, H-6'), 6.83(1H, d, *J*= 8.4 Hz, H-5'), 6.39(1 H, d, *J*=1.6Hz, H-8), 6.19 (1H, d, *J*=1.6 Hz, H-6), 5.51 (1H, d, *J*=7.6 Hz, H-1''), 5.05 (1H, brs, H-1''''), 4.35 (1H, brs, H-1'''), 3.8-3.00 (m, remaining sugar protons), 0.95 (3H,d, *J*=6.4 Hz, CH_3 -6''''), 0.8 (3H, d, *J* =6.4 Hz, CH_3 -6'''). ^{13}C-NMR spectral data (100 MHz, DMSO-d_6): δ_{ppm} 177.52(C-4), 164.42(C-7), 161.49(C-5), 157.54 (C-2) 156.72 (C-9), 148.67 (C-4`), 145.07 (C-3'), 132.96 (C-3), 121.54 (C-1'), 121.52 (C-6'), 116.38 (C-5'), 115.45 (C-2'), 104. 2 (C-10), 101.96 (C-1''), 100.99 (C-1'''), 99.05 (C-1''''), 98.86 (C-6), 93.98 (C-8), 77.69 (C-3''), 77.33 (C-5''), 74.2(C-2''), 70.66 (C-4''), 68.87(C-6''), 17.94 (C-6'''), 17.45 (C-6'''').

Compound 3: Yellow amorphous powder; R_f values 0.4 (S_1), 0.76 (S_2), deep purple under UV light, turned to yellow fluorescence with ammonia vapour, yellow with NP/PE and gave green color with ($FeCl_3$). UV spectral data, λ_{max}(nm): (MeOH): 265, 315(sh), 350; (+NaOMe): 269, 301 (sh), 389; (+NaOAc): 265, 318 (sh), 358; (+NaOAc/H_3BO_3): 265, 319 (sh), 352; (+$AlCl_3$): 255 (sh), 274, 301 (sh), 354; (+$AlCl_3$ /HCl): 274, 298 (sh), 348. ^{1}H-NMR spectral data (400 MHz, DMSO-d_6): δ_{ppm} 8.01 (2H, d, *J* = 8.4 Hz, H2'/6'), 7.3 (2H, d, *J* = 8.4 Hz, H-3'/ 5'), 6. 6 (1H, d, *J* = 2 Hz H-8), 6.2 (1H, d, *J* = 2 Hz, H-6), 5.4 (brs, H-1''), 5.1 (1H, d, *J*=6.9 Hz, H-1'''), 3.8 (3H, s, OCH_3), 1.23 (3H, d, *J*=6Hz, H-6''). ^{13}C- NMR spectral data (100 MHz, DMSO-d_6: δ_{ppm} 176.4 (C-4), 162.44 (C-7), 161.26 (C-5), 155.6 (C-2), 133.3 (C-3), 130.9 (C-6'/2'), 115.21 (C-3'/5'), 104.09 (C-10), 104 (C-1''), 103.8 (C-1'''), 56.47 (4'-OCH_3), 18.2 (3H, C-6'').

Compound 4: Yellow amorphous powder; R_f values 0.41 (S_1), 0.55 (S_2), deep purple under UV light, turned to yellow fluorescence with amm. vapour, yellow with Naturstoff and gave green color with ($FeCl_3$). UV spectral data, λ_{max}(nm): (MeOH): 255(sh), 265, 315(sh), 366; (+NaOMe): 275, 316(sh), 410; (+NaOAc): 274, 318 (sh), 388; (+NaOAc/H_3BO_3): 275, 319 (sh), 378; (+$AlCl_3$): 265(sh), 274, 321(sh), 400; (+$AlCl_3$ /HCl): 264 (sh), 268, 319(sh), 398. ^{1}H-NMR spectral data (400 MHz, DMSO-d_6): δ_{ppm} 8.02 (2H, d, *J* = 7.2 Hz, H2'/6'), 6.9 (2H, d, *J* = 8 Hz, H-3'/ 5'), 6.73 (1H, s, H-8), 5.0 (H-1'''), 4.76 (1H, d, *J*=9.6 Hz, H-1''). ^{13}C-NMR spectral data (100 MHz, DMSO-d_6): δ_{ppm} 175.64 (C-4), 166.40 (C-7), 148.15 (C-2), 134.3 (C-3), 127.2 (C-6'/2'), 114.29 (C-3'/5'), 109 (C-6), 105.46 (C-1'''), 82.21 (C-5''), 79.23 (C-3'''), 73.91 (C-1''), 73.9 (C-3''), 72.77 (C-2''), 71.37 (C-5'''), 71.09 (C-4'''), 70.88 (C-2'''), 70.51 (C-4''), 67.1 (C-6''), 63.73 (C-6''').

Compound 5: Yellow needles; R_f values 0.6 (S_1), 0.58 (S_2), deep purple under UV light, turned to yellow fluorescence with ammonia vapour, yellow with NP/PE and gave green color with ($FeCl_3$). UV spectral data, λ_{max}(nm) (MeOH): 266, 300 (sh), 352; (+NaOMe): 273, 324, 405; (+NaOAc): 273, 308 (sh), 377; (+NaOAc/H_3BO_3): 267, 300 (sh), 350; (+$AlCl_3$): 260 (sh), 288, 303 (sh), 348, 398; (+$AlCl_3$ /HCl): 284, 304 (sh), 346, 396. ^{1}H-NMR spectral data (400 MHz, DMSO-*d*$_6$): δ_{ppm} 8.04 (2H, d, *J* = 8.8 Hz, H2'/6'), 6.92 (2H, d, *J* = 8.8 Hz, H-3'/ 5'), 6.43 (1H, d, *J* = 2 Hz, H-8), 6.33 (1H, d, *J* = 2.4 Hz, H-6), 5.36 (1H, brs, H-1"), 3.896 (3H- OCH_3).^{13}C-NMR spectral data (100 MHz, DMSO-*d*$_6$): δ_{ppm} 177.56 (C-4), 164.44 (C-7), 157.12 (C-2), 133.96 (C-3), 129.41 (C-6'/2'), 116.13 (C-3'/5'), 109.18 (C-1"), 79.06 (C-2"), 79.76 (C-4"), 72.93 (C-3"), 60.82 (C-5"), 56.5 (4'-OCH_3).

Compound 6: Yellow needles, R_f values 0.61(S_1), 0.56 (S_2), deep purple under UV light, turned to yellow fluorescence with amm. vapour, yellow with NP/PE and gave green color with ($FeCl_3$). UV spectral data, λ_{max}(nm): (MeOH): 260, 295 (sh), 346; (+NaOMe): 269, 325, 396; (+NaOAc): 268, 305 (sh), 372; (+NaOAc/H_3BO_3): 264, 300 (sh), 348; (+$AlCl_3$): 271, 304 (sh), 348, 398; (+$AlCl_3$ /HCl): 273, 302 (sh), 343, 396. Negative ESI-MS. m/z 432 [M^+- H]$^-$. ^{1}H-NMR spectral data (400 MHz, DMSO- *d*$_6$); δ_{ppm} 13.87(1Hs, OH-5), 8.05 (2H, d, *J* = 8.8 Hz, H2'/6'), 6.90 (2H, d, *J* = 8.4 Hz, H-3'/ 5'), 6.8 (1H, d, *J* = 2.1 Hz, H-8), 6.4 (1H, d, *J* = 2.1 Hz, H-6), 5.4 (1Hs, H-1"),1.24 (3H, d, *J*=6.1 Hz, H-6").

Compound 7: Pale yellow amorphous powder, mp 314 ºC, R_f 0.62 (S_1) and 0.59 (S_2). It showed dark purple fluorescent spot under UV light (365 nm) turned to deep yellow on exposure to NH_3 vapours. UV spectral data λ_{max} nm MeOH: 258, 301 (sh), 362; NaOMe: 272, 330 (sh), 402; NaOAc: 273,325 (sh), 395; NaOAc/H_3BO_3: 262, 310 (sh), 381; $AlCl_3$: 268, 310 (sh), 360; $AlCl_3$/HCl: 269, 311 (sh), 362. Negative ESI/MS: m/z 463 [M^+-H]$^-$. ^{1}H-NMR spectral data (400 MHz, DMSO-*d*$_6$): δ_{ppm} 12.657 (1H, s, H-bond OH-5), 7.59 (1H, d, *J*=2 HZ, H-2'), 7.58 (1H, dd, *J*=8, 2 Hz, H-6`), 6.87(1H, d, *J*= 8.8 Hz, H-5'), 6.43(1 H, d, *J*=1.6Hz, H-8), 6.22 (1H, d, *J*=2Hz, H-6), 5.47 (1H, d, *J*= 7.2 Hz, H-1"), 3.8-3.00 (m, remaining sugar protons). ^{13}C-NMR spectral data (100 MHz, DMSO-*d*$_6$), δ_{ppm} 176.40(C-4), 164.22 (C-7), 161.19 (C-5), 156.29 (C-2) 156.13 (C-9), 148.48 (C-4'), 144.30 (C-3'), 133.27 (C-3), 121.69 (C-1'), 121.11 (C-6'), 115.2 (C-5'), 115.16(C-2'), 104.62 (C-10), 103.9 (C-1"), 99.67 (C-6), 93.50 (C-8) 69.88 (C-4"), 76.46 (C-3"), 74.02 (C-2") 77.53 (C-5"), 60.91 (C-6").

Compound 8: Pale yellow amorphous powder, mp 314 ºC, R_f 0.61 (S_1) and 0.58 (S_2). It showed dark purple fluorescent spot under UV light (365 nm) turned to deep yellow on exposure to NH_3 vapours. Response to spray reagents: green color ($FeCl_3$) and yellow fluorescence UV, (NP/PE). UV spectral data λ_{max} nm MeOH: 258, 290 (sh), 353; NaOMe: 270, 325 (sh), 398; NaOAc: 270,324 (sh), 399; NaOAc/H_3BO_3: 267, 391; $AlCl_3$: 269, 313 (sh), 362, 425; $AlCl_3$/HCl: 269, 312, 362, 395. Negative ESI/MS: m/z 447.39 [M^+-H]$^-$.^{1}H-NMR spectral data (300 MHz, DMSO-*d6*) Fig. (20): δ_{ppm} 12.65 (1H, s, H-bond OH-5), 7.30(1H, d, *J*=1.8 HZ, H-2`), 7.26 (1H, dd, *J*=8.4, 1.8 Hz, H-6'), 6.87(1H, d, *J*= 8.4 Hz, H-5'), 6.39(1 H, d, *J*=2.1Hz, H-8), 6.21 (1H, d, *J*=2.1Hz, H-6), 5.26 (1H, brs, H-1"), 3.98 (1H, brs, H-2″) 3.8-3.00 (m, remaining sugar protons), 0.82 (3H, d, *J* =5.7 Hz, CH_3 -6″).
^{13}CNMR spectral data (75 MHz, DMSO-*d*$_6$): δ_{ppm} 175.37(C-4), 164.76(C-7), 161.90 (C-5), 158.0 (C-2) 156.99 (C-9), 147.9 (C-4'), 145.79 (C-3'), 134.88 (C-3), 121.69 (C-1'), 120.2(C-6'), 116.24 (C-5'), 116.04 (C-2'),104.62 (C-10), 102.54 (C-1"), 99.24 (C-6), 94.20 (C-8) 71.14 (C-4"), 70.98 (C-3"), 70.89 (C-2") 70.61 (C-5″), 18.1 (C-6").

Compound 9: Dark yellow amorphous powder, gives negative Molisch's test, with mp 312ºC its chromatographic properties showed R_f values: 0.67 (S_1), 0.03 (S_2); it was observed as deep yellow fluorescence under UV- light, turned to bright yellow with ammonia vapour, orange fluorescence with NP/PE reagent and green with $FeCl_3$ spray reagent. UV spectral data λ_{max} (nm): (MeOH) 255, 297.5 (sh), 301 (sh), 370; (+NaOMe) 274, 297, 324 (sh), 398; (+NaOAc) 257, 274, 321 (sh), 388; (+NaOAc/H_3BO_3) 262, 326 (sh), 391; (+$AlCl_3$) 269, 295 (sh), 314 (sh), 445; (+$AlCl_3$/HCl) 255 (sh), 268, 299 (sh), 438. EI-MS: m/z 301 [M]$^+$, 100% ^{1}H-NMR spectral data (300 MHz, DMSO-*d*$_6$) δ_{ppm} 7.66 (1H, d, *J*=2.1Hz, H-2'), 7.52 (1H, dd, *J*=8.4, 2.1 Hz, H-6'), 6.89(1H, d, *J*=8.4 Hz, H-5'), 6.41 (1H, d, *J*=2.1 Hz, H-8), 6.18 (1H, d, *J*=2.1 Hz, H-6). ^{13}C-NMR spectral data (75 MHz, DMSO-*d*$_6$) δ_{ppm} 175.72 (C=O), 164.02 (C-7), 160.58 (C-5), 156 (C-9), 148.1 (C-2), 147.6 (C-4'), 145.02 (C-3'), 135.6 (C-3), 121.8 (C-1'), 119.85(C-6'), 115.57(C-5'), 114.92 (C-2'), 102.8 (C-10), 98.17 (C-6), 93.33 (C-8).

Compound 10: Yellow amorphous powder, R_f values 0.79 (S_1) 0.042 (S_2); dull Yellow fluorescence under UV light, turned to shine yellow with amm. vapour, yellow with NP/PE and gave green color with ($FeCl_3$). UV spectral data, λ_{max}(nm) (MeOH): 253 (sh), 266, 294 (sh), 322 (sh), 367; (+NaOMe): 278, 316, 416; (+NaOAc): 274, 303, 387; (+NaOAc/H_3BO_3): 267, 297(sh), 320 (sh), 372; (+$AlCl_3$): 260 (sh), 268, 303 (sh), 350, 424; (+$AlCl_3$ /HCl): 256 (sh)269, 303 (sh), 348, 424. Negative ESI/MS *m/z* 285.0 [M^+-H]$^-$, ^{1}H-NMR spectral data, (300 MHz, Acetone–*d*$_6$), δppm 7.90 (2H, d, *J* = 8.4 Hz, H2'/6'), 6.99 (2H, d, *J* = 8.4 Hz, H-3'/ 5'), 6.48 (1H, d, J = 2.1 Hz H-8), 6.26 (1H, d, *J* = 2.1 Hz H-6), [δ-values of Acetone-d_6 must be corrected by +0.13]. ^{13}C-NMR spectral data, (75 MHZ, DMSO-*d*$_6$), δppm 164.7 (C-7), 146.9 (C-2), 134.9 (C-3), 129.68 (6', 2'), 116.04 (C-3'/5').

4. Biological study

4.1 Hypoglycemic activity

Antidiabetic activity of aqueous methanol extract of *Thunbergia grandiflora* Roxb leaves (100 mg/Kg b.wt) was evaluated in comparable to metformin as reference drug (100 mg/Kg b.wt). Male albino rats were injected intra-peritoneal with alloxan (150 mg/KG b.wt. Sigma Co.) to induce diabetes mellitus [9]. Hyperglycemia was assessed after 72 hr by measuring blood glucose [10] and after 1 and 2 months intervals. At the end of each study period, blood samples were collected from the retro orbital venous plexus through the eye canthus of aneasthetized rats after an overnight fast. Serum was isolated by centrifugation and the blood glucose level was measured.

4.2 Antioxidant activity

Antioxidant activity of aqueous methanol extract of *T. grandiflora* Roxb leaves (100 mg/kg b.wt) was determined by two methods:

4.2.1 Glutathione method

Male albino rats were injected intra-peritoneal with alloxan (150 mg/kg b.wt) to induce diabetes mellitus [9]. Hyperglycaemia was assessed after 72 hr by measuring blood

glucose [10]. After 7 days, blood samples were collected. Fresh heparinised blood used for estimation of blood glutathione level as oxidative stress marker [11] and by using biodiagnostic kit in comparison with effect of vitamin E (Pharco Pharmaceutical Co) as reference drug, the absorbance measured at λ_{max} 405 nm. $GSH_{blood} = A_{sample}$ X 66.66 mg/dl.

4.2.2 *In vitro* DPPH radical scavenging activity

DPPH-free radical scavenging activity of *T. grandiflora* Roxb leaves extract was measured following Yen and Duh 1994 [12] using ascorbic acid as reference drug. DPPH-free radical scavenging activity was calculated as percentage inhibition (PI) according to the formula: PI= {(AC-AT)/AC} X100 where: AC= Absorbance of the control at t=0 min, AT=Absorbance of the sample + DPPH at t=16min

DPPH-free radical scavenging activity was calculated and expressed as IC_{50} that is the concentration of *T. grandiflora* required to scavenge 50% of DPPH used.

4.3 Hepatoprotective activity

Hepatoprotectiv activity of aqueous methanol extract of *Thunbergia grandiflora* Roxb leaves (100 mg/Kg b.wt) was evaluated in comparable to silymarin as reference drug (25 mg/Kg b.wt) according to method described by Sadeghi *et al.* 2008 [13]. Liver damage in rats was induced according to the method of Klassen and Plaa [14] by intraperitoneal injection of 5ml/Kg of 25% carbon tetrachloride in liquid paraffin. Serum was isolated by centrifugation. ALT and AST in serum were determined by method described by Thewfweld [15] while ALP was determined according to method of Kind and King [16]. The data obtained were analyzed using the Students t test [17].

4.4 Method for evaluation of antimicrobial activity

The antimicrobial activity of some isolated compounds **1, 5, 6** and **9** from *T. grandiflora* Roxb leaves were investigated *in vitro* against different bacteria and fungi using suitable media. The test was done using the diffusion agar technique, well diameter 6 mm [18-20].

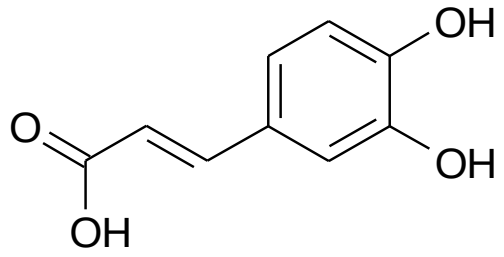
5. Results and Discussion

5.1 Total phenolic and flavonoid contents

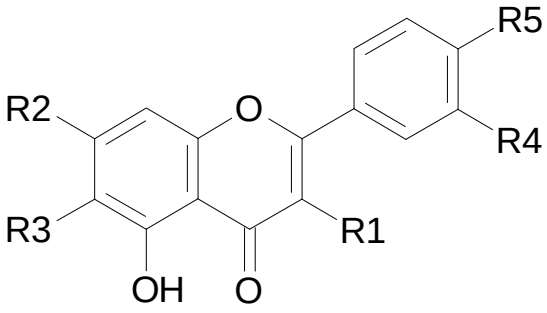
Quantitative estimation of total phenolic content of *T. grandiflora* Roxb leaves was determined as 119.8 mg/g expressed as GAE (Gallic Acid Equivalents), while those of total flavonoids content yielded 36.8mg/g extract as quercetin equivalents.

5.2 Identification of the isolated compounds

The defatted methanol extract of *T. grandiflora* Roxb leaves was fractionated by repeated column chromatographic separations to obtain compounds 1 – 10, identified based on chemical and physicochemical analyses. Spectral data of the known compounds were in good accordance with those previously published [22, 34]; as: Caffeic acid 1 [4, 21], Quercetin-3-*O*-rutinoside-7-*O*-α-L-rhamnopyranoside 2 [22, 23], Kaempferol-3-*O*-α-L-rhamnopyranosyl-7-*O*-β-D-arabinopyranoside -4'-methyl ether 3 [24], Kaempferol-6-C-sophoroside 4 [25-27], Kaempferide 3-*O*- α-L-arabinofuranoside 5 [24], Kaempferol-3-*O*-α-L-rhamnopyranoside (Afzelin) 6 [22, 24], Isoquercetrin 7 [28, 29], Quercetrin 8 [22, 30, 31, 34], Quercetin 9 [30-34]. and Kaempferol 10 [29, 31-32]. All these compounds were isolated and identified for the first time from *T. grandiflora* Roxb leaves.



Compound 1: Caffeic acid



Compound **2**	R1= *O*- rutinose, R2= *O*- -α-L- rhamnose, R3=H, R4, R5 =OH
Compound **3**	R1= *O*- -α-L- rhamnose, R2= *O*- β-D- arabinopyranose, R3=H, R4=H, R5=OCH_3
Compound **4**	R1= R2= R5=OH, R3= sophoroside (C-glycoside), R4=H
Compound **5**	R1= *O*--α-L- arabinofuranose, R2=OH, R3=H, R4=H R5=OCH_3,
Compound **6**	R1= *O*-α-L- rhamnose, R2= R5=OH, R4=H, R3=H
Compound **7**	R1= *O*-β-D- glucose, R2=OH, R3=H, R4, R5 =OH
Compound **8**	R1= *O*- -α-L- rhamnose, R2=OH, R3=H, R4, R5 =OH
Compound **9**	R1=R2=OH, R3=H, R4, R5 =OH
Compound **10**	R1= R2= R5=OH, R3=H, R4=H

5.3 Antidiabetic activity

Aqueous methanol extract of *T. grandiflora* Roxb leaves at the dose of 100 mg/Kg b.wt. received by diabetic rats (high blood glucose level) significantly reduce the blood glucose level, as there was 25.6 % reduction in glucose level after two weeks and 49.9 % after four weeks. The activity was comparable to metformin (reference drug) which reduce the blood glucose level by 44.5% after two weeks and 67.1% after four weeks (Table 1& Figure 1).

5.4 Antioxidant activity

5.4.1 *Glutathione method:*

Table 2 and Figure 2 showed that 38.9 % glutathione level oxidative stress in blood of diabetic rats. Aqueous methanol extract of *T. grandiflora* Roxb leaves (100 mg/Kg b.wt.) and vitamin E (7.5 mg/kg) kept glutathione level in blood of diabetic rats around the normal level as they possess significant antioxidant activity.

5.4.2 *In-vitro* antioxidant activity (Free radical scavenging activity)

Antioxidant activity of aqueous methanol extract of *T. grandiflora* Roxb leaves (Table 3 and Figure 3) was calculated as the percent of change of absorbance of 2, 2' diphenyl-1-picrylhydrazyl (DPPH) radical using ascorbic acid as standard solution. The percent inhibition versus concentration of the extract was drawn. The highest radical scavenging effect was observed 640µg/mL (70.4%). IC_{50} was recorded at values 263 µg/ml. (the concentration of the sample causing 50% inhibition of DPPH is obtained from the calibration curve).

5.5 Hepatoprotective activity

Aqueous methanol extract of *T. grandiflora* Roxb leaves showed significant decrease in serum level of AST, ALT and ALP in rats after 72 hr and more reduction in their level after 7 days as it reduces level of AST by 48% after 72 hr and 65.9% after 7 days, while reduces level of ALT by 47.2% after 72 hr and 56.5% after 7 days and reduces level of ALP by 45.9% after 72 hr and 67.6 % after 7 days, which maintain the functional integrity of hepatic cells that indicating its hepatoprotective effect. The activity was comparable to silymarin which reduces level of ALT by 66.7 % after 72 hr and 76% after 7 days, reduces level of AST by 65% after 72 hr and 76.3% after 7 days and reduces level of ALP by 62.5% after 72 hr and 89% after 7 days (Table 4 and Figure 4).

5.6 Antimicrobial activity

Evaluation of antimicrobial activity of compounds **1, 5, 6** and **9** (Caffeic acid, Kaempferol-3- *O*-α-**L**-arabinofuranoside 4'methyl ether, Kaempferol-3- *O*-α-**L**-$^1C_4$-rhamnopyranoside and, quercetin respectively) against fungi, Gram positive and Gram negative bacteria revealed marked antimicrobial activity against *Aspergillus fumigatus*, *Candida albicans*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli* but they have no effect on the growth of *Pseudomonas aeruginosa*. Compound 5 and compound 9 were the most active compounds so determination of MIC (minimum inhibitory concentration) were determined Table 5.

Table 1: Antidiabetic activity of aqueous methanol extract of *Thunbergia grandiflora* Roxb leaves

Sample	Diabetic rats non treated	Diabetic rats treated with aqueous methanol extract (100 mg/kg)		Diabetic rats treated with metformin (100 mg/kg)	
	M ± S.E	M ± S.E	% of change	M ± S.E	% of change
Zero	259.6 ± 8.4	263.8 ± 9.2	-	254.6 ± 7.8	-
2 weeks	261.3 ± 5.1*	196.4 ± 6.5*	25.6	141.3 ± 3.5*	44.5
4 weeks	264.2 ± 7.8*	132.3 ± 4.2*	49.9	83.8 ± 2.1*	67.1

Table 2: Antioxidant activity of aqueous methanol extract of *Thunbergia grandiflora* Roxb leaves using glutathione method

Groups	Blood glutathione (mg/dl) Mean ± S.E	% of changes
Control	36.3 ± 1.2	-
Diabetic rats	22.2 ± 0.5*	38.9
Diabetic rats treated with Vitamin E (7.5 mg/ kg)	35.8 ± 1.1	1.4
Diabetic rats treated with aqueous methanol extract (100 mg/kg)	35.3 ± 0.9	2.8

* Statistically significant from control group at $p < 0.01$

Table 3: Antioxidant activity of aqueous methanol extract of *Thunbergia grandiflora* Roxb leaves using DPPH scavenging

Sample conc.(µg/ml)	DPPH scavenging %
640	70.40
320	57.88
160	35.45
80	20.51
40	11.92
20	5.35
0	0

All the determinations were performed in three replicates and averaged

Table 4: Hepatoprotective activity of aqueous methanol extract of *Thunbergia grandiflora* Roxb leaves

Groups	AST (U/L)				ALT (U/L)				ALP (KAU)			
	Zero	7 day	72hr after CCl_4	7 day after CCl_4	Zero	7 day	72hr after CCl_4	7 day after CCl_4	Zero	7 day	72hr after CCl_4	7 day after CCl_4
Control	47.6 ± 2.1	46.9 ± 1.8	166.3 ± 4.8*	184.4 ± 5.4●*	38.9 ± 1.3	38.4 ± 1.6	149.4 ± 4.2*	158.9 ± 5.1●*	7.3 ± 0.1	7.4 ± 0.1	48.8 ± 1.8*	69.7 ± 2.1●*
Aqueous methanol extract	46.2 ± 1.9	45.7 ± 1.6	86.4 ± 2.9*	62.9 ± 2.6●*	41.1 ± 1.4	42.7 ± 1.2	78.9 ± 2.9*	69.1 ± 2.3*	7.2 ± 0.1	7.1 ± 0.1	26.4 ± 0.9*	22.6 ± 0.4●*
Silymarin	45.3 ± 1.6	44.9 ± 1.9	58.2 ± 2.3*	43.7 ± 1.5●*	39.8 ± 1.2	38.4 ± 1.3	49.8 ± 1.6*	38.2 ± 1.1●*	7.4 ± 0.1	7.2 ± 0.1	18.3 ± 0.4*	7.6 ± 0.1●*

* Statistically significant from zero time at $p < 0.01$, • Statistically significant from 72 hr after CCl_4 at $p < 0.01$

Table 5: Antimicrobial activity of some isolated compounds of *Thunbergia grandiflora* Rox leaves

Sample Tested microorganism Tested microorganisms	Compound 1	Compound 5	MIC 5	Compound 6	Compound 9	MIC 9	Standard	MIC
FUNGI							*Amphotericin B*	
Aspergillus fumigatus (RCMB 02568)	16.3± 0.58	23.3± 0.58	0.98	12.3 ± 0.36	21.8 ± 0.32	1.95	23.7± 1.2	0.98
Candida albicans (RCMB 05036)	18.1± 1.5	21.4± 1.2	1.95	14.1 ± 0.35	20.4 ± 1.5	3.9	25.4± 0.58	0.49
Gram Positive Bacteria:							*Ampicillin*	
Streptococcus pneumoniae (RCMB 010010)	16.4± 0.58	23.1± 1.5	0.98	15.4± 0.55	21.3 ± 0.19	1.95	23.8± 1.2	*0.98*
Staphylococcus aureus (RCMB 010028)	18.1± 0.63	24.2± 1.2	0.98	15.9 ± 0.52	23.4 ± 0.37	0.98	27.4± 0.72	*0.49*
Gram Negative Bacteria:							*Ciprofloxacin*	
Pseudomonas aeruginosa (RCMB 010043)	NA	NA	NA	NA	NA	NA	20.6± 1.2	1.95
Escherichia coli (RCMB 010052)	16.8± 1.2	25.3± 0.58	0.98	14.3± 0.58	21.3± 0.24	1.95	23.4± 0.63	0.98

NA: No activity, RCMB: Regional Centre for Mycology and Biotechnology/ Antimicrobial unit test organisms, data are expressed in the form of mean ± SD

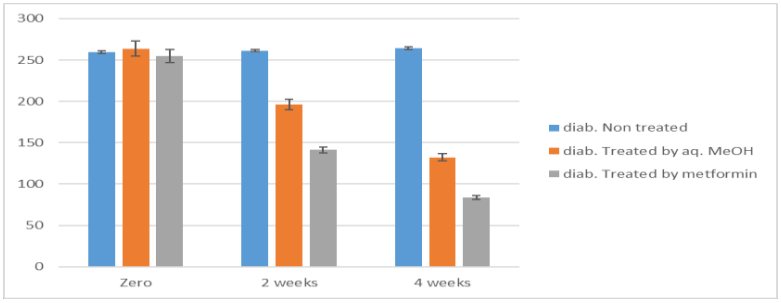


Fig 1: Antidiabetic activity of aqueous methanol extract of *Thunbergia grandiflora* Roxb leaves

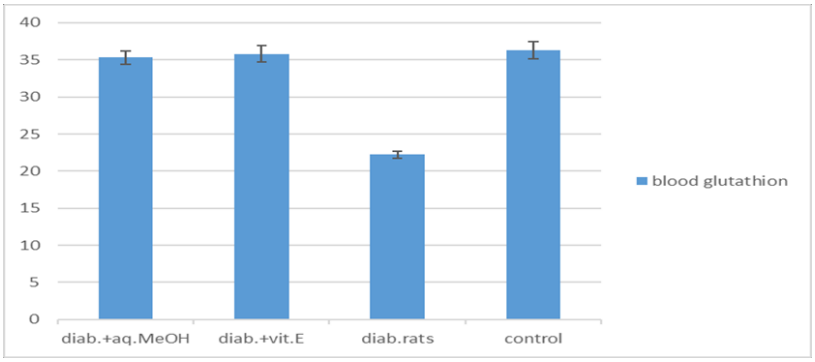


Fig 2: Antioxidant activity of aqueous methanol extract of Thunbergia grandiflora Roxb leaves using glutathione method

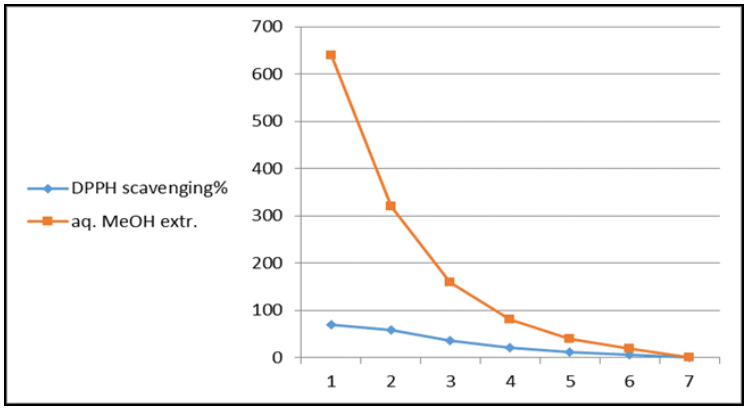
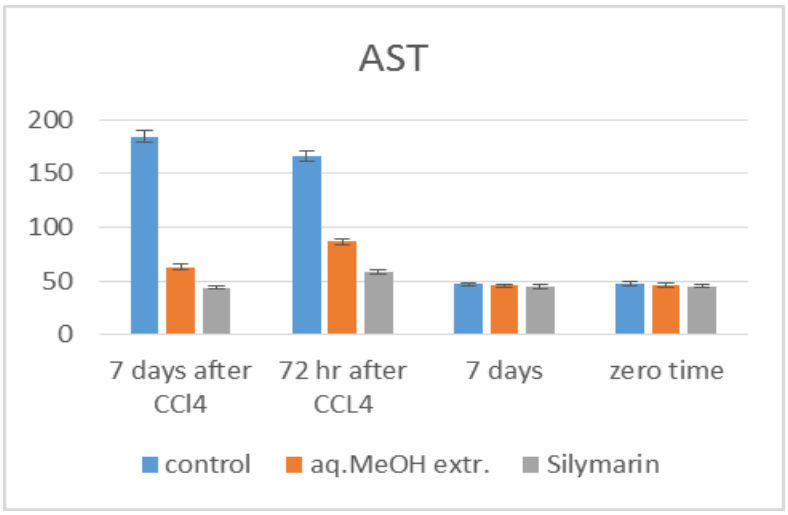
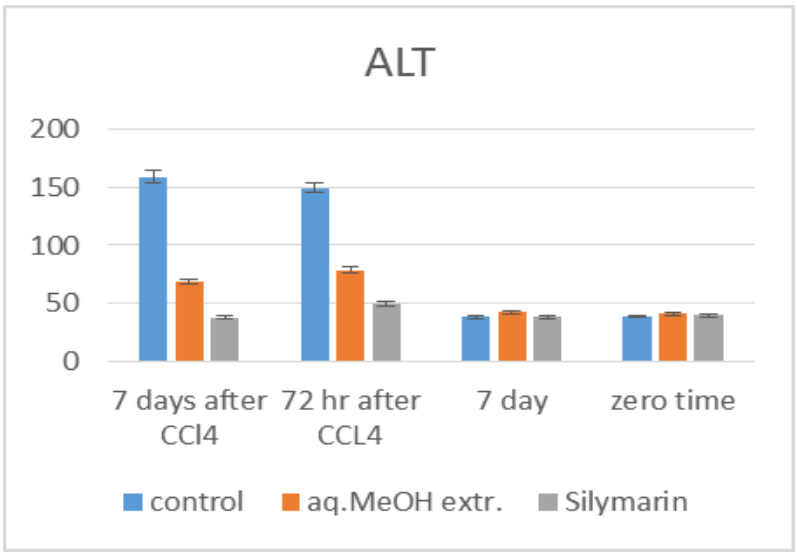


Fig 3: Antioxidant activity of aqueous methanol extract of Thunbergia grandiflora Roxb leaves using DPPH scavenging





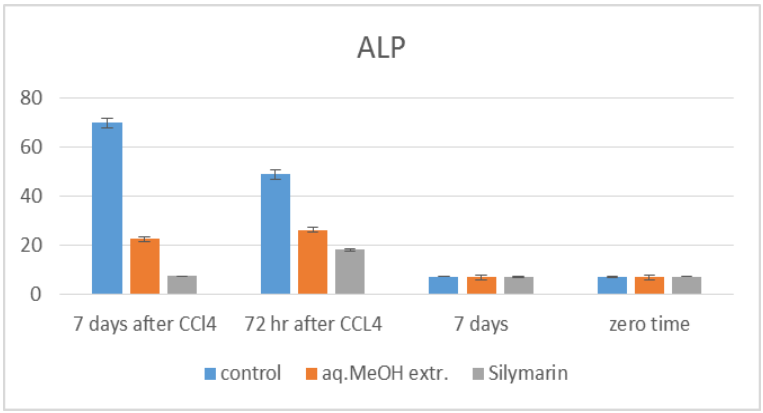


Fig 4: Effect of aqueous methanol extract of *Thunbergia grandiflora* Roxb leaves on serum liver function parameters (AST, ALT& ALP) in liver damaged rats

6. Conclusion

Phenolic acid and nine flavonoids; kaempferol, quercetin and their derivatives were isolated for the first time from the dried leaves of *Thunbergia grandiflora* Roxb extract, The extract has significant antioxidant, antidiabetic and hepatoprotective activities in addition to marked antimicrobial activity which give *Thunbergia grandiflora* Roxb great potential and supporting the use of the plant as a source of natural raw material for phytopharmaceuticals preperations.

This work was taken from the PHD Thesis of Salwa A Abu El wafa, Pharmacognosy Department, Faculty of Pharmacy "Girls", Al Azhar University.

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