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Phytochemical Analysis and Therapeutic Evaluation of *Ehretia anacua* Against Streptozotocin-Induced Diabetes in Rats

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Abstract
Diabetic patients have a higher risk of health problems, including heart attack, stroke, kidney failure, and permanent vision loss. *Ehretia anacua* is a plant used in folk medicine for the management of diabetes. The current study investigated the phytochemical profile of *Ehretia anacua* and evaluated its antioxidant activity, acute toxicity, and therapeutic potential against streptozotocin-induced diabetes in a rat model. Additionally, we evaluated the phenolic and flavonoid content either colorimetrically or by HPLC analysis. Eleven compounds were isolated namely, phytyl palmitate (**1**), *β*-sitosterol tetracosanoate (**2**), palmitic acid (**3**), isophytol (**4**), *β*-sitosterol (**5**), *β*-sitosterol-3-*O*-*β*-D-glucopyranoside (**6**), methyl rosmarinate (**7**), caffeic acid (**8**), (6*R*,7*E*,9*R*)-9-hydroxy-megastigma-4,7-dien-3-one 9-*O*-D-glucopyranoside (**9**), roseoside (**10**) and rosmarinic acid (**11**). These results revealed that *E. anacua* is a rich source of phenolic compounds. The ethyl acetate and *n*-butanol fractions have potent antioxidant activity with value of IC_{50} is 25.16 ± 0.09 and 87.84 ± 0.39 µg/mL, respectively. Moreover, the ethyl acetate fraction exhibited superior antidiabetic efficacy by significantly reducing blood sugar levels and lipid profiles and modulating related biochemical parameters. These results suggest that *E. anacua* leaves could serve as a potential source of antidiabetic and antioxidant medications, possibly offering new avenues for combination therapy for diabetes treatment.
Keywords: *Ehretia anacua*;antidiabetic;antioxidant; phytochemical analysis; total phenolic; total flavonoids; HPLC.

1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia, resulting from defects in insulin secretion, insulin action, or both [1]. The prevalence of diabetes is increasing worldwide, and it is estimated that by 2045, approximately 700 million people will be affected by this disease [2]. Ehretia comprises approximately 50 species belonging to the Boraginaceae family. It is widely distributed in the tropical and subtropical regions of the world. It contains approximately 23 species reported in Africa, 12 species in Asia, and some species present in Australia and Mexico [3]. The biological effects associated with various phytochemicals found in these plants, such as alkaloids, polyphenolics, flavonoids, tannins, and phytosterols, are primarily synthesized as a defense mechanism against various stressors [4]. Additionally, Ehretia plants have been traditionally used to treat various diseases, such as diabetes, hypertension, respiratory tract problems, gastrointestinal diseases, wound healing, skin disease, malaria, epilepsy, and liver disease [5-7]. They are also recommended for the treatment of fever, pain, rheumatism, menstrual disorders, and uterine hemorrhage [8, 9]. The leaves of *Ehretia anacua* (Teran & Berlandier) Ivan Murray Johnston (*E. anacua*) have been traditionally used for the treatment of wounds, edema, skin rash, itching, fungal infection, menstrual pain, hemorrhage, contraception, and weight loss, while *E. anacua* roots have been used for the treatment of gastrointestinal pain and diarrhea [10, 11]. The use of medicinal plants has been a traditional practice for the treatment of diabetes, and *E. anacua* is one such plant that has been used in Nigerian folk medicine for its antidiabetic properties [12]. Recently, biological studies on different Ehretia species confirmed their traditional and folk uses for antimicrobial, anti-inflammatory, antioxidant, antidiabetic, anticancer, antimalarial, hepatoprotective, and wound healing activities [13]. Although the Ehretia genus has undergone important phytochemical and biological studies, the phytochemical study of *E. anacua* species has not been explored and only few biological studies including antimicrobial and antidiabetic activities were reported [12, 14].

Consequently, the current study aimed to investigate the phytochemical profile of *E. anacua* and evaluate its antioxidant activity, acute toxicity, and therapeutic potential against streptozotocin-induced diabetes in adult male Wistar albino rat model. Through systematic phytochemical analysis and pharmacological evaluation, we seek to provide scientific validation for the traditional use of *E. anacua* in diabetes management and improvement of related biochemical parameters. Such

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insights may contribute to the development of novel therapeutic strategies to combat diabetes and its associated complications, thus offering new avenues for the discovery of effective antidiabetic agents from natural sources.

2. Experimental:

2.1. Plant material:

E. anacua leaves were collected in October 2020 from the Aswan Botanical Garden, Aswan, Egypt (24°05′37″N–32°53′13″E). The plant was kindly identified by Dr. Hafeez Rafael, Director of the Aswan Botanical Garden Herbarium. The herbarium has been deposited in the Pharmacognosy Department, Al-Azhar University, Assiut (voucher specimen E.A. 1 2020), and authentic reference material is available at the Aswan Botanical Garden Herbarium. The materials were shade-dried and powdered to a yield of 5 kg.

2.2. Chemicals, reagents, and equipment's

Organic solvents *n*-hexane, methylene chloride, ethyl acetate, acetone, *n*-butanol, and methanol were purchased from El-Nasr Pharmaceutical and Chemical Co., Egypt. Silica gel 70–230 mesh (Sigma-Aldrich Chemicals Co., Germany) and Sephadex LH-20 (Sigma-Aldrich Chemicals Co., Germany) were used for the column chromatography. Pre-coated silica gel plates GF_{254} (E. Merck, Darmstadt, Germany) was used for TLC analysis. $DPPH^{\cdot}$, ascorbic acid, quercetin and streptozotocin (STZ) were purchased from Sigma-Aldrich Chemicals Co., Germany.

NMR spectra were recorded at 600 MHz (14.1 T) using a Bruker Avance Neo console equipped with a TCI 5-mm cryoprobe for ^{1}H and 150 MHz for ^{13}C or Bruker Ascend Aeon 400 MHz for ^{1}H and 100 MHz for ^{13}C, Germany. HPLC-ESI-MS was conducted on an Agilent 6120 Quadrupole MSD mass spectrometer (Agilent Technologies, USA) equipped with an Agilent 1200 Series quaternary LC system and Eclipse XDB-CD18 column (5 µm, 150 × 4.6 mm). Total phenolic and flavonoids contents were assayed using microplate reader (FluoStar Omega, BMG, LABTECH, Germany).

2.3. Experimental animals:

Adult male Wistar albino rats were purchased from the animal house, Faculty of Pharmacy, Al- Azhar University, Cairo, Egypt. The animals were kept under standard laboratory conditions fed and water ad-libitum throughout the experimental period. Rats were maintained in a friendly environment of 12-h/12-h light–dark cycle at room temperature (22–25 ℃). The animals weighed about 180 ± 20 gm just before the experiment. The procedures for this animal studies were approved by Ethical approval number: AZ-AS/PH-REC/19/2024.

2.4. Extraction and fractionation:

The air-dried powdered (5 kg) of *E. anacua* leaves were extracted by maceration in MeOH–H_2O (80:20, *v/v*) at room temperature till exhaustion. The total extract was concentrated using a rotary evaporator (Heidolph WB 4000; Germany). at 45 ℃ under reduced pressure, until a constant weight (500 g) was reached. Dry hydromethanolic extract was digested with distilled H_2O (500 mL). The suspension was transferred to a separating funnel and successively partitioned between the aqueous layer and *n*-hexane (500 mL × 3), methylene chloride (500 mL × 3), ethyl acetate (500 mL × 3) and *n*-butanol (500 mL × 3) until exhaustion. The obtained fractions were dried under reduced pressure in a rotary evaporator to give *n*-hexane (55 g), methylene chloride (19.60 g), ethyl acetate (14.22 g), *n*-butanol (60 g), and aqueous (332 g) fractions.

2.5. Phytochemical studies:

2.5.1. Determination of total phenolic content:

The total phenolic content of *the E. anacua* methanolic extract was determined using the Folin–Ciocalteu reagent [15]. Different concentrations of gallic acid (25, 50, 100, 200, 400, 600, 800, and 1000 µg/mL) were prepared in methanol for the calibration curve. Briefly, 10 µL of *E. anacua* methanol extract/standard mixed with 100 µL of Folin-Ciocalteu reagent (1: 10 distilled water) in a 96-well microplate. Then, 80 µL of 1M Na_2CO_3 was added and incubated at room temperature (25 ℃) for 20 min in the dark. At the end of incubation time the resulting blue complex colour was measured at 630 nm using a microplate reader (FluoStar Omega). The results of the extract were presented as µg gallic acid equivalent to mg of extract using the following equation based on the standardization curve: $y=0.0027\times-0.0421$, $R^2=0.9994$.

2.5.2. Determination of total flavonoid content

The total flavonoids content of *E. anacua* were estimated from the total methanolic extract using the aluminum chloride method [16]. Stock solutions of rutin standard different concentrations were prepared at concentrations of 5, 10, 20, 40, 80, 100, 120, 160, and 200 µg/mL for standard curve preparation. Briefly, 15 µL of *E. anacua* methanol extract/standard was placed in a 96-well microplate, then, 175 µL of methanol was added followed by 30 µL of 1.25 % $AlCl_3$. Finally, 30 µL of 0.125 M sodium acetate was added and incubated for 5 min. At the end of incubation time the resulting yellow colour was measured at 420 nm and recorded using a microplate reader (FluoStar Omega). The total flavonoid content of the extract was presented as µg rutin equivalent to mg of extract using the following equation due to the standardization curve: $y=0.0009\times+0.0182$, $R^2=0.9994$.

2.5.3 High performance liquid chromatography analysis of flavonoid and phenolic content

The phenolic and flavonoid constituents of *the E. anacua* methanol extract was analyzed by HPLC and identified by matching their retention times and UV spectra with an external standard [17, 18]. All standards namely gallic acid, chlorogenic acid, catechin**,** methyl gallate, caffeic acid, syringic acid, pyro catechol, rutin, ellagic acid, coumaric acid, vanillin, ferulic acid, naringenin, daidzein, quercetin, cinnamic acid, apigenin, kaempferol and hesperetin were purchased from Sigma-Aldrich. HPLC analysis was performed using an Agilent 1260 series equipped with an Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B), at a flow rate of 0.9 mL per minute. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A);

5-12 minute (60% A); 12-20 minute (82% A). A multiwavelength detector was used to monitor the absorbance at 280 nm. The injection volume was 5 µL for each sample. The column temperature was maintained at 40 ℃.

2.5.4. Isolation and purification of major phytochemicals

A portion of the *n*-hexane-soluble fraction (35 g) was chromatographed on a silica gel column (1000 g), and elution was started with *n*-hexane 100% followed by *n*-hexane-acetone gradient; 100 mL eluates were collected and monitored by TLC. Similar fractions were grouped together and concentrated under reduced pressure to yield seven fractions, labeled EaH-I - EaH-VII. Fraction EaH-II (13.122 g) was chromatographed on silica gel C.C. (400 g) and eluted with *n*-hexane–acetone (98:2, *v/v*). The eluted fractions were collected according to TLC monitoring to give ten sub-fractions EaH-II-1 - EaH-II-10. The sub-fraction EaH-II-3 (5.040 g) was re-chromatographed on silica gel C.C. (150 g) and isocratically eluted with *n*-hexane–ethyl acetate (99:1, v/v) to afford seven subfractions (A-G). The sub-fraction EaH-II-3-B (1.605 g) was re-chromatographed on silica gel C.C. (50 g) and eluted with *n*-hexane–acetone (99.5:0.5, *v/v*) isocratically to afford 13 sub-fractions (1-13).The sub-fraction EaH-II-3-B-3 (283 mg) was re-chromatographed on silica gel C.C. and eluted with *n*-hexane 100% several times to afford two pure compounds, **1** (12 mg) and **2** (8 mg). The sub-fraction EaH-II-3-E (763 mg) was re-chromatographed on silica gel C.C. (25 g) and eluted with *n*-hexane–ethyl acetate (99.5:0.5, *v/v*) isocratically to afford two pure compounds **3** (6 mg) and **4**(5 mg). Sub-fraction EaH-II-6 (1.543 g) was recrystallized in methanol to afford pure crystals of compound **5** (380 mg). Fraction EaH-V (4.036 g) was chromatographed on silica gel C.C. (130 g) and eluted with *n*-hexane–acetone (70:30, *v/v*). The eluted fractions were collected according to TLC monitoring to give ten sub-fractions EaH-V-1 - EaH-V-10. The sub-fraction EaH-V-7 (989 mg) was re-chromatographed on silica gel C.C. (30 g) and isocratically eluted with *n*-hexane: acetone (80:20, v/v) to afford compound **6** (107 mg), which was washed with methylene chloride and methanol, respectively.

Apart from ethyl acetate soluble fraction (9 g) was chromatographed on a silica gel column (300 g), started with CH_2Cl_2 100% followed by a CH_2Cl_2−MeOH gradient; 100 mL of each eluate was collected and monitored by TLC. Similar fractions were grouped together and concentrated under reduced pressure to obtain 12 fractions, labeled EaE-I - EaE-XII. Fraction EaE-VII (595 mg) was eluted with CH_2Cl_2–MeOH (90:10, *v/v*) chromatographed on silica gel C.C. (18 g) and eluted with CH_2Cl_2–MeOH (95:5, *v/v*) isocratic to afford five sub-fractions EaE-VII-1 - EaE-VII-5. Sub-fraction EaE-VII-2 (104 mg) was subjected several times to Sephadex LH-20 [CH_2Cl_2–MeOH, 50:50 *v/v*] to obtain compound **7** (6 mg). The sub-fraction EaE-VII-4 (240 mg) was re-chromatographed on silica gel C.C. (10 g) and eluted with CH_2Cl_2–MeOH (97:3, *v/v*) isocratic to afford compound **8** (15 mg). The fraction EaE-X (2.564 g) eluted with CH_2Cl_2–MeOH (80:20, *v/v*) chromatographed on silica gel C.C. (80 g) and eluted with CH_2Cl_2–MeOH (95:5, *v/v*) isocratically to afford sixteen sub-fractions EaE-X-1 - EaE-X-16. The sub-fraction EaE-X-8 (130 mg) was chromatographed using Sephadex LH-20[CH_2Cl_2–MeOH, 50:50 *v/v*] and Sephadex LH-20 MeOH (100%) to afford compound **9** (20 mg). Fraction EaE-X-11 (210 mg) was re-chromatographed on Sephadex LH-20 [CH_2Cl_2–MeOH, 50:50 *v/v*], affording three sub-fractions, Fr-1, Fr-2, and Fr-3. Subfraction Fr-2 (117 mg) was subjected to Sephadex LH-20 MeOH (100%)to obtain pure compound **10** (23 mg). Sub-fraction Fr-3 (78 mg) was re-chromatographed on silica gel C.C.(4 g) and eluted with CH_2Cl_2–MeOH (95:5, *v/v*)isocratically to afford compound **11**(4 mg).

2.5.5: Spectroscopic data of isolated compounds

Compound (**1**): yellow oily residue, R*f*: 0.74 using system *n*-Hexane–Acetone (97:3, *v/v*). EIMS $[M]^+$ ion peak at *m/z* = 534 corresponds to molecular formula [$C_{36}H_{70}O_2$], base peak at *m/z* = 57 and diagnostic peaks at *m/z* = 296, 278, 123, 95 and 71. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.33 (1H, t, *J*= 7.2 Hz, H-2), 4.58 (2H, d, *J*= 7.2 Hz, H-1), 2.29 (2H, t, *J* = 7.5 Hz, H-2′), 2.00 (2H, m, H-4), 1.69 (3H, s, H-20), 1.61 (2H, m, H-3′), 1.04-1.40 (43 H, m, H 4′-15′ and H-5-15), 0.88 (3H, t, *J*= 7.2 Hz, H-16′), 0.86 (6H, d, *J*= 6.6 Hz, H-16, 17), 0.85 (3H, d, *J*= 6.6 Hz, H-18), 0.84 (3H, d, *J*= 6.6 Hz, H-19). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 173.97 (C-1′), 142.63 (C-3), 118.17 (C-2), 61.21 (C-1), 39.86 (C-4), 39.38 (C-14), 37.44 (C-10), 37.37 (C-8), 37.30 (C-12), 36.64 (C-6), 34.41 (C-2′), 32.80 (C-11), 32.68 (C-7), 31.94 (C-14′), 29.70-29.17 (C-4′-13′), 27.98 (C-15), 25.07 (C-3′), 25.04 (C-5), 24.81 (C-13), 24.47 (C-9), 22.72 (C-17), 22.70 (C-15′), 22.63 (C-16), 19.75 (C-18), 19.71 (C-19), 16.37 (C-20), 14.13 (C-16′).

Compound (**2**): white amorphous powder, R*f*: 0.68 using system *n*-hexane–acetone (97:3, *v/v*). ESIMS $[M+K]^+$ ion at *m/z* = 803.67 represent of molecular formula [$C_{53}H_{96}O_2$] also peak ions at *m/z* = 414.30 and 391.35 represent steroidal nucleus and $[C_{24}H_{48}O+K]^+$ respectively. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.37 (1H, d, *J*=4.2 Hz, H-6), 4.61 (1H, m, H-3), 2.30 (2H, m, H-4), 2.26 (2H, t, *J*= 7.2 Hz, H-2′), 2.00 (1H, m, H-12), 1.85 (3H, m, H-1, 2), 1.66 (1H, m, H-3′), 1.31 – 1.21 (42H, m, H-3′-23′, 7, 12), 1.16 (1H, m, H-1), 1.11 (1H, m, H-17), 1.06 (1H, m, H-14), 1.02 (3H, s, H-19), 0.95 (1H, m, H-24), 0.92 (3H, d, *J*= 6 Hz, H-21), 0.88 (3H, t, *J*= 6.6 Hz, H-24′), 0.85 (3H, d, *J*= 8.4 Hz, H-29), 0.83 (3H, d, *J*= 7.2 Hz, H-26), 0.81 (3H, d, *J*= 6.6 Hz, H-27), 0.68 (3H, s, H-18). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 173.52 (C-1′), 139.88 (C-5), 122.73 (C-6), 73.83 (C-3), 56.83 (C-14), 56.18 (C-17), 50.17 (C-9), 45.98 (C-24), 42.46 (C-13), 39.87 (C-12), 38.31 (C-4), 37.15 (C-1), 36.75 (C-10), 36.30 (C-20), 34.88 (C-2′), 34.08 (C-22), 32.08 (C-22′), 32.04 (C-7), 32.01 (C-8), 29.86 – 29.29 (C-4′-21′), 29.26 (C-25), 28.40 (C-16), 27.96 (C-2), 26.21 (C-23), 25.22 (C-15), 24.44 (C-3′), 23.21 (C-28), 22.85 (C-23′), 21.17 (C-11), 19.97 (C-26), 19.47 (C-19), 19.17 (C-27), 18.92 (C-21), 14.27 (C-24′), 12.13 (C-29), 12.00 (C-18).

Compound (**3**): white crystals, MP 38-40 ℃, R*f*: 0.53 using system *n*-Hexane–Acetone (80:20, *v/v*). EIMS $[M]^+$ ion peak at *m/z* = 256 corresponding to molecular formula [$C_{16}H_{32}O_2$] and $[M\text{-}COOH]^+$ at *m/z* = 213. LCMS $[M+H]^+$ ion peak at *m/z* = 257. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 11.93 (1H, s, COO<u>H</u>), 2.17 (2H, t, *J* = 7.2 Hz, H-2), 1.46 (2H, m, H-3), 1.26 (2H, m, H-14), 1.23 (20H, m, H-4-13), 1.20 (2H, m, H-15), 0.85 (3H t, *J* = 7.2 Hz, H-16). ^{13}C NMR (DMSO-d_6, 150 MHz): δ 174.98 (C-1), 34.14 (C-2), 31.75 (C-14), 29.01-29.50 (C-4-13), 24.96 (C-3), 22.56 (C-15), 14.43 (C-16).

Compound (**4**): colourless oily droplets, R*f*: 0.45 using system *n*-Hexane–Acetone (85:15, *v/v*).

^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.92 (1H, dd, *J*= 17.3, 10.8 Hz, H-2), 5.20 (1H, d, *J*= 17.3 Hz, H-1a), 5.04 (1H, d, *J*= 10.8 Hz, H-1b), 1.28 (3H, s, H-20), 0.86 (6H, d, *J*= 6.5 Hz, H-16, 17), 0.85 (3H, d, *J*= 1.8 Hz, H-19), 0.84 (3H,d, *J*= 3 Hz, H-18). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 145.43 (C-2), 111.63 (C-1), 73.50 (C-3), 42.84 (C-4), 39.51 (C-14), 37.56 (C-10, 12), 37.54 (C-8), 37.43 (C-6), 32.94 (C-7), 32.90 (C-11), 28.12 (C-15), 27.86 (C-20), 24.94 (C-13), 24.62 (C-9), 22.87 (C-16), 22.77 (C-17), 21.50 (C-5), 19.90 (C-18), 19.84 (C-19).

Compound (**5**): white needles, MP 134-136 ℃, R*f*: 0.55 using system *n*-Hexane–Acetone (85:15, *v/v*). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.34 (1H, d, *J* = 5.2 Hz, H-6), 3.51 (1H, m, H-3), 1.00 (3H, s, H-19), 0.92 (3H, d, *J* = 6.5 Hz, H-21), 0.85 (3H, t, *J*= 7 Hz, H-29), 0.83 (3H, d, *J* = 7.1 Hz, H-27), 0.81 (3H, d, *J* = 6.8 Hz, H-26), 0.67 (3H, s, H-18). ^{13}C NMR ($CDCl_3$, 100 MHZ): δ 140.89 (C-5), 121.84 (C-6), 71.92 (C-3), 56.90 (C-14), 56.20 (C-17), 50.27 (C-9), 45.96 (C-24), 42.46 (C-13), 42.42 (C-4), 39.91 (C-12), 37.39 (C-1), 36.63 (C-10), 36.28 (C-20), 34.08 (C-22), 32.04 (C-2), 32.04 (C-8), 31.78 (C-7), 29.29 (C-25), 28.38 (C-16), 26.22 (C-23), 24.44 (C-15), 23.20 (C-28), 21.22 (C-11), 19.95 (C-26), 19.53 (C-19), 19.18 (C-21), 18.92 (C-27), 12.12 (C-29), 11.99 (C-18).

Compound (**6**): white granular powder, MP 276-278 ℃, R*f*: 0.51 using system Methylene chloride–Methanol (90:10, *v/v*). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 5.31 (1H, d, *J*= 4.8 Hz, H-6), 3.46 (1H, m, H-3), 2.36 (1H, m, H-4), 2.12 (1H, m, H-4), 1.93 (3H, m, H-12, 7), 1.79 (3H, m, H-28, 16, 1), 1.62 (1H, m, H-25), 1.49 (6H, m, H-28, 16, 15, 11, 2), 1.38 (2H, m, H-11, 8), 1.32 (1H, m, H-20), 1.30 (1H, m, H-22), 1.16 (3H, m, H-23, 12), 1.10 (1H, m, H-14), 1.06 (1H, m, H-15), 1.01 (1H, m, H-1), 1 (1H, m, H-22), 0.98 (1H, m, H-17), 0.94 (3H, s, H-19), 0.89 (1H, m, H-24), 0.89 (3H, d, *J*= 6.4 Hz, H-21), 0.88 (1H, m, H-9), 0.83 (3H, d, *J*= 6.8 Hz, H-29), 0.81 (3H, d, *J*= 6.8 Hz, H-26), 0.78 (3H, d, *J*= 7.2 Hz, H-27), 0.64 (3H, s, H-18); glucose: 4.21 (1H, d, *J*= 8 Hz, H-1′), 3.40 (1H, m, H-6′), 3.11 (1H, m, H-3′), 3.06 (1H, m, H-5′), 3.02 (1H, m, H-4′), 2.89 (1H, m, H-2′). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 140.46 (C-5), 121.20 (C-6), 100.83 (C-1′), 76.97 (C-3), 76.74 (C-3′, 5′), 73.47 (C-2′), 70.08 (C-4′), 61.10 (C-6′), 56.20 (C-17), 55.46 (C-14), 49.63 (C-9), 45.17 (C-24), 41.86 (C-12, 13), 38.33 (C-4), 36.86 (C-1), 36.22 (C-10), 35.51 (C-20), 33.37 (C-22), 31.44 (C-7), 31.40 (C-8), 29.28 (C-28), 28.72 (C-2), 27.81 (C-25), 25.46 (C-16), 23.88 (C-23), 22.63 (C-15), 20.61 (C-11), 19.72 (C-29), 19.10 (C-19), 18.94 (C-27), 18.62 (C-21), 11.79 (C-18),11.68 (C-26).

Compound (**7**): pale-yellow amorphous powder, R*f*: 0.74 using system Methylene chloride–Methanol (90:10, *v/v*). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.56 (1H, d, *J*= 15.8 Hz, H-7), 7.05 (1H, d, *J*= 2.1 Hz, H-2), 6.96 (1H, dd, *J*= 8.2, 2.1 Hz, H-6), 6.79 (1H, d, *J*=8.2 Hz, H-5), 6.72 (1H, d, *J*=2 Hz, H-2′), 6.69 (1H, d, *J*= 8 Hz, H-5′), 6.58 (1H, dd, *J*=8, 2 Hz, H-6′), 6.27 (1H, d, *J*= 15.8 Hz, H-8), 5.20 (1H, dd, *J*= 7.5, 5.3 Hz, H-8′), 3.71 (3H, s, H-1″), 2.96-3.11 (2H, m, H-7′). ^{13}C NMR (CD_3OD, 100 MHz): δ 172.18 (C-9′), 168.33 (C-9), 149.84 (C-4), 147.96 (C-7), 146.84 (C-3), 146.22 (C-3′), 145.39 (C-4′), 128.75 (C-1′), 127.58 (C-1), 123.21 (C-6), 121.78 (C-6′), 117.53 (C-2′), 116.51 (C-5′), 116.31 (C-5), 115.23 (C-2), 114.14 (C-8), 74.67 (C-8′), 52.67 (C-1″), 37.90 (C-7′).

Compound (**8**): pale-yellow amorphous powder, R*f*: 0.54 using system Methylene chloride–Methanol (85:15, *v/v*). ^{1}H NMR (DMSO-d_6, 600 MHz): δ 7.41 (1H, d, *J*=15.86 Hz, H-7), 7.03 (1H, d, *J*=1.8Hz, H-2), 6.97 (1H, dd, *J*= 8.11, 1.8 Hz, H-6), 6.76 (1H, d, *J*=8.11 Hz, H-5), 6.17 (1H, d, *J*=15.86 Hz, H-8). ^{13}C NMR (DMSO-d_6, 150 MHz): δ 167.87 (C-9), 148.04 (C-4), 145.47 (C-3), 144.60 (C-7), 125.71 (C-1), 121.17 (C-6), 115.72 (C-8), 115.10 (C-5), 114.62 (C-2).

Compound (**9**): pale-yellow amorphous powder, R*f*: 0.51 using system Methylene chloride– Methanol (85:15, *v/v*). ESIMS $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions at *m/z* = 371.27, 393.26 and 409.21 respectively which determine a molecular formula [$C_{19}H_{30}O_7$]. ^{1}H NMR (CD_3OD, 400 MHz): δ 5.88 (1H, s, H-4), 5.78 (1H, dd, *J* = 15.6, 6.4 Hz, H-8), 5.64 (1H, dd, *J* = 15.6, 9.2 Hz, H-7), 4.40 (1H, m, H-9), 2.68 (1H, d, *J* = 9.2 Hz, H-6), 2.43 (1H, d, *J* = 16.8 Hz, H-2a), 2.05 (1H, d, *J* = 16.8 Hz, H-2b), 1.94 (3H, s, H-13), 1.29 (3H, d, *J* = 6.4 Hz, H-10), 1.03 (3H, s, H-11), 1.01 (3H, s, H-12); glucose: 4.36 (1H, d, *J* = 7.6 Hz, H-1′), 3.82 (1H, dd, *J* = 12, 2.4 Hz, H-6′), 3.66 (1H, dd, *J* = 12, 5.6 Hz, H-6′), 3.33 (1H, m, H-3′), 3.31 (1H, m, H-4′), 3.22 (1H, m, H-5′), 3.18 (1H, dd, *J* =8.8, 8 Hz, H-2′). ^{13}C NMR (CD_3OD, 100 MHz): δ 202.2 (C-3), 166.03 (C-5), 138.42 (C-8), 129.02 (C-7), 126.35 (C-4), 102.65 (C-1′), 78.29 (C-3′), 78.14 (C-5′), 77.18 (C-9), 75.45 (C-2′), 71.72 (C-4′), 62.90 (C-6′), 56.96 (C-6), 48.52 (C-2), 37.31 (C-1), 28.27 (C-12), 27.76 (C-11), 23.97 (C-13), 21.26 (C-10).

Compound (**10**): pale-yellow amorphous powder, R*f*: 0.46 using system Methylene chloride–Methanol (85:15, *v/v*). ESIMS $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions at *m/z* = 387.26, 409.24 and 425.24 respectively which determine a molecular formula [$C_{19}H_{30}O_8$]. ^{1}H NMR (CD_3OD, 400 MHz): δ 5.85 (3H, m, H-8, 7, 4), 4.41 (1H, m, H-9), 2.51 (1H, d, *J*= 16.4 Hz, H-2), 2.14 (1H, d, *J*= 16.4 Hz, H-2), 1.91 (3H, d, *J*= 1.6 Hz, H-13), 1.28 (3H, d, *J*= 6.4 Hz, H-10), 1.02 (6H, s, H-12, 11); glucose: 4.33 (1H, d, *J*= 7.6 Hz, H-1′), 3.84 (1H, dd, *J*= 11.6, 2 Hz, H-6′), 3.61 (1H, dd, *J*= 11.6, 5.6 Hz, H-6′), 3.35 – 3.23 (3H, m, H-3′ – 5′), 3.16 (1H, dd, *J*= 9.2, 7.6 Hz, H-2′). ^{13}C NMR (CD_3OD, 100 MHz): δ 201.19 (C-3), 167.21 (C-5), 135.27 (C-8), 131.54 (C-7), 127.17 (C-4), 102.73 (C-1′), 79.99 (C-6), 78.09 (C-5′), 77.99 (C-3′), 77.27 (C-9), 75.23 (C-2′), 71.65 (C-4′), 62.83 (C-6′), 50.96 (C-2), 42.40 (C-1), 24.68 (C-12), 23.43 (C-11), 21.19 (C-10), 19.54 (C-13).

Compound (**11**): pale-yellow amorphous powder, R*f*: 0.35 using system Methylene chloride–Methanol (70:30, *v/v*). ^{1}H NMR (CD_3OD, 600 MHz): δ 7.50 (1H, d, *J*= 15.9 Hz, H-7), 7.01 (1H, d, *J*=1.2 Hz, H-2), 6.91 (1H, dd, *J*= 7.8, 1.2 Hz, H-6), 6.76 (2H, d, *J*= 7.8 Hz, H-5, 2′), 6.67 (1H, d, *J*= 7.8 Hz, H-5′), 6.61 (1H, dd, *J*= 7.8, 1.2 Hz, H-6′), 6.25 (1H, d, *J*= 15.9 Hz, H-8), 5.10 (1H, dd, *J*= 8.9, 3 Hz, H-8′), 3.09 (1H, dd, *J*= 14.2, 3 Hz, H-7′), 2.95 (1H, dd, *J*= 14.2, 9.3 Hz, H-7′). ^{13}C NMR (CD_3OD, 150 MHz): δ 176.84 (C-9′), 168.92 (C-9), 149.52 (C-4), 147.09 (C-7), 146.74 (C-3), 146.01 (C-3′), 144.95 (C-4′), 130.44 (C-1′), 127.77 (C-1), 123 (C-6′), 121.74 (C-6), 117.52 (C-5′), 116.46 (C-5), 116.21 (C-2′), 115.13 (C-2), 115.11 (C-8), 76.61 (C-8′), 38.40 (C-7′).

2.6. Biological studies:

2.6.1 Antioxidant radical scavenging capacity (RSC)

2.6.1.1. Preparation of extracts for antioxidant assay

Different fractions (*n*-hexane, methylene chloride, ethyl acetate, *n*-butanol, and aqueous) and total methanolic extract from *E. anacua* were dissolved in methanol to obtain different concentrations of (1.95, 3.90, 7.81, 15.62, 31.25, 62.50, and 125 µg/mL) [19]. Natural antioxidant ascorbic acid and quercetin were used as positive control by dissolving in methanol solution to obtain the same concentration of the tested samples [20, 21]. The DPPH$^{\cdot}$ stock solution was prepared in methanol as 0.1 mM DPPH$^{\cdot}$.

2.6.1.2. DPPH· radical scavenging assay:

The DPPH radical scavenging activity was determined following Blois and Olarewaju et. al. method with some modification [22, 23]. DPPH is stable free radical used for determination of antioxidant activity *in vitro* of plant extracts. The principle of using DPPH is by measuring the ability of tested materials to donate hydrogen to the free radical that causes colour quenching

from purple to yellow colour. The more active antioxidant material that being the more percentage of inhibition. Briefly, 0.2 mL of standard and extract of different concentrations (1.95 – 125 µg/mL) were added to 1.8 mL of 0.1 mM $DPPH^{\cdot}$ solution, then the mixture was vigorously shaken and allowed to stand for 30 minutes in dark at room temperature. After incubation, the solution absorbance was measured against blank (1.8 mL $DPPH^{\cdot}$ and 0.2 mL methanol only without sample which express 100% free radicals) at 517 nm spectrophotometer. The extract activities were determined on the base of reduction of $DPPH^{\cdot}$ absorbance. The change in absorbance was used for calculating the antioxidant scavenging activity percentage with respect to the blank. The analysis was done in triplicate for each fraction and standard. The percentage of inhibition (1%) was calculated on the base of radical scavenging activity as follows:

$$\text{Inhibition \%} = \frac{(\text{Abs.of control} - \text{Abs. of sample})}{\text{Abs.control}} \times 100$$

2.6.1.3. Inhibition concentration IC_{50} values:

It is defined as the amount of sample required to decrease 50% of the $DPPH^{\cdot}$ absorbance. The IC_{50} calculation was done by using (MLA- "Quest Graph™ IC_{50} Calculator." AAT Bioquest, Inc, 27 Jan, 2024, https://www.aatbio.com/tools/ic50-calculator) [24].

2.6.2. Acute toxicity study:

The acute toxicity study followed the reported protocol of Lorke's method 1983 [25]. The method was conducted in two phases using twenty male Wistar albino rats. In the first phase, twelve rats were divided into three groups (four rats per each) and given single oral dose of crude methanol extract at concentrations of 10, 100 and 1000 mg/kg of body weight single oral dose for the assessment of the acute toxicity or death produced by the extract at different doses according to the used protocol. In addition, a group of four rats was kept as a control group and did not administer any treatment. In the second phase, oral administration of a single dose of 1600, 2900 and 5000 mg/kg of body weight to three rats (one rat per dose) and one rat kept as control. The animals were observed for twenty-four hours to record any signs of acute toxicity and mortality rates.

2.6.3. Antidiabetic activity of *E. anacua* leaves on streptozotocin induced diabetic rats:

2.6.3.1. Induction of type II diabetes mellitus:

Induction of type-II diabetes mellitus was done by single intraperitoneal dose (50 mg/kg) of freshly prepared of streptozotocin (STZ) dissolved in 0.1 mol/L cold sodium citrate buffer (pH 4.5) [26]. Rats were allowed free access to food and water for four days then blood sugar level was measured from tail vein. The rats with blood glucose levels over 225 mg/dL were considered diabetic [27].

2.6.3.2. Experimental design for antidiabetic activity:

The crude methanol extract and two other fractions (methylene chloride and ethyl acetate) of *E. anacua* leaves were investigated for their anti-diabetic effect in STZ-induced diabetic rats following the reported protocol of (Mondal et al. 2012) with some modification [27]. The rats were divided into six groups (six rats per group).

- Group I: Normal control rats received water.
- Group II: Non treated diabetic rats.
- Group III, IV and V: Diabetic rats treated with methylene chloride, ethyl acetate and methanol extracts at dose 200 mg/kg (P.O.) respectively dissolved in 1mL of 0.04% DMSO in water.
- Group VI: Diabetic rats treated with glibenclamide (SANOFI, Egypt) at dose of 0.5 mg/kg, P.O.as standard antidiabetic drug [28].

  Groups (III-VI) take the treatment once daily at 9:00 a.m. for 15 days, blood glucose levels were measured on days 0, 10 and 15 and results were reported as mg/dL. Blood glucose level determination was done by using a GlucoDr® Super Sensor test strips (All Medicus Co., Germany) based on the glucose oxidase principle [29].

2.6.3.3. Estimation of serum lipid and kidney physiological profile in STZ induced type II diabetes mellitus:

After day 15 and completion of the antidiabetic assay, the blood samples were collected by cardiac puncture for studies of biochemical parameters and the experimental animals were anesthetized by using phenobarbital sodium (35 mg/kg) and euthanized by cervical dislocation [30]. The collected blood samples were centrifugated for 15 minutes at 2000 rpm for serum separation. The lipid profile as total cholesterol and triglycerides were determined using ready-made kit produced by Spinreact S.A.U, Spain and BioMed, Egypt respectively, and assayed spectrophotometrically using the Sharma et.al. method [31]. The kidney function profile as serum urea was assayed using ready-made kit produced by Diamond Diagnostics, Egypt following the method of Thomas [32], whereas serum uric acid was estimated by the kit of Spectrum diagnosis, Egypt following the Fossati et.al method [33] and serum creatinine was measured by using a ready-made kit produced by Diamond Diagnostics, Egypt and estimated by using the Thomas method [34].

2.7. Statistical analysis

All the values of blood glucose and biochemical parameters were represented as means ± SD and the groups were compared by using one way ANOVA followed by Tukey's for multiple comparison test. Differences between groups were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Graphpad Prism® software was used for statistical data analysis.

3. Results:

3.1. Phytochemical study:

3.1.1. Total phenolic content:

The total phenolic content of methanol extract of *E. anacua* leaves revealed a high amount of phenolic content. The phenolic content gave absorbance reading at 1.631667 which is equivalent to 137.76 ± 4.39 µg gallic acid equivalent per mg of extract. The high phenolic content of methanol extract in agreement with previously reported literature [13].

3.1.2. Total flavonoid content:
The results revealed the presence of remarkable amount of flavonoid content. The flavonoids content gave absorbance reading at 0.113 equivalent to 1.1663 ± 0.07 µg rutin equivalent per mg of extract.

3.1.3. HPLC analysis of total phenolic and flavonoid contents:
The HPLC analysis of total phenolic and flavonoid contents of *E. anacua* leaves extract revealed the presence of various components which are identified comparing with standard compounds. The results of retention time in minutes and concentrations were listed in table 1 and the HPLC chromatogram shown in figure 1.

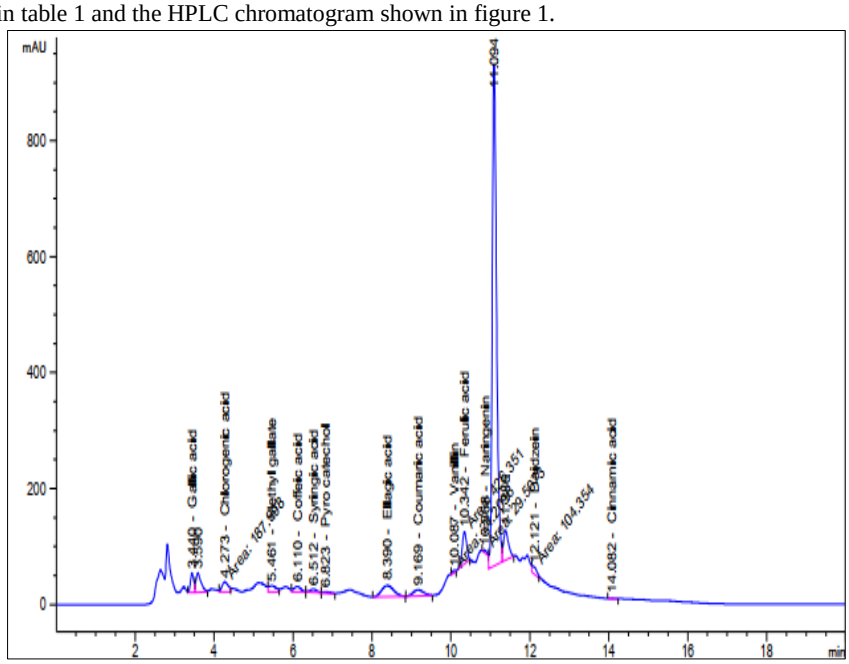

Figure 1. HPLC chromatogram of identified phenolic and flavonoid components of *E. anacua* leaves extract.

The analysis findings reveal that the plant extract contains a variety of polyphenolic compounds and flavonoids. These include ellagic acid (10512.86 µg/gm), ferulic acid (1167.48 µg/gm), chlorogenic acid (1039.79 µg/gm), gallic acid (721.61 µg/gm), caffeic acid (357.68 µg/gm), methyl gallate (351.08 µg/gm), coumaric acid (250.12 µg/gm), pyrocatechol (227.96 µg/gm), syringic acid (190.13 µg/gm), vanillin (42.37 µg/gm), and cinnamic acid (6.11 µg/gm), alongside flavonoid contents like daidzein (300.66 µg/gm) and naringenin (141.66 µg/gm). These compounds contribute to the antioxidant properties of the extract and potentially offer health benefits. Their concentrations were determined through HPLC analysis.

Table 1. Results of phenolic and flavonoid contents assigned in *E. anacua* leaves extract analyzed by HPLC**.**

No.	Compounds	Retention time (min)	Conc.(µg/g)
1	Gallic acid	3.44	721.61
2	Chlorogenic acid	4.27	1039.79
3	Methyl gallate	5.46	351.08
4	Caffeic acid	6.11	357.68
5	Syringic acid	6.51	190.13
6	Pyro catechol	6.82	227.96
7	Ellagic acid	8.39	10512.86
8	Coumaric acid	9.16	250.12
9	Vanillin	10.08	42.37
10	Ferulic acid	10.342	1167.48
11	Naringenin	10.868	141.66
12	Daidzein	12.121	300.66
13	Cinnamic acid	14.082	6.11

3.1.4. Identification of isolated compounds:
The *n*-hexane and ethyl acetate fractions were subjected to several chromatographic techniques which led to isolation and identification of six compounds (**1-6**) from *n*-hexane fraction in addition to five compounds (**7-11**) from ethyl acetate fraction. The identification of isolated compounds was done by comparing their spectroscopic data (1D NMR, 2D NMR, LCMS, ESIMS and EIMS) with previously published data. Accordingly, the compounds isolated from *n*-hexane fraction were identified as Phytyl palmitate (**1**) [35, 36], *β*-Sitosterol tetracosanoate (**2**) [37-39], Palmitic acid (**3**) [40, 41], Isophytol (**4**) [42, 43], *β*-Sitosterol (**5**) [44, 45], *β*-Sitosterol-3-*O*-*β*-D-glucopyranoside (**6**) [44, 46]. Additionally, the ethyl acetate fraction

isolated compounds were identified as Methyl rosmarinate (**7**) [47], Caffeic Acid (**8**) [48, 49], Dearabinosyl pneumonanthoside ((6*R*,7*E*,9*R*)-9-Hydroxy-megastigma-4,7-dien-3-one 9-*O*-D-glucopyranoside) (**9**) [50-52], Roseoside (blumenyl A *β*-D-glucopyranoside) (**10**) [51, 53] and Rosmarinic acid (**11**) [54, 55]. Compounds **1**, **2**, **4** and **9** were first isolated from Boraginaceae family while compounds **3** and **10** were first isolated from genus Ehretia. Compounds **5**. **6**, **7**, **8** and **11** are first isolated from *E. anacua* species. The chemical structure of the isolated compounds was shown in figure 2.

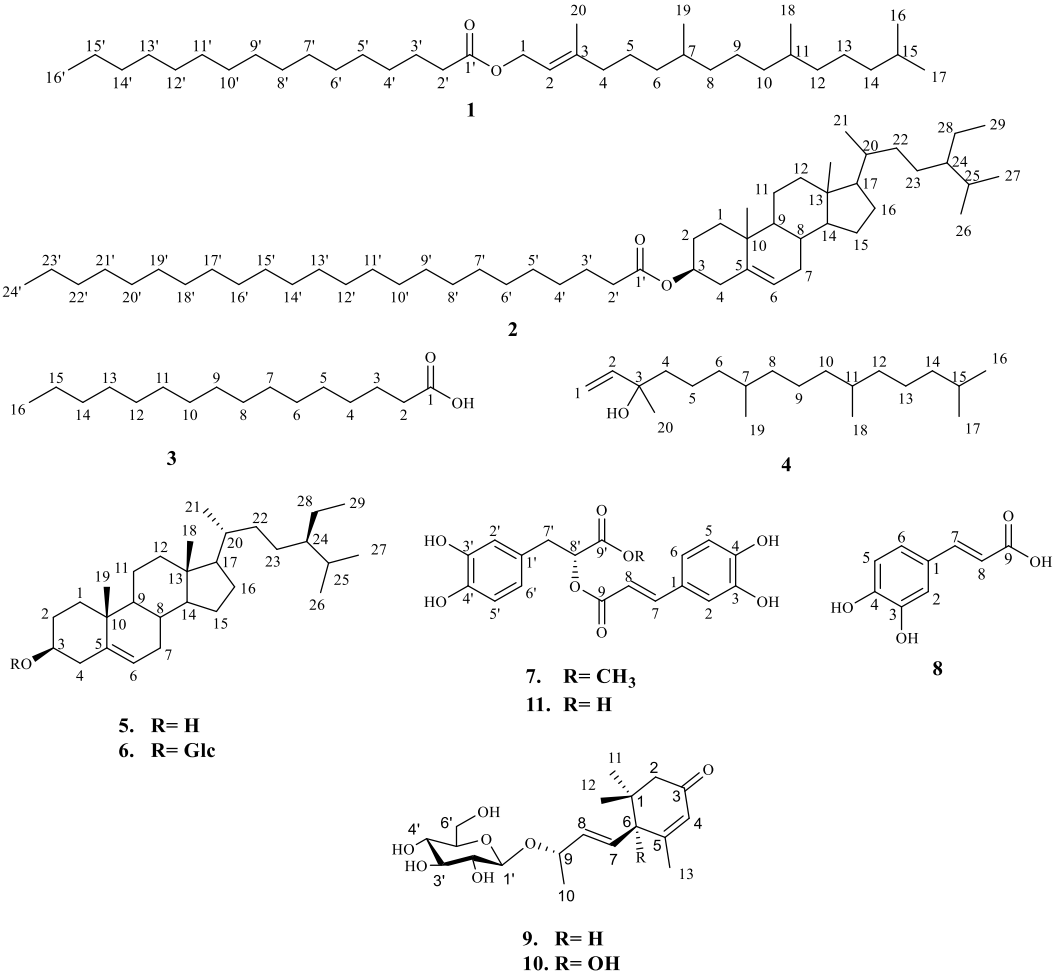

Figure 2. Chemical structures of isolated compounds from *E. anacua* leaves extract.

3.2. Pharmacological study:

3.2.1. Antioxidant assay using DPPH radical scavenging capacity (RSC):

The crude methanol extract of *E. anacua* leaves in addition to different fractions (*n*-hexane, methylene chloride, ethyl acetate and *n*-butanol) were investigated at different concentrations for their antioxidant activity along with standard antioxidant ascorbic acid and quercetin. The percentage of DPPH scavenging activity is used to determine the degree of each fraction activity. The antioxidant activity of different fractions of *E. anacua* leaves extract were listed in table 2 and illustrated in figure 3.

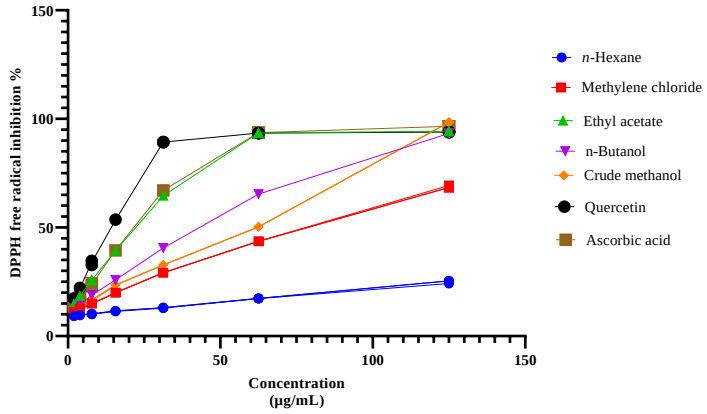

Figure 3. Antioxidant activity of different fractions of *E. anacua* leaves extract.

Table 2. DPPH radical scavenging activity (%) of different fractions of *E. anacua* leaves extract.

Conc. (µg/mL)	1.95	3.90	7.81	15.62	31.25	62.50	125.00	IC_{50} (µg/mL)
***n*-Hexane**	9.16± 0.00	9.63±0.08	10.16±0.12	11.49±0.09	13.00±0.09	17.33±0.04	25.03±0.71	289.50±20.97
DCM	10.83 ± 0.21	12.53±0.24	14.92±0.05	19.96±0.04	29.14±0.00	43.60±0.04	68.74±0.53	531.90±18.85
Ethyl acetate	14.54 ± 0.33	18.42±0.17	25.72±0.16	39.30±0.04	64.60±0.09	93.50±0.09	94.35±0.14	25.16±0.09
***n*-Butanol**	12.61±0.09	14.54±0.05	18.87±0.14	25.85±0.04	40.53±0.00	65.34±0.04	93.31±0.08	87.84±0.39
Crude MeOH	12.26±0.55	13.48±0.09	16.51±0.44	23.46±0.09	32.75±0.04	50.27±0.04	98.37±0.02	782.00±18.47
Quercetin	17.25±0.18	22.13±0.08	34.00±1.10	53.48±0.09	89.30±0.09	93.47±0.00	93.92±0.09	15.76±0.12
Ascorbic acid	14.01±0.41	17.38±0.92	23.83±0.78	39.49±0.00	67.04±0.00	93.63±0.00	96.58±0.00	24.5±0.23

Each value represents mean ± SD

Determination of IC_{50} of different fractions

The activity of the fractions in comparison with standards quercetin and ascorbic acid were indicated by the $IC_{50\%}$. The plotting of different fractions concentration against their percentage of inhibition and by using statistical analysis, that help to determine and identify the concentration that scavenge DPPH free radicals by 50% as shown in table 2 and figure 4. Comparison with standard quercetin and ascorbic acid(Vit. C)the ethyl acetate fraction showed the highest activity with IC_{50} is 25.16 ± 0.09 µg/mL followed by *n*-butanol with IC_{50} 87.84 ± 0.39 µg/mL. On the other hand, the crude methanol and methylene chloride showed the lowest activity with IC_{50} 782 ± 18.47 and 531.9 ± 18.85µg/mL respectively table 2 & figure 4.

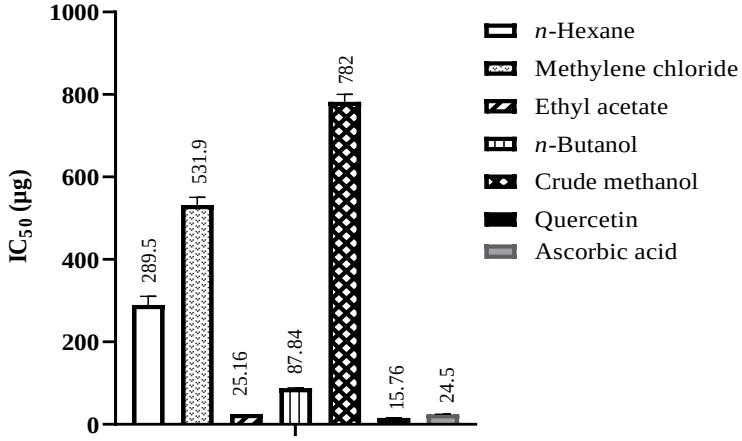

Figure 4. IC_{50} of different fractions.

3.2.2. Acute toxicity study:

The results of the acute toxicity study of *E. anacua* leaves methanol extract showed that there were no signs of toxicity observed, and no death recorded within 24 hours. Since in first phase no death was recorded, these doses of 1600, 2900 and 5000 mg/kg per body weight of *E. anacua* leaf extract administrated orally to a new animal (one animal per each dose) in the second phase. All animals were observed for 24 hours for signs of acute toxicity and death. The results showed no signs of toxicity or death at concentrations up to 5000 mg/kg of body weight. The results revealed that the LD_{50} of *E. anacua* leaves extract is greater than 5000 mg/kg of body weight orally as suggested by Lorke's (1983) method [25].

3.2.3. Anti-diabetic activity of *E. anacua* leaves extracts on STZ induced diabetic rats:

3.2.3.1. Effects of *E. anacua* leaves extracts on blood glucose level in STZ induced diabetic rats:

The change in fasting blood glucose level in normal, diabetic control and diabetic groups taking daily single oral dose of *E. anacua* crude methanol extract, methylene chloride fraction and ethyl acetate fraction in addition to glibenclamide as a standard antidiabetic drug, were presented in table 3 and shown in figure 5. The diabetic group treated with STZ exhibited significant (P<0.001) elevation in blood glucose level in comparison with the normal control group. The antihyperglycemic effect of groups treated with crude methanol extract and different fractions were compared with the diabetic control group. The results showed that oral administration of crude methanol extract daily makes high significant (P<0.001) reduction of blood glucose level at day 10 and 15 in comparison to diabetic control. The crude methanol extract treated group showed decreased blood sugar level from 412.8 ± 131.4 to 141 ± 20.95 and 138.7 ± 32.65 on days 10 and 15, respectively.

Also, ethyl acetate fraction exhibited high significant (P<0.001) reduction in blood glucose level in compared to diabetic control on days 10 and day 15. The reduction in ethyl acetate fraction from 486 ± 79.04 to 239.3 ± 67.88 and to 189.5 ± 60.82 mg/dL on days 10 and 15 respectively. In addition, group treated with the standard antidiabetic drug glibenclamide at a daily oral dose exhibited high significant (P<0.001) reduction in blood glucose level compared to diabetic control at day 10 and day 15 from 377.3 ± 53.5 to 223.7 ± 67.88 and to 97 ± 3.92 mg/dL respectively. On the other hand, the group treated with methylene chloride fraction did not express any significant (P<0.05) reduction compared with the diabetic control group.

3.2.3.2. Effects of *E. anacua* leaves extracts on lipid profile in STZ induced diabetic rats:

The effect of *E. anacua* methanol extract and the two fractions (methylene chloride and ethyl acetate) and control groups on the lipid profile, total cholesterol and triglycerides after 15 days of treatment were depicted in table 4 and shown in figure 6.

Table 3. Effect of daily oral administration of *E. anacua* leaves extracts on fasting blood glucose level.

Group (*n*= 6)	Treatment	Fasting plasma glucose concentration (mg/dL)		
		Day 0	Day 10	Day 15
I	**Normal control**	109.7 ± 12.5***	105.0 ± 7***	95.0 ± 5.66***
II	**Diabetic control**	426.8 ± 44.32	378.0 ± 119.7	331 ± 65.96
III	**Diabetic + Methylene chloride**	477.3 ± 51.5	429.3 ± 58.8	312.3 ± 55.19
IV	**Diabetic + Ethyl acetate**	486.0 ± 79.04	239.3 ± 67.88***	189.5 ± 60.82***
V	**Diabetic + Crude methanol**	412.8 ± 131.4	141.0 ± 20.95***	138.7 ± 32.65***
VI	**Diabetic + Glibenclamide**	377.3 ± 53.5	223.7 ± 67.88***	97 ± 3.92***

Reading values represent mean ± SD. *n* = number of animals in each group. The significant difference ***P <0.001 when compared at the same time with diabetic control (one way ANOVA followed by Tukey's for multiple comparison test)

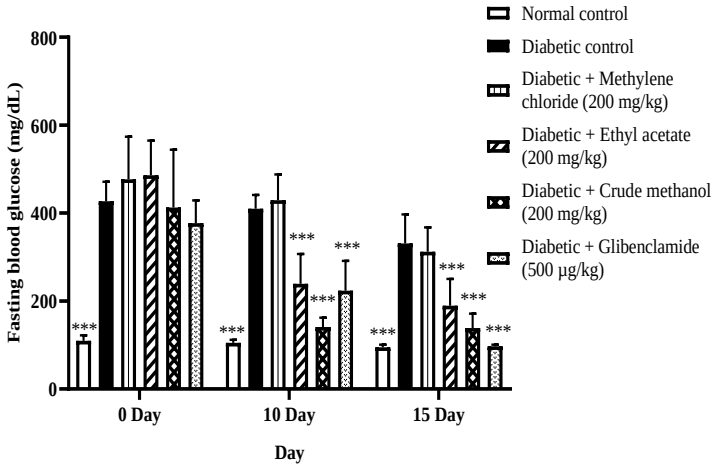

Figure 5. Effect of *E. anacua* leaves extract and different fractions (200 mg/kg) and control groups on blood glucose level in STZ induced diabetic rats, Glibenclamide (0.5 mg/kg), Control group take normal saline as vehicle. Each column represent mean ± SD. Significant difference *P <0.05, **P <0.01, ***P <0.001 when compared with diabetic control at the same time (one way ANOVA followed by Tukey's for multiple comparison test)

In the present study diabetic group treated with STZ resulted in a significant (P<0.01 and P<0.001) elevation in total cholesterol and triglycerides levels when compared to the normal control group. The daily oral administration of crude methanol extract showed significant (P<0.05 and P<0.01) reduction in serum triglycerides and total cholesterol level in comparison to diabetic control. Also, the group treated with ethyl acetate fraction showed significant (P<0.001) reduction in triglycerides and total cholesterol levels. In addition, administration of the antidiabetic drug glibenclamide daily for 15 days showed a significant (P<0.001) reduction in both triglycerides and total cholesterol. On the other hand, the methylene chloride fraction did not exhibit a significant change in lipid profile parameters, triglycerides or total cholesterol.

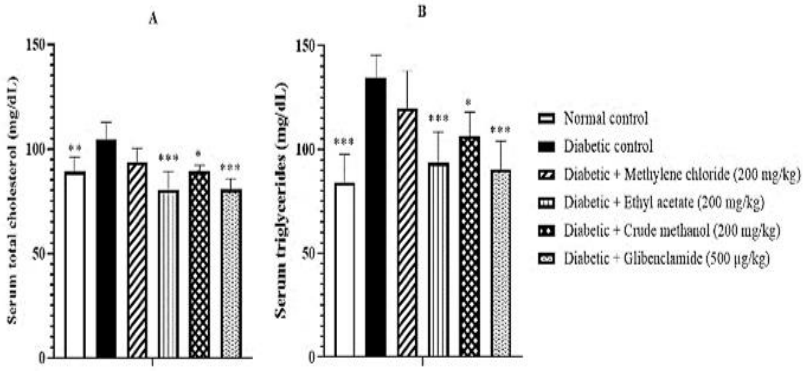

Figure 6. Effect of *E. anacua* leaves extract and different fractions (200 mg/kg) and control groups on lipid parameters total cholesterol (A), triglycerides (B) in STZ induced diabetic rats, Glibenclamide (0.5 mg/kg), Control group take normal saline as vehicle. Each column represent mean ± SD. Significant difference *P <0.05, **P <0.01, ***P <0.001 when compared with diabetic control (one way ANOVA followed by Tukey's for multiple comparison test)

3.2.3.3. Effects of *E. anacua* leaves extracts on kidney function in STZ induced diabetic rats:

The kidney function markers including serum urea, uric acid and creatinine after 15 days of daily oral administration of *E. anacua* methanol extract and the two fractions (methylene chloride and ethyl acetate) in addition to control groups were depicted in table 4 and shown in figure 7. In the present study, the serum urea, uric acid and creatinine levels exhibited significant (P<0.001) elevation in STZ-induced diabetic rats in comparison to normal control. Also, the results showed that crude methanol and ethyl acetate fraction exhibited significant (P<0.001) reduction in urea, uric acid and creatinine parameters in comparison to diabetic group. In addition, the group treated with methylene chloride fraction showed a significant (P<0.001) reduction in serum urea and uric acid and significant (P<0.05) increase in serum creatinine level. Moreover, group treated with Glibenclamide showed significant (P<0.01 and P<0.001) reduction in serum parameters creatinine, urea and uric acid levels

Table 4. Effect of *E. anacua* leaves extract and different fractions on different serum biochemical parameters.

Group n= 6	Treatment	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)	Creatinine (mg/dL)
I	Normal control	89 ± 7**	84 ± 13.45***	46 ± 2.58***	10.60 ± 2.13***	0.88 ± 0.09***
II	Diabetic control	104.3 ± 8.39	134.8 ± 10.63	78 ± 3.16	18.85 ± 3.07	1.17 ± 0.04
III	Diabetic + Methylene chloride	93.5 ± 6.95	119.8 ± 18.04	54.75 ± 3.3***	12.35 ± 2.55***	0.98 ± 0.17*
IV	Diabetic + Ethyl acetate	80.25 ± 8.99***	93.33 ± 15.04***	48.75 ± 3.3***	10.63 ± 1.11***	0.84 ± 0.13***
V	Diabetic + Crude methanol	89.5 ± 2.65**	106.5 ± 11.39*	49.0 ± 2.94***	9.23 ± 1.50***	0.81 ± 0.08***
VI	Diabetic + Glibenclamide	80.5 ± 5.26***	90.25 ± 13.7***	44.75 ± 7.5***	10.18 ± 0.51***	0.94 ± 0.06**

Reading values represent mean ± SD. *n* = number of animals in each group. Significant difference **P* <0.05, ***P* <0.01, ****P* <0.001 when compared with diabetic control (one way ANOVA followed by Tukey's for multiple comparison test)

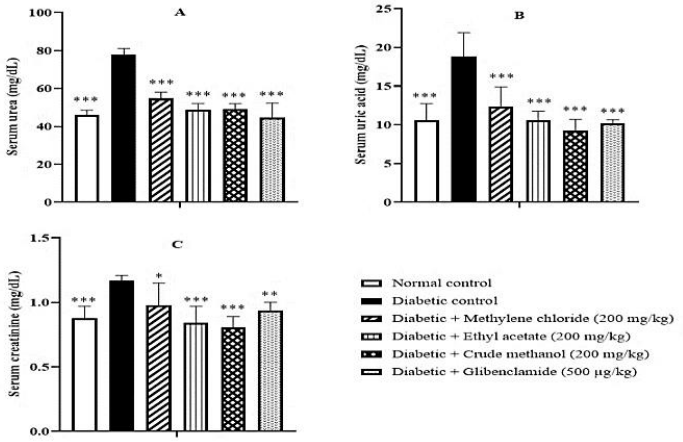

Figure 7. Effect of *E. anacua* leaves extract and different fractions (200 mg/kg) and control groups on serum kidney parameters urea (A), uric acid (B), creatinine (C) in STZ induced diabetic rats, Glibenclamide (0.5 mg/kg), Control group take normal saline as vehicle. Each column represent mean ± SD. Significant difference **P* <0.05, ***P* <0.01, ****P* <0.001 when compared with diabetic control (one way ANOVA followed by Tukey's for multiple comparison test)

4. Discussion

Diabetes is a common, chronic disease with a high prevalence of morbidity and mortality worldwide. Antioxidants have been reported to reduce the risk of diabetes, improve glucose disposal, and alleviate complications associated with diabetes. This is due to their ability to counteract increased oxidative stress [56]. Enzymatic antioxidants as glutathione peroxidase and non-enzymatic antioxidants as ascorbic acid have the ability to detoxify ROS and repair the resulting damage in the biological systems [57, 58]. The antioxidant has great importance for scavenge these reactive species that prevent the onset and progression of the diseases associated with oxidative stress [59]. Despite the availability of numerous known medications for diabetes management, there is considerable interest in medications derived from natural origin such as herbal drugs due to their lower side effects and toxicity when compared to synthetic medications [60, 61].

In our research, we examined the total phenolic and flavonoid content of the methanol extract of *E. anacua*. Our results revealed a significant amount of phenolic content and a substantial quantity of flavonoid content. Furthermore, we used HPLC investigation of *E. anacua* leaves and identified various phenolic acids, including ellagic acid, ferulic acid, chlorogenic acid, and gallic acid. These compounds have been previously reported to have antioxidant properties, which aligns with our findings that the ethyl acetate and *n*-butanol fractions of *E. anacua* exhibit potent antioxidant activity due to their high polyphenolic content. This content has been shown to scavenge free radicals and exhibit antioxidant properties [62]. These results were further supported by the isolation of three phenolic compounds, namely caffeic acid, rosmarinic acid, and methyl rosmarinate, from the ethyl acetate fraction, which also possess potential antioxidant activity [63]. Previous studies have shown that other species within the Ehretia genus contain significant levels of polyphenolic and flavonoid content, which also exhibit antioxidant activity and DPPH radical scavenging activity [64, 65].

Type II diabetes mellitus is characterized by an elevation in blood glucose levels. The treatment with STZ (50 mg/kg) in rats resulted in irreversible destruction of pancreatic β-cells, which led to impaired insulin release and consequently hyperglycemia, which can be used as a model for antidiabetic activity evaluation [66]. The continuous administration of *E. anacua* leaves methanol extract and ethyl acetate fraction at a dose of 200 mg/kg for 15 days results in a reduction of blood glucose levels in STZ-induced diabetic rats. Glibenclamide was used as a standard antidiabetic drug for comparing activity in STZ-induced diabetic rats [67]. The possible explanation for the antidiabetic activity demonstrated by the ethyl acetate fraction of *E. anacua* is the presence and identification of megastigmane glucoside (6R,7E,9R)-9-Hydroxy-megastigma-4,7-dien-3-one 9-*O*-D-glucopyranoside (9) and roseoside (10). Previous research has shown that megastigmane can stimulate insulin release from the β-cell line INS-1 [68]. Additionally, the fraction contains phenolic compounds such as caffeic acid, rosmarinic acid, and methyl rosmarinate, which have been reported to possess antidiabetic properties [69-71]. The plant extract showed high levels of total phenolics and flavonoids contents. HPLC analysis revealed the presence of various polyphenolic compounds such as ellagic acid, ferulic acid, chlorogenic acid, and gallic acid. These compounds are known for their potential antidiabetic activity according to previous reports. [72]. These findings align with previous studies on the aqueous extract of *E. anacua* in alloxan-induced diabetic rats [12] and support the traditional and recent pharmacological investigation of the Ehretia genus as a potential source for antidiabetic drugs [13].

It is well established that the increase in blood glucose in STZ-induced diabetes mellitus is linked with higher levels of serum triglycerides and cholesterol [26]. The STZ-induced diabetic rats impaired fat metabolism and caused elevations of serum lipids such as triglycerides and total cholesterol levels [73]. Normally, insulin facilitates the breakdown of triglycerides by activating the lipoprotein lipase enzyme. However, in diabetes mellitus, this enzyme remains inactive, resulting in hypertriglyceridemia [74]. Oral administration of the methanol extract and ethyl acetate fraction of *E. anacua* leaves at a dose of 200 mg/kg for 15 days improved lipid parameters (total cholesterol and triglycerides) in diabetic rats. The presence of phenolics, flavonoids, and tannins in the plant was attributed to these improvements [75]. The assessment of the total phenolic and flavonoid content of crude methanol extract indicated a high level of phenolics. The HPLC analysis also revealed the presence of various phenolic compounds, such as ellagic acid, ferulic acid, chlorogenic acid, and gallic acid. The antihyperlipidemic activity of the ethyl acetate fraction may be attributed to the presence of protective phenolic compounds, such as caffeic acid, rosmarinic acid, and methyl rosmarinate, which have been reported to have a beneficial effect on lipid parameters [76, 77].

Additionally, diabetes induces an elevation of serum urea, uric acid, and serum creatinine as a result of renal dysfunction [66]. Increased urea production in diabetes might be due to enhanced catabolism of both liver and plasma proteins. Also, uric acid increased in diabetics as a result of metabolic disturbance and high activity of lipid peroxidation, xanthine oxidase, and increased cholesterol and triglyceride levels [78, 79]. The administration of daily doses of 200 mg/kg of *E. anacua* leaves methanol extract and different fractions (ethyl acetate and methylene chloride) resulted in a significant reduction in kidney markers such as serum urea, uric acid, and creatinine, which returned to near normal levels. This activity may be related to the high phenolic content confirmed by HPLC analysis and the total phenolic and flavonoid estimation of the crude methanol extract. Additionally, the isolation and identification of protective phenolics, such as caffeic acid and rosmarinic acid, from the ethyl acetate fraction may contribute to this activity [77, 80].

5. Conclusion:

From our investigation, there is a significant antidiabetic effects in both methanol extract and ethyl acetate fraction of *E. anacua* leaves. These extracts not only reduced blood sugar levels but also improved lipid profiles and kidney function parameters. Interestingly, the methylene chloride fraction showed notable efficacy in maintain kidney function parameters. Moreover, the ethyl acetate and *n*-butanol fractions exhibited strong antioxidant activity, which attribute to the high phenolic contents identified through HPLC analysis. Our phytochemical analysis led to the isolation and identification of eleven compounds, four of which were firstly isolated from Boraginaceae family, two from Ehretia genus and five from *E. anacua* species These compounds primarily consist of phenolic acids, sterols, and megastigmane derivatives. Given the results, it suggests that *E. anacua* could hold promise as a potential lead for the development of new antidiabetic drug.

6. Conflicts of interest:

The authors declare no conflict of interest.

7. Acknowledgments:

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