Determination of content analysis of *Achillea arabica* Kotschy. plant flower ethanolic extract by LC-MS/MS and NMR and revealing the curative effects of the extract in diabetic rats

Hanife Ceren Hanalp
Van Yuzuncu Yil University: Van Yuzuncu Yil Universitesi
Abdulahad Dogan (ahadobi@hotmail.com)
Van Yuzuncu Yil University https://orcid.org/0000-0002-5438-8560
Tuba Kusman Saygi
Van Yuzuncu Yil University: Van Yuzuncu Yil Universitesi

Research Article

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Abstract

Background *Achillea arabica* is a perennial aromatic herb widely used in folk medicine for the treatment of the stomachache and abdominal pain, wound healing, gout, cancer, diabetes and such. The aim of this study was to determine the photochemical content of the ethanolic lyophilized extract of *A. arabica* flower by LC-MS/MS and NMR and to reveal the antidiabetic and antioxidant effects of this extract against streptozotosine (STZ)-induced in diabetic rats.

Methods and Results After toxicity test, 35 female rats were divided into 5 groups. Control, Diabetes mellitus (DM), *A.arabica* (400 mg/kg) extract, DM +*A.arabica* (400 mg/kg) extract and DM+Glibenclamide (2 mg/kg). According to LC–MS/MS results of *A. arabica* flower extract, quinic acid (2439.9 µg/g), cyranoside (858.4 µg/g), chlorogenic acid (698.7 µg/g) and cosmosin (347.8 µg/g) were determined as major compounds, respectively. In addition, two new compounds were determined in this plant according to NMR analyzes and these compounds were named edremitine and achillosine, respectively. It was determined that while diabetic rats fed *A. arabica* plant extract significantly decreased blood glucose level, serum glucose, HbA1c, liver and kidney damage biomarker levels, and malondialdehyde (MDA) content compared to the DM group, it caused fluctuations in antioxidant enzyme levels.

Conclusion We conclude that *A. arabica* flower plant extract may be a good addition to the well established pharmacological approach of DM by contributing to the reduction of overall oxidative load.

Introduction

The genus *Achillea*, which belongs to the Asteraceae family, consists of more than 100 species, The *Achillea* species are used for treatment for many diseases in folk medicine. They are used for gynecological and hepatobiliary disorders, rheumatic pain, spasmodic gastrointestinal, pneumonia, hemorrhages, inflammation, wounds healing etc. Members of this genus contain volatile oil and other secondary metabolites, including terpenes (especially sesquiterpene lactones), flavonoids, polyphenols and others [1]. Furthermore, clinical studies have demonstrated that some *Achillea* species potential against irritable bowel syndrome (IBS), multiple sclerosis (MS), episiotomy wound, ulcerative colitis, primary dysmenorrhea and oral mucositis [1].

A. millefolium subsp. *millefolium* is one of the species with medicinal plant which has anti-inflammatory effect and is used as treat wounds and burns [2]. *Achillea millefolium* are used in folk medicine against gastrointestinal disorders due to their antiphlogistic, spasmolytic and haemostyptic activities [3]. It was shown that these activities are due to the sesquiterpene lactones and phenolics compounds in the structure of the plant [4].

The genus *Achillea* L. (Compositae) commonly used as medicinal plant have various pharmacological effects because of presence of active compounds since a long time. These plants contain bioactive compounds like terpenes, flavonoids, alkaloids, tannins, lignins [5]. Obtained a new chlorine-containing

sesquiterpene lactone together with two new epimeric secoguaianolides of tanaphallin and the known vomifoliol from aerial parts of *Achillea cretica* L. has been reported [6]. In a study, It has been reported that a new sesquiterpene compound, namely as santolinoidol, has been found by HPLC analysis of the *Achillea santolinoides* subsp *wilhelmsii* [7]. In another study, three new sesquiterpene lactones and eight known compounds were reported from the aerial parts of *Achillea clavennae*. One of the new compounds has been reported to exhibit higher cytotoxicity than cisplatin on partially apoptotic death in human U251 and rat C6 glioma cell lines [8].

Achillea arabica Kotschy. (synonym: *Achillea biebersteinii* Afanasiev), which is known as Hanzabel in Turkey. In folk medicine, *A. arabica* is used for the treatment of stomachache and abdominal pain, and antinociceptive action [9], inflammatory and spasmodic gastrointestinal disorders, hepatobiliary and cardiovascular ailments [10], DNA damage [11], antiulcer and hypoglycemic [12]. *A. arabica* flowers contains various chemical components such as sabinene, *p*-cymene, α-terpenine, 1.8-cineol, linalool, trans-sabinene, terpinen-4-ol, nerol, ascaridole, cis-piperitone epoxide, trans-piperitone epoxide, trans-sesquisabinene hydrate, thymol, carvacrol, iso-ascaridole, eugenol, lavandulyl-2-methylbutanoate, hexadecene, γ-eudesmol, octadecene, hexadecenoic acid methyl ester, hexadecenoic acid, tricosane (N-) and pentacosane (N-) [9]. In addition, ethanolic extracts of *A. arabica* have been reported to contain flavonoids, quercetin, routine and myrisetin [13].

Nowadays, the interest of medicinal plants as therapeutic agents against diseases such as neurodegenerative, cardiovascular, skin disorders and chronic disturbance like diabetes mellitus (DM) is growing [14]. DM is the most common disorder of the endocrine system and the levels of fasting glucose (≥ 126 mg/dL) and/or glycolyzed hemoglobin % (HbA1c %) ≥ 6.5 are used for hyperglycaemic diagnosis [15]. There are two common types of diabetes, Type 1 and Type 2. Type 2 make up for approximately 90-95% of current patients, and here lifestyle, nutrition and stress play an important role. In addition, DM prevalence is increasing in the world, with 336 million cases reported, and it is expected that by 2030 the number will be increased to 552 million, in both developing and developed countries [16]. Complications of DM are including such as nephropathy, neuropathy, foot amputation and chronic diseases cause both quality of life and serious economic losses for countries. Because of the possible negative side effects of drugs, medicinal plants have traditionally been widely used in the world for the control of DM and it's complications [17].

One of our aims in this study was to identify the major compounds in the plant by LC-MS/MS and to reveal new compounds by 1H-, 13C-NMR spectrometric analyses. Our other purpose was to determine the role of *A.arabica* flower ethanolic lyophilized extract on hyperglycemia, hyperlipidemia, lipid peroxidation content, oxidative biochemical markers and antioxidant enzyme alterations in serum and tissues of in STZ (50 mg/kg) induced diabetic rats.

Materials And Methods

Chemicals and reagents

The streptozotocin (STZ), oxidised glutathione, reduced glutathione (GSH), ethylenediaminetetraacetic acid (EDTA), trihydroxymethyl aminomethane, butylated hydroxytoluene, 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), trichloroacetic acid (TCA), metphosphoric acid, β-Nicotinamide adenine dinucleotide phosphate (NADPH), sodium chloride (NaCl), 5,5'-dithiobis-(2-nitrobenzoic acid), potassium dihydrogen phosphate (KH_2PO_4), and sodium dihydrogen citrate anhydrous used in the experiments were all of technical grade and purchased commercially from Sigma-Aldrich (St. Louis, MI, USA). Kits for the GPx and SOD analyses were purchased from Randox Laboratories Ltd. (Shanghai, China).

Plant material

The *A.arabica* plants collected by Hanife Ceren HANALP and Associate Professor Abdulahad DOGAN in the Edremit district (GPS coordinates: 38° 25' 09 " N; 43° 15' 54" E), Van, Turkey, during May 2018. The samples were authenticated by Associate Professor Süleyman Mesut Pinar (Department of Botany, Van Yuzuncu Yil University, Turkey). The voucher specimens were placed into the Van Yuzuncu Yil University, Faculty of Science, Department of Biology (VANF) herbarium (herbarium code:164098).

Preparation of lyophilized extract

The plant ethanolic lyophilized extract was prepared according to the method of Dogan et al [18], with some modifications. The flower samples were dried in the shade and crushed into a fine powder. The aqueous extracts were prepared using a magnetic stirrer, by adding 50 g of flower powder into 1L of 80 % ethanol of distilled water (dH_2O) in shaker for 2 hours. Then it was passed through cheesecloth and strainer, liquid part was centrifuged at 10 000 rpm for 20 min. Supernatants were passed through a 0.45 µm hydrophilic filter with the help of an injector. The combined supernatants were evaporated with a rotary evaporator at 37°C (Rotavapor R-205; Buchi, Switzerland). After the liquid extracts were evaporated, they were placed in beakers and stored at -80 °C for 48 hours. The frozen samples were allowed to dry under a vacuum in a lyophilizer device at -51 °C.

LC-MS/MS analysis

The LC-MS/MS phytochemical content analysis of the *A.arabica* flower extract was performed based on 53 phytochemical compounds previously optimized and validated by Yilmaz [19]. In addition, in the preparation of the standard solutions, the dry extracts were spiked with ferulic acid D1 (20 mg/L), rutin D2 (1 mg/L), and quercetin D3 (5 mg/L), diluted to 1000 mg/L with methanol and filtrated through 0.2 µm syringe filter prior to the LC-MS/MS analysis.

Mass spectrometer and chromatography conditions

A Shimadzu-Nexera model ultrahigh performance liquid chromatograph (UHPLC) (Shimadzu Corp., Kyoto, Japan) coupled with a tandem mass spectrometer was used to conduct the quantitative evaluation of the 53 phytochemicals. A reversed phase Agilent Poroshell 120 EC-C18 model (2.1 mm, 2.7 µm × 150 mm) analytical column (Agilent Technologies, Santa Clara, CA, USA) was used for the chromatographic

separation. During the analysis, the column temperature was 40 °C. A gradient flow was used for the elution, in which eluent A (water + 0.1% formic acid + 5 mM ammonium formate) and eluent B (methanol + 0.1% formic acid + 5 mM ammonium formate) were used. The following gradient elution profile was used: 20%–100% B (0–25 min), 100% B (25–35 min), and 20% B (35–45 min). The solvent flow rate was 0.5 mL/min and the injection volume was 5 µL, respectively. The mass spectrometric detection was carried out using a Shimadzu LCMS-8040 model tandem mass spectrometer (Shimadzu Corp.) equipped with an electrospray ionization (ESI) source operating in both positive and negative ionization modes. The LC-ESI-MS/MS data were obtained and conducted using LabSolutions software (Shimadzu Corp.). For quantitative analysis in the extract, the multiple reaction monitoring (MRM) mode was used. In order to acquire optimal phytochemical fragmentation and maximal transmission of product ions, the collision energies (CE) were optimized. The MS conditions comprised the following: drying gas (N2) flow, 15 L/min; nebulizing gas (N2) flow, 3 L/min; DL temperature, 250 °C; heat block temperature, 400 °C, and interface temperature, 350 °C [19].

Compounds isolation from Achillea arabica extract

The total crude ethanol extract (2.16 g) was subjected to column chromatography over silica gel eluted with chloroform and gradually increasing the polarity, by addition of MeOH (up to 20%) to give 2 fractions. Then MeOH (up to 40%) to give 3 fractions. When 50% methanol was added give 2 fractions were taken, finally 100% methanol was added to collect at total of 12 fractions. Similar fractions were combined to obtain in total 10 fractions and a flavone glycoside was obtained from fraction 4 and a sesquiterpene lactone was obtained from fraction 5 and purifed on prep. silica gel TLC plates using from a solvent system (CH_3Cl /MeOH) (9.5:0.5).

NMR and Mass analysis

The NMR spectra were measured on a Bruker Avance NEO NMR Specrometer at 500 and 125 MHz (^1H at 500 MHz, ^{13}C at 125 MHz) instrument at 24 C. The TMS resonance was used as internal standard. Chemicals shifts are given in parts per million (ppm) and coupling constants (J) in Hertz. The LC/HRMS spectral analyses were obtained on Thermo Orbitrap Q-exactive was used for mass analyses of the isolated compounds.

Animals

A total of 40 *Wistar albino* female rats, which were 2–3 weeks old and weighed 100–350 g, were acquired from the Experimental Animal Research Center of Van Yuzuncu Yil University. The animal used, and all of the experimental procedures during the study, were approved of by the Animal Experiments Ethics Committee of Van Yuzuncu Yil University for ethical concerns (YUHADYEK-2019/06). During the experiments, the ethical regulations that were followed were in line with the institutional and national guidelines for animal welfare protection. The rats were housed in cages made of plastic at a steady room temperature of 25 ± 2 °C and a 12-h light/dark photoperiod. They were supplied with drinking water and a diet that was wheat and soybean meal-based *ad libitum*.

Acute toxicity test

For evaluation of the acute toxicity of the lyophilized ethanolic *A.arabica* extract on the rats, increasing dosages of 25, 50, 100, 250, 500, 1000, and 2000 mg/kg of body weight (bw) were given via oral gavage for 5 rats. Signs or symptoms of toxicity and mortality were not determined for thefirst 72 h. Thereafter, the favorable dosage of the extract used in the experimental study was ascertained to be 400 mg/kg.

Induction of diabetes and experimental design

Cold and fresh citrate buffer (0.1 M, pH 4.5) containing STZ was prepared for the rats that were fasted for 12 hours. Then, a single dose of 50 mg/kg was given intraperitoneally (i.p.) to each rat. 72 hours after STZ administration, fasting blood glucose levels were measured with Accu-Chek Go (Roche) glucometer device strips from the tails of the rats. At the end of the determined period, it was observed that all rats given STZ had blood glucose levels above 200 mg/dL and they were diagnosed with diabetes.

The rats were randomly divided into 5 groups, each containing 7 rats.

Control group: The rats in this group were fed only with feed and water *ad libitum* during the 21-day experiment period.

*Diabetes mellitus (DM) group:*The rats in this group with diabetes mellitus with STZ (50 mg/kg, bw) were fed only with feed and water *ad libitum* during the 21-day experiment period.

A. arabica (400 mg/kg) extract group: During the 21-day experiment period the rats fed with feed and water *ad libitum*, and *A. arabica* (400 mg/kg) extract was given as gavage once a day.

DM+A. arabica (400 mg/kg) extract group: During the 21-day experiment, diabetic rats were fed with feed and water *ad libitum*, and *A. Arabica* (400 mg/kg) extract was given as gavage once a day.

DM+Glibenclamide (2 mg/kg) group: During the 21-day experiment, diabetic rats were fed with food and water *ad libitum*, and glibenclamide (2 mg/kg) was administered orally once a day.

Additionally, the weekly live body weights (LBW) and blood glucose values of the rats were examined during the experiment.

The obtained blood and preparation of tissues supernatant

At the end of the experiment, the rats were anesthetized by ketamine (10 mg/kg, ip), and blood was drawn from rat's heart with injectors. Blood put into hemogram tubes were used for HbA1c % measurement. The blood taken into biochemistry tubes was centrifuged at 3000 g for 15 minutes at 4 °C and biochemical parameters were measured in the serum samples obtained.

Tissue samples taken from rats were placed in tubes after washing with physiological water and stored in a deep freezer at at –78 °C until analysis. The tissues were homogenized for 3 min in 50 mM of ice-

cold KH_2PO_4 solution (1:5 w/v) using a stainless steel probe homogenizer (SONOPULS HD 2200, Bandelin, Berlin, Germany), and then subsequently centrifuged at 9000 rpmfor 30 min. All of the processes were carried out at 4 °C. Then, supernatants were used to determine malondialdehyde contents and antioxidant defence systems constituents as described previously method [20].

Measurement of HbA1c and biochemical parameters

The glycosylated haemoglobin % (HbA1c %) were determined using an auto analyser device (Cobas 6000 c501, Germany) with kits Roche Cobas kits. Alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatinine (CREA), uric acid (UA), urea, glucose (GLU), triglyceride (TRIG), cholesterol (CHOL), high density lipoprotein cholesterol (HDL_c) and low density lipoprotein cholesterol (LDL_c) were measured using a auto analyzer (ARCHITECT 16200, Abbott park, IL 60064, USA) and Abbott biochemistry kits.

Lipid peroxidation and antioxidant enzyme analyses

Total protein content of the tissues were determined using the Bradford method [21]. The level of reduced glutathione (GSH) in tissues was measured using the method described by Beutler et al [22]. Tissue MDA content based on TBA activity was determined according to the method of Buege and Aust [23]. Glutathione peroxidase (GPx) activity was measured using a method of the Paglia and Valentine [24]. Superoxide dismutase (SOD) activity was measured at 505 nm by calculating the inhibition percentage of formazan dye formation [25]. Glutathione *S*-transferase (G*S*T) was assayed by following the conjugation of glutathione with CDNB at 340 nm, as described by Mannervik and Guthenberg [26]. Catalase (CAT) activity was determined using the method described by Aebi [27].

Statistical analysis

The statistical analyses were performed using the Minitab 13 for Windows package program.All datawere expressed as the mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) statistical test was used to determine differences between the means of the experimental groups with statistical significance accepted at $P < 0.05$. The student-t test was used for determination of the differences in body weight changes.

Results And Discussion

Phytochemical content analysis of A. arabica plant extract by LC-MS/MS

Achillea arabica is a perennial aromatic herb widely used in folk medicine for the treatment of the stomachache and abdominal pain, wound healing, gout, cancer, diabetes and such [9, 28]. In studies on the *A. arabica* plant, it has been reported that the plant contains antioxidant compounds and major

essential fatty acids such as *p*-cymene, piperitone, camphor and 1,8-cineole [29,30]. In addition, *Achillea* species are very rich in phenolic and sesquiterpene lactone compounds [1].

Therefore, in this study, phenolic compounds of *A. arabica* species were determined by LC-MS/MS and detailed information of this phenolics were given (Fig.1 and Table 1). *A. arabica* were investigated by LC–MS/MS and the major compounds were detected to be quinic acid (2439.9 µg/g), cyranoside (858.4 µg/g), chlorogenic acid (698.7 µg/g) and cosmosiin (347.8 µg/g), respectively. The analytical method validation parameters in this study were made according to the LC-MS/MS method developed by Yilmaz [19].

Quinic acid and its derivatives, which are compounds rich in polyphenols, are an important compound abundantly found in various plant products. Quinic acid is a normal component of our diet and can be converted to tryptophan and nicotinamide via the gastrointestinal tract microflora, thus providing humans with an *in situ* physiological source of these essential metabolic components [31]. Chlorogenic acid, an ester of caffeic acid and quinic acid, has been reported to have potent antioxidant and multiple antiviral activities against HIV [32,33]. Plants of the genus *Achillea*, which are rich in protocatechuic, vanilic, chlorogenic, ferulic and quinic acid contents, have been reported to have antiinflammatory, analgesic, antimicrobial, antitrypanosomal, antidiabetic and antitumor effects [34].

It has been stated that *Achillea* species may have the potential to develop new functional and nutraceutical products due to their strong antioxidant and anticytotoxic effects [35]. In addition, It has been reported that cyranoside and cosmosilin compounds determined by LC-MS/MS in various plant extracts may have antidiabetic effects due to their antioxidant, antimicrobial and *in vitro* diabetic enzyme inhibition effects [36].

NMR analysis findings of *A. arabica* flower extract

In the present study, ethanol extract of *Achillea arabica* afforded a new sesquiterpene lactone and a new phenolic glycoside. Compound **1** is a sesquiterpene which has a lactone ring.

The ^{1}H NMR spectra of compound **1** displayed. Looking at the ^{1}H NMR spectrum δ 5.06 and 5.36 doublet signals (*J*= 8.2 Hz) indicate the presence of an isolated double bond in the structure.

The ^{13}C NMR spectra of compound **1** showed 3 carbonyl carbon atoms and two methylene carbons one of them is isolated methylene between C-15 and C-16 (δ 126.0 and 117.0, respectively) and the other one pentacyclic methylene with C-3 and C-4 (δ 136.8 and 136.3). HRMS spectrum of compound **1** confirmed its molecular structure ($C_{17}H_{18}O_6$) molecular ion peak at m/z 318. HRMS; Exact Mass: 318.11034, Calculated m/z $[M+H]^+$: 319.11816, Experimental m/z $[M+H]^+$: 319.11496. According to literature research, it was determined that this substance was found for the first time and named as edremitine **(1).** The other new compound **2** is a glycoside which has phenolic skeleton were determined (Fig. 2). The ^{1}H NMR spectra of compound **2** displayed 2 aromatic singlet protons (this protons appeared at δ 7.68 and 7.69 at H-2’ and H-6’ respectively) and 2 aromatic doublet protons signals (δ 7.69 and 7.05 at protons 7

and 8 with *J*=8.3 Hz). The ^{13}C-NMR spectra of compound **2** showed 3 methoxyl carbons, 1 carbonyl carbon and 10 quaternary carbons. The methoxyl carbons appeared at δ 56.11, 60.17 and 60.93. HRMS spectrum of compound **2** approved its molecular structure ($C_{29}H_{34}O_{16}$) molecular ion peak at m/z 638; [M-glc]: 360. HRMS; Exact Mass: 638.18469, Calculated m/z $[M-H]^+$: 637.17686, Experimental m/z $[M-H]^+$: 637.30078.

In fact, the literature search showed that similarity between structure of compound **2** and quercetin 3-O-vicianoside which was previously isolated [37]. The compound **2** name is 6, 4' two hydroxy, 5, 3', 5' three methoxy 3-O-vicianoside. Herewith compound **2** was isolated from nature for the first time and named as achillosine. Structures of these compounds were elucidated based on the ^{1}H-, ^{13}C-NMR spectrometric analyses.

(1): ^{1}H-NMR (600 MHz, CD_3OD): 5.98 (1H, d, H-3), 5.62 (1H, d, H-4), 3.42 (1H, m, H-5), 3.87 (1 H, d, *J* = 12.0, 10.1 Hz, H-6), 3.79 (1H, m, H-7), 4.07 (1H, m, H-8), 2.19 (2H, dd, *J* = 11.6; 8.4 Hz, H-11), 4.83 (1H, s, H-13), 5.06 (1H, d, *J*= 8.2 Hz, H-15), 5.36 (1H, d, *J*= 8.2 Hz, H-16), 2.95 (3H, m, H-17). ^{13}C-NMR (150 MHz, CD_3OD): 126.2 (C-1), 190.0 (C-2), 136.8 (C-3), 136.3 (C-4), 49.0 (C-5), 78.0 (C-6), 66.4 (C-7), 73.6 (C-8), 42.0 (C-9), 134.4 (C-10), 41.0 (C-11), 180.0 (C-12), 24.4 (C-13), 144.0 (C-14), 126.0 (C-15), 117.0 (C-16), 22.5 (C-17).

(2): ^{1}H-NMR (600 MHz, $CDCl_3$): 7.69 (1H, d, *J*= 8.3Hz, H-7), 7.05 (1H, d, *J*= 8.3Hz, H-8), 7.68 (1H, s, H-2'), 7.69 (1H, s, H-6'), 3.98 (3H, s, 5-O-CH_3), 3.85 (3H, s, 3'-O-CH_3), 3.64 (3H, s, 5'-O-CH_3), 5.24 (1H, d, H-1"), 3.57-5.15 (4H, m, H-2"-6"and 2'"-5'"). ^{13}C-NMR (150 MHz, CD_3OD): 156.0 (C-2), 151.79 (C-3), 179.0 (C-4), 169.80 (C-5), 169.90 (C-6), 122.76 (C-7), 127.90 (C-8), 156.00 (C-9), 106.19 (C-10), 110.83 (C-1'), 122.44 (C-2'), 146.36 (C-3'), 148.38 (C-4'), 114.61 (C-5'), 130.00 (C-6'), 56.11 (5-O-CH_3), 60.17 (3'-O-CH_3), 60.93 (5'-O-CH_3), 93.08 (C-1").

Acute toxicity test and body weight

Animal showed good tolerance to testing seven (25, 50, 100, 250, 500, 1000 and 2000 mg/kg) doses of the ethanolic lyophilized *A.arabica* flower extracts. No any noticeable signs of toxicity and mortality were observed after daily administration of the extract orally at the end of the 3rd day (Data not shown).

The body weight (bw) of each rat was recorded every week during the 21-day experimental period. DM and treatment with extract/drug groups bw were shown in Fig. 3. The the final week bw of the DM group was significantly decreased compared to the first week; however, final week bws of the other groups were a significantly increased compared to the first week. In patients with DM, circulating blood glucose cannot/or little be taken into the cell in the absence or insufficiency of insulin, and when this happens, the body starts burning fat and muscle for energy, causing a decrease in total body weight. According to the findings obtained in our study, DM body weight caused a significant decrease compared to other groups. On the other hand, 400 mg/kg dose of *A. arabica* extract and glibenclamide (2 mg/kg) prevented STZ-induced diabetes as demonstrated by significant reduction of bw levels in DM-treatments (*A. arabica*

extract and glibenclamide) groups. Our findings are consistent with changes in body weight caused by previous STZ-induced diabetes studies [38,39].

Effect of *A. arabica* extract on glucose, HbA1c and biochemical parameters

During the 21-day treatment, weekly the differences in blood glucose levels within the group and between the groups are given in Table 2. Blood glucose levels in DM group were significantly increased compared to all other groups (except the 1st day of DM+*A.arabica* 400 mg / kg). In addition, the 21th day blood glucose level of the DM group was increased according to the first three measurements. On the other hand, blood glucose levels of DM+*A.arabica* (400 mg/kg) and DM+Glibenclamide (2 mg/kg) groups were significantly decreased as weeks progressed, and these glucose levels were down by about half when compared to the first measurement.

As shown in Table 3. HbA1c and Glucose levels were significantly increased in DM group compared to all other groups while DM-treatment (extract and drug) groups showed a significant decrease compared to DM group. AST, ALT, ALP, CREA and urea biochemical parameters were significantly increased in the DM group compared to the control and *A. arabica* groups. On the other hand, AST, ALT, ALP, CREA (only, DM+Glibenclamide group) and urea levels were significantly decreased in DM-treatment (extract + drug) groups compared to DM group. Among the lipid profile parameters, TRIG and LDL levels were significantly decreased in *A.arabica* and DM-treatment (extract and drug) groups compared to control and DM groups. HDL level was significantly increased in DM+*A.arabica* (400 mg / kg) group compared to control, DM and DM + Glibenclamide (2 mg / kg) groups (Table 3).

Due to the low or absolute absence of insulin released from the β-cells of the pancreas, circulating glucose cannot be taken into the cell and the glucose level continues to be present in a high rate of circulation. There are many factors that cause pancreatic β-cells damage and insulin release disorder, such as oxidative stress, sedentary lifestyle, malnutrition, genetic predisposition, ATP synthesis disorder in the electron transport system, etc. If the ATP/ADP ratio is not sufficient in mitochondria, the K^+ channels cannot depolarize and closed, so the Ca^{2+} channels cannot open and the Ca^{2+} cannot enter cell, and if the Ca^{2+} level is not at a sufficient in the cell, the insulin release will not circulate or little release into the circulation [40]. In another study, STZ application caused histopathological changes in islets, significantly decreased islet diameter/area, β-cell index and blood insulin and C-peptide levels; however, they reported that *Achillea arabica* extract application caused improvement in islet histology, diameter/area, β-cell index values, blood insulin and C-peptide levels, compared to diabetic rats, similar to rats treated with glibenclamide [41].

As shown in Table 2, our results identified that there was a significant decrease in weekly blood glucose level in the treatment extract or drug groups compared to the DM group. Similarly, as shown in Table 3, glucose and HbA1c, liver and kidney damage biomarker parameters, and TRIG and LDL levels from lipid profile parameters showed significant decrease in treatment groups (*A. arabica* extract and glibenclamide) compared to DM group. Decrease in the treatment groups of blood glucose, HbA1c,

liver and kidney damage biomarkers indicate that the plant extract has an antidiabetic and antioxidant role. In previous studies reported that similar results from *Achillea* species on blood glucose and biochemical parameters levels in rats [12,42]. In another study, it have been reported that *Achillea arabica* (synonymous: *Achillea biebersteinii*) ethanolic extract may have α-amylase enzyme inhibition, hypoglycemia and therapeutic effects on pancreatic damage in diabetes mellitus [28]. In liver and kidney damage, AST, ALT, ALP, LDH, urea and CREA parameters leak into the circulation and increase their levels. Similarly, diabetes-induced oxidative stress causes an increase in lipid profile parameters LDL, CHOL, TRIG and a decrease in HDL levels [43]. *Achillea arabica* essential oil (0.2 ml/kg/day) has been reported to cause a decrease in AST, ALT, GGT, ALP, bilirubin, cholesterol, triglycerides LDL and VLDL levels and an increase in HDL level against CCl_4-induced toxicity in rats over 21 days [44]. In another study, it was reported that 25 mg/kg/day or 100 mg/kg/day *Achillea millefolium* extract had a significant reduction in blood sugar, serum liver enzymes, triglycerides, and total CHOL and LDL cholesterol levels compared to the diabetic groups [42].

Effect of *A. arabica* extract on MDA content, GSH level and antioxidant enzyme activities

As shown in Fig. 4A. Liver and kidney (except *A.arabica* group) MDA contents were significantly increased in DM group compared to all other groups. The decrease in liver GSH level in DM group was significant determined compared to all other groups (Fig. 4B). The differences in kidney tissue GSH levels were not statistically significant between the groups.

As shown in Table 4. Liver GPx enzyme activity was significantly decreased in DM group compared to *A.arabica* group. The liver CAT enzyme activity of DM and control groups were significant decreased compared to *A.arabica* and DM+Glibenclamide groups. Also, liver CAT enzyme activity was significantly increased in the *A.arabica* group compared to the DM+*A.arabica* (400 mg/kg) group. Similarly, liver GST enzyme activity was significantly increased in the *A.arabica* group compared to the DM+Glibenclamide (2 mg/kg) group. Kidney CAT enzyme activity was significantly increased in DM+Glibenclamide (2 mg/kg) group compared to DM+*A.arabica* (400 mg/kg) group. In addition, kidney GST enzyme activity was significantly increased in DM group compared to all other groups (Table 4).

Free radicals such as hydroxyl ($HO^{\cdot}$), hydroperoxyl ($HOO^{\cdot}$), superoxide ($O_2^{\cdot}$-), lipid ($L^{\cdot}$), lipid peroxyl ($LOO^{\cdot}$), peroxy ($ROO^{\cdot}$), lipid alkoxyl ($LO^{\cdot}$), nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$), and protein ($P^{\cdot}$) play an important role in the formation of diabetes [45]. Increasing free radicals cause an increase in MDA content, liver and kidney damage biomarkers, and a decrease in antioxidant defense system enzymes such as SOD, GPx, CAT and GR [38]. These enzymes protect cells against ROS, including CAT, H_2O_2 breaks down into H_2O and O_2; SOD, which removes $O_2^{\cdot -}$; GST, which can remove xenobiotics by forming thiol groups; GR, which converts oxidized glutathione (GSSG) into reduced GSH [46]. According to our results, diabetes caused a considerable increase in liver and kidney levels of MDA contents while treatment with *Achillea arabica* extract or glibenclamide drug significantly mitigated these elevations (Fig. 4A). Also, our results found that *Achillea arabica* extract or glibenclamide led to a significant increase in GSH level (Fig. 4B) and antioxidant enzyme activities in liver and kidney tissues (Table 4). The treatment

of gastric ulcer by the *A. arabica* (200 mg/kg) extract showed significant decrease in oxidative stress markers by 84.61, 54.43 and 64.80 % for GSH, MDA and SOD, respectively, while ranitidine drug exhibited significant decrease in the OS markers by 79.80, 33.54, and 52.51 % [12]. In another study, it was reported that the ethanolic lyophilized extract of *A.arabica* leaf showed significant antioxidant activity and DNA protective effect, as well as, the plant extract was not cytotoxic even at the highest dose studied (512 µg/ml) and inhibited H_2O_2-induced cell toxicity [11]. Isolated all compounds from *A.arabica* plant were examined for their anti-inflammatory activity to inhibit lipopolysaccharide-induced NO production in RAW264.7 macrophage cells, and compounds **3** and **4** produced a promising anti-inflammatory effect (76% and 80% inhibition, respectively) [47]. It was reported that the AST, ALT, GGT, ALP enzymes and bilirubin concentrations as well as the level of MDA, nonprotein sulfhydryl and total protein contents in liver tissues were significantly reinstated towards normalization by the *A. arabica* essential oil (0.2 mL/kg) against CCl_4-induced hepatotoxicity in rats [44]. It is known that *A. arabica* plant extract have major compounds such as α-terpinene (41%), p-cymene (13%) [48], 1,8-cineole (9–37%), camphor (16–30%) [49], piperitone (35%) and eucalyptol (13%) [50].

Conclusion

As a result, ethanolic extracts of *A. arabica* were found to be rich in phenolic compounds and sesquiterpenoids. Among phenolics, mainly quinic acid, cyranoside, chlorogenic acid and cosmosiin were found to be high amounts in the plant. Additionally, a new sesquiterpene lactone namely ((3ab, 9ba)- 6-(hydroxymethyl)- 2,7-dioxo-4a- (2-butenoyloxy)- 2,3,3a,4,5,7,9a,9b-octahydroazuleno [4,5-b] furan) **(1)** and a new glycoside which has phenolic skeleton as namely 3,6,4'-trihydroxy-5,3',5'-trimethoxyflavone-3-O-α-L-rhamnopyranoside **(2)** were isolated and characterized by NMR. Thus, *A. arabica* should be considered as a rich source for these compounds with their antioxidant, antihyperglycemic and insulin secretion properties. Therefore, we propose that *A. arabica* extract may be useful in curing patients suffering from diabetes and its complications. However, since *in vivo* studies are negligible, further studies are required to determine the optimal doses of *A. arabica* plant.

Declarations

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Authors contributions A. Dogan: Study design, writing-review & editing. H. C. Hanalp: Performing *in vivo* analyzes. T. Kusman Saygı**:** *In vitro* analyzes to be performed.

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Data availability The data and material used and analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest The authors declare that they have no competing interest

Ethical approval The procedures conducted herein were in line with those of the National and Institutional Regulations for the Protection of Animal Welfare. The animal used, and all of the experimental procedures during the study, were approved of by the Animal Experiments Ethics Committee of Van Yuzuncu Yil University for ethical concerns (YUHADYEK-2019/06).

ORCIDs:

Hanife Ceren Hanalp https://orcid.org/0000-0001-5235-4167

Abdulahad Dogan http://orcid.org/0000-0002-5438-8560

Tuba Kuşman Saygı http://orcid.org/0000-0002-1771-1453

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Tables

Table 1. Quantitative screening of phytochemicals in ethanol extract of *Achillea arabica* by LC–MS/MS.

No	Compounds	Retention time	Amount (μg analyte/ g extracts)
1	Quinic acid	3.0	2439.9
2	Fumaric aid	3.9	44.1
3	Aconitic acid	4.0	55.2
4	Gallic acid	4.4	2.6
5	Protocatechuic acid	6.8	103.1
6	Gentisic acid	8.3	9.4
7	Chlorogenic acid	8.4	698.7
8	Protocatechuic aldehyde	8.5	8.6
9	4-OH Benzoic acid	10.5	13.6
10	Caffeic acid	12.1	43.3
11	Vanillin	13.9	14.8
12	*p*-Coumaric acid	17.8	14.2
13	Ferulic acid	18.8	7.7
14	Salicylic acid	21.8	43.3
15	Cynaroside	23.7	858.4
16	Miquelianin	24.1	79.5
17	Rutin	25.6	1.4
18	isoquercitrin	25.6	326.8
19	Hesperidin	25.8	1.9
20	Genistein	26.3	0.3
21	Cosmosiin	28.2	347.8
22	Astragalin	30.4	37.3
23	Quercetin	35.7	165.2
24	Naringenin	35.9	23.1
25	Luteolin	36.7	264.0
26	Kaempferol	37.9	8.4
27	Apigenin	38.2	60.0
28	Amentoflavone	39.7	0.1

29	Chrysin	40.5	0.4
30	Acacetin	40.7	0.8

Table 2. Effects of *A.arabica* extracts on rat blood glucose levels during the application period

Groups	Day	Glucose level (mg/dL)
Control	1	89.33 ± 6.62^{Aa}
	7	92.67 ± 4.32^{Aa}
	14	83.83 ± 11.43^{Aa}
	21	85.33 ± 6.38^{Aa}
DM	1	462.67 ± 63.11^{ABbc}
	7	441.50 ± 68.49^{ABbc}
	14	423.83 ± 37.40^{Bbc}
	21	508.50 ± 57.27^{Abc}
A. arabica (400 mg/kg) extract	1	93.83 ± 6.79^{Aa}
	7	91.33 ± 17.61^{ABa}
	14	82.00 ± 10.24^{Ba}
	21	84.17 ± 7.07^{Ba}
DM + *A. arabica* (400 mg/kg) extract	1	480.60 ± 46.83^{Abc}
	7	354.75 ± 25.08^{Bb}
	14	358.00 ± 80.61^{Bbc}
	21	244.00 ± 83.48^{Cb}
DM + *Glibenclamide* (2 mg/kg)	1	281.40 ± 32.85^{Ab}
	7	127.50 ± 28.68^{Bb}
	14	167.67 ± 41.52^{Bb}
	21	141.83 ± 33.15^{Bb}

Two way ANOVA was used to determine whether the means were significantly different or not between the days and hours ($p < 0.05$). Following analysis of variance, the Tukey test was used to determine whether the means were significantly different or not between the days and groups ($p < 0.05$).

A, B, C: Capital letters represent the difference between days in the same group ($p < 0.05$).

a, b, c: Lowercase letters represent the same day difference between different groups ($p < 0.05$).

Table 3. Effects of *A.arabica* extract on biochemical parameters during the application period

Parameters	Groups				
	Control	DM	*A. arabica* (400 mg/kg) extract	DM + *A. arabica* (400 mg/kg) extract	DM + Glibenclamide (2 mg/kg)
AST (U/L)	167.33 ± 17.92	1093.80 ± 255.28^{a}	101.17 ± 15.17^{ab}	205.67 ± 70.69^{bc}	100.00 ± 22.99^{abd}
ALT (U/L)	67.17 ± 11.29	887.00 ± 83.93^{a}	44.50 ± 8.62^{ab}	77.17 ± 18.80^{bc}	61.20 ± 13.03^{bc}
LDH (U/L)	912.50 ± 202.96	917.00 ± 126.13	851.17 ± 163.29	1111.17 ± 157.08^{bc}	928.60 ± 133.22
ALP (U/L)	313.67 ± 65.29	1222.20 ± 230.74^{a}	221.67 ± 51.68^{ab}	775.60 ± 153.34^{abc}	577.00 ± 139.68^{abc}
HbA1c (%)	3.79 ± 0.16	7.61 ± 0.48^{a}	3.51 ± 0.11^{ab}	5.38 ± 1.03^{abc}	5.16 ± 1.44^{bc}
CREA (mg/dL)	0.32 ± 0.03	0.44 ± 0.03^{a}	0.33 ± 0.03^{b}	0.37 ± 0.09	0.37 ± 0.05^{ab}
Urea (mg/dL)	50.00 ± 4.34	81.80 ± 4.15^{a}	42.83 ± 6.37^{ab}	52.00 ± 10.26^{b}	43.80 ± 7.66^{b}
GLU (mg/dL)	123.00 ± 20.83	755.20 ± 39.12^{a}	163.83 ± 22.34^{ab}	531.17 ± 82.22^{abc}	446.40 ± 110.79^{abc}
TRIG (mg/dL)	111.50 ± 18.06	120.80 ± 9.23	91.50 ± 11.50^{ab}	72.33 ± 17.50^{abc}	74.40 ± 17.87^{ab}
CHOL (mg/dL)	67.00 ± 11.24	75.80 ± 15.35	67.50 ± 15.40	66.67 ± 12.32	61.00 ± 8.75
HDL (mg/dL)	31.92 ± 6.67	33.44 ± 4.30	38.70 ± 5.86	42.57 ± 6.12^{ab}	35.10 ± 3.49^{d}
LDL (mg/dL)	40.25 ± 7.75	47.44 ± 6.45	30.37 ± 2.93^{ab}	25.90 ± 4.69^{ab}	29.10 ± 5.63^{ab}

Data are expressed as the mean ± SD. One way ANOVA was followed by the Tukey test, when appropriate (n=7). Differences between the groups were considered significant ($p < 0.05$).

[a] Difference between the Control group and the other groups was significant ($p < 0.05$).

[b] Difference between the DM group ant the other groups wassignificant ($p < 0.05$).

[c] Difference between the *A.arabica* group and the other groups was significant ($p < 0.05$).

[d] Difference between the DM + *A. arabica* group and the DM + Glibenclamide group was significant ($p < 0.05$).

AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; ALP: alkalane phosphatase; HbA1c :glycosylated haemoglobin; CREA: creatinine; GLU: glucose; TRIG: Triglyceride; CHOL: cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein.

Table 4: Effects of *A. arabica* extracts on antioxidan defence system parameters during the application period

		Groups				
Tissue	Enzym	Control	DM	*A. arabica* (400 mg/kg) extract	DM + *A. arabica* (400 mg/kg) extract	DM + Glibenclamide (2 mg/kg)
Liver	SOD U/g Prt	56.92 ± 11.84	43.47 ± 12.47	60.38 ± 14.76	55.59 ± 13.50	46.14 ± 12.58
	GPx U/g Prt	18.34 ± 1.35	15.80 ± 4.43	22.53 ± 4.03[b]	21.68 ± 4.40	17.00 ± 3.91
	CAT nmol/g Prt	8.15 ± 2.33	10.48 ± 2.24	17.02 ± 5.21[ab]	10.63 ± 4.70[c]	14.20 ± 2.06[ab]
	GST nmol/g Prt	18.29 ± 3.99	18.05 ± 2.72	22.85 ± 6.57	17.28 ± 2.38	16.34 ± 1.76[c]
Kidney	SOD U/g Prt	92.89 ± 15.57	83.98 ± 9.89	87.84 ± 10.53	80.82 ± 9.08	76.68 ± 16.59
	GPx U/g Prt	32.33 ± 8.68	27.33 ± 6.92	34.40 ± 6.51	26.98 ± 5.46	28.18 ± 3.10
	CAT nmol/g Prt	13.29 ± 3.32	14.27 ± 3.70	15.07 ± 3.91	12.13 ± 3.27	16.24 ± 2.88[d]
	GST nmol/g Prt	1.70 ± 0.66	3.31 ± 0.96[a]	1.85 ± 0.61[b]	1.96 ± 0.56[b]	2.29 ± 0.46[b]

Data are expressed as the mean ± SD. One way ANOVA was followed by the Tukey test, when appropriate (n=7). Differences between the groups were considered significant ($p < 0.05$).

[a] Difference between the Control group and the other groups was significant ($p < 0.05$).

[b] Difference between the DM group ant the other groups wassignificant ($p < 0.05$).

[c] Difference between the *A.arabica* group and the other groups was significant ($p < 0.05$).

[d] Difference between the DM + *A. arabica* group and the DM + Glibenclamide group was significant ($p < 0.05$).

SOD: superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase. GST: glutathione *S*-transferase; Prt: protein.

Figures

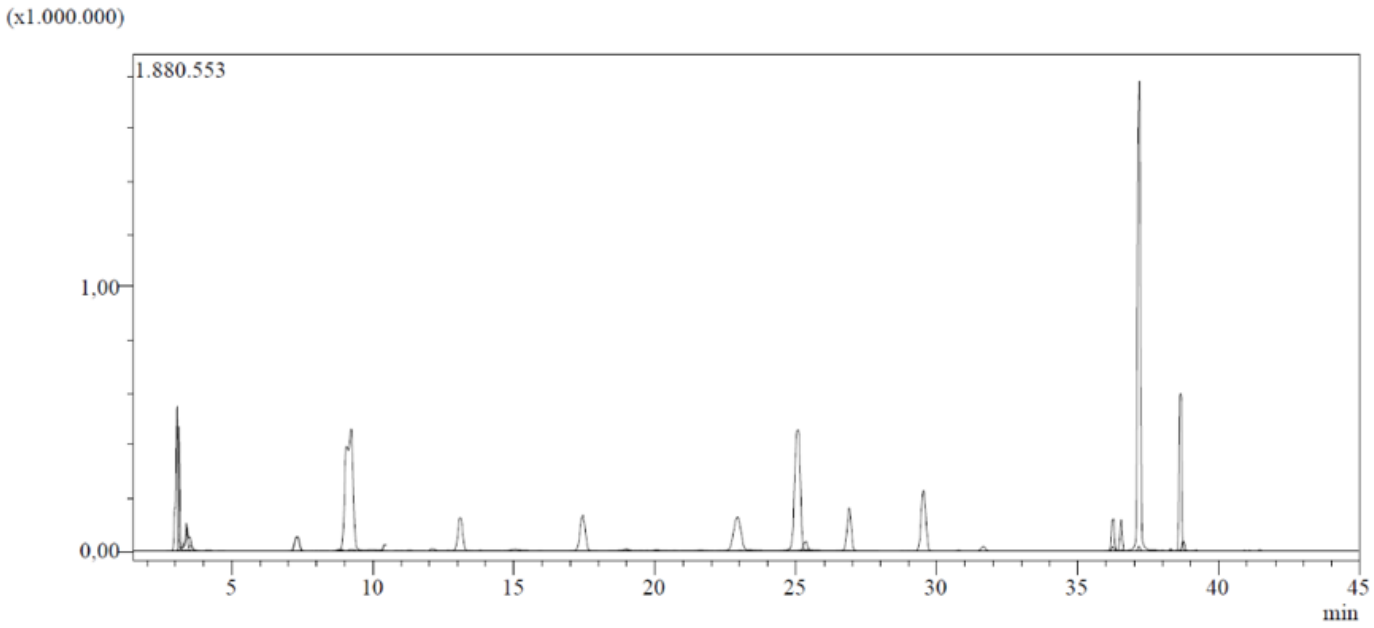

Figure 1

Total Ion Chromatogram (TIC) of ethanolic extract of *Achillea arabica* analysed by the developed LC–MS/MS method

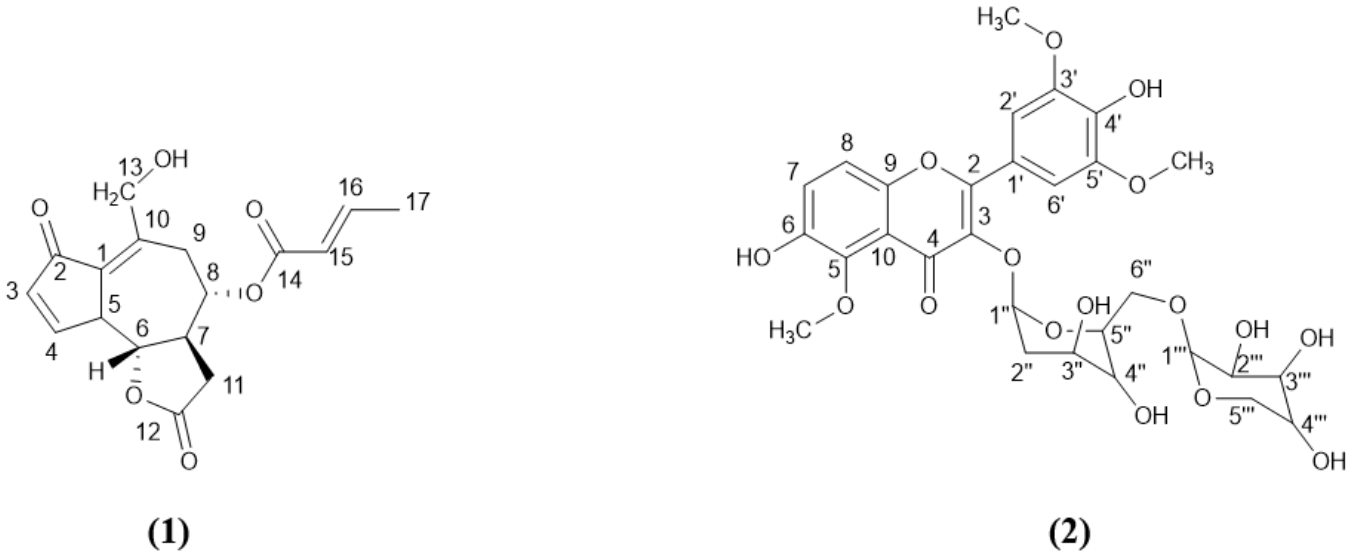

Figure 2

Structures of the new compounds isolated from *Achillea arabica*

(1) Edremitine **(2)** Achillosine

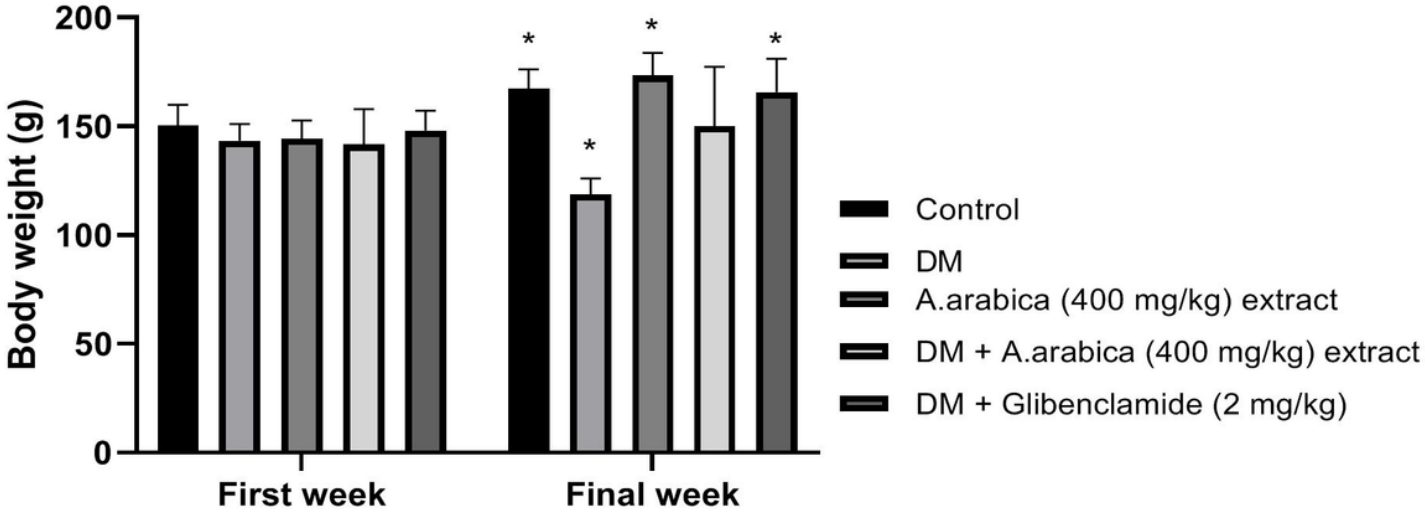

Figure 3

Effects of *A.arabica* extracts on rat body weight levels during the application period

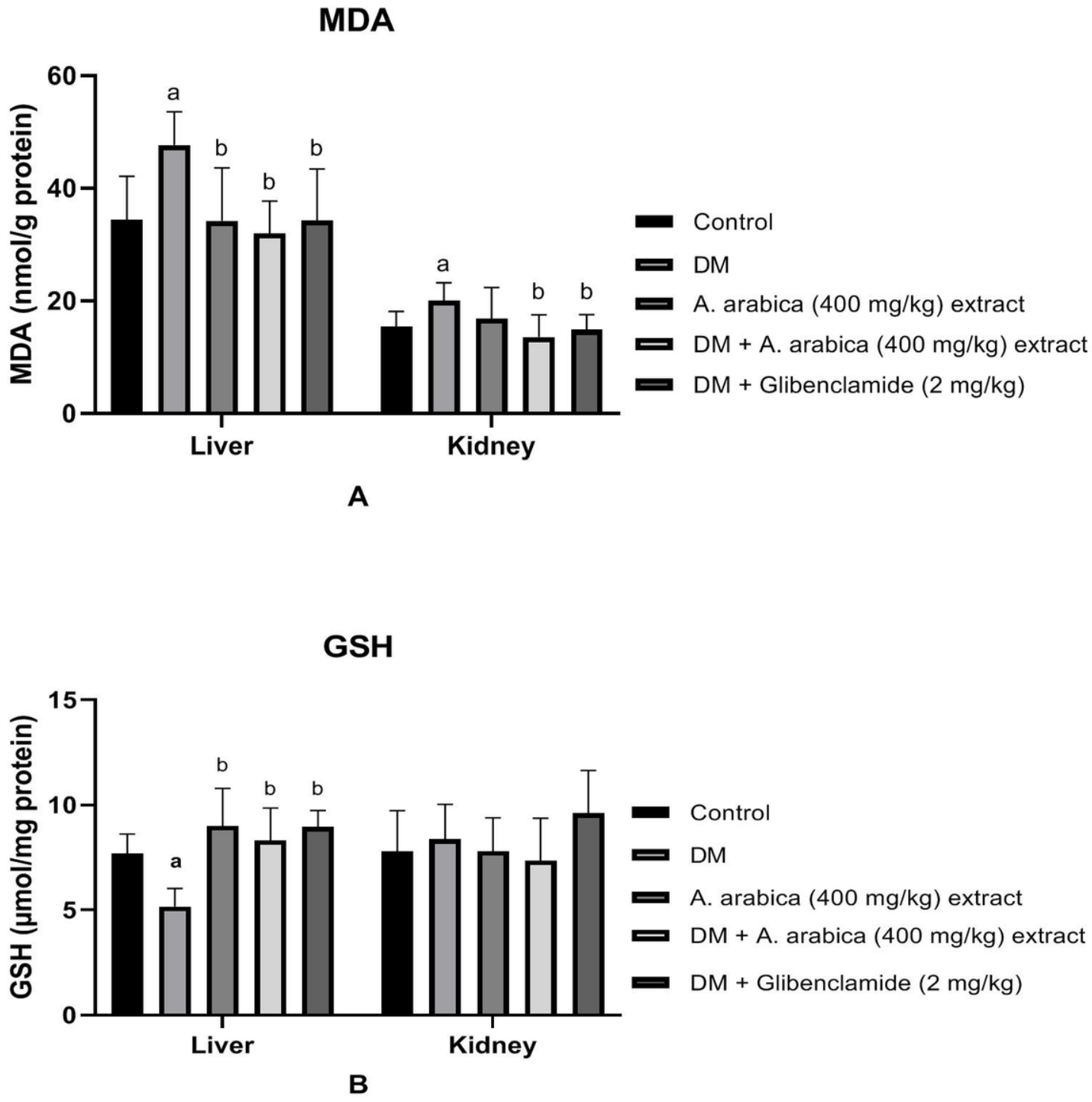

Figure 4

Effects of *A.arabica* extracts on MDA and GSH levels during the application period

A:MDA, Malondialdehyde; **B**:GSH, Reduced glutathione.

Supplementary Files

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- GraphicalAbstract.pdf
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