Research Article

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# Phytochemicals profiling, *in vitro* and *in vivo* antidiabetic activity, and *in silico* studies on *Ajuga iva* (L.) Schreb.: A comprehensive approach

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**Abstract:** *Ajuga iva* (L.) Schreb. is a well-known antidiabetic medicinal plant used for several traditional medicine aspects in different areas of the world, including Libya. This study includes phytochemical analysis, antidiabetic evaluation, and *in silico* studies of the plant, *A. iva*, growing in Libya. The constituents of the plant were profiled using LC-MS/MS-QTOF analysis, and a total of 28 compounds were tentatively identified, including engeletin, pyrocatechol, eriodyctiol-7-hexoside, and 3,4-dihydroxybenzaldehyde, as major constituents. In addition, the steroidal compounds, i.e., 20-hydroxyecdysone, 24-dehydroprecyasterone, makisterone A, and ajugasterone D, which are considered chemomarkers for the plant, were also annotated by LC-MS analysis. The plant extract induced inhibition of α-amylase and α-glucosidase enzymes at $IC_{50}$ values of 0.18 and 0.12 mg/mL, compared to the $IC_{50}$ of the standard acarbose at 0.11 and 0.09 mg/mL, respectively. Fasting blood glucose (FBG, 360.7 mg/dL) levels were significantly reduced by the treatment of streptozotocin (STZ)-diabetic animals with 400 mg/kg (140.5 mg/dl) and 500 mg/kg (112.3 mg/dL) doses of the plant extract. The plant extract also induced a significant ($p < 0.01$) increase in insulin serum level compared to the untreated diabetic rats; however, the higher dose of the plant induced similar insulin induction compared to glibenclamide. Histopathological examination of the pancreatic and liver tissues indicated that *A. iva* extract induced regeneration in the islets of Langerhans and liver cells compared to the untreated diabetic rats. Docking analysis demonstrated that eriodyctiol-7-hexoside, echinacoside, and 2″-galloylhyperin showed the lowest binding energies to the target sites of α-amylase and α-glucosidase enzymes, indicating their potential role in *A. iva* antidiabetic bioactivities. The results support the recorded traditional bioactivity of *A. iva* as an antidiabetic herb, whereas its contents of polyphenols play a major role in the plant's antidiabetic effect.

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# 1 Introduction

Diabetes mellitus is one of the metabolic disorders characterized by hyperglycemia caused by insulin deficiency. This disease is distinguished by a change in fuel utilization from carbohydrates to lipids. Diabetes has been identified as a prevalent condition that affects millions of individuals worldwide and causes chronic illnesses in different body organs, including the cardiovascular, renal, and nervous systems, that are implicated in diseases like nerve damage, kidney failure, and blood vessel diseases, including retinopathy [1]. The incidence of diabetes was predicted to reach 2.8% in 2000 and 4.4% in 2030 among all age groups [2]. Furthermore, long-term hyperglycemia may cause the body to produce more reactive oxygen species (ROS) and raise their concentration in a variety of bodily tissues, including soft tissues that are particularly vulnerable to ROS, such as the liver, heart, and brain [3]. The ROS overproduction was also reported to induce substantial emergence of diabetic complications, metabolic stress, and cell death [4,5]. In complementary and alternative medicine, medicinal plants have been used and are still used as part of diabetes management [6–8]. In that context, several cross-sectional-based reports have proven that the prevalence of medicinal plant use in diabetes is high, which might reflect the perception of the patients and the effectiveness of the plants in disease management. For example, most people in Saudi Arabia used medicinal plants and also preferred their use in the management of diabetes and obesity [9–13]. In addition, the reports listed several medicinal plants used in the treatment of diabetes all over the world [14–17]. Several species of antidiabetic plants have been phytochemically, biologically, and clinically investigated in numerous articles [18–20]. The pure natural products obtained from these plants have been utilized as promising candidates for antidiabetic drug discovery.

The Lamiaceae plant *Ajuga iva* (L.) Schreb., often known as "Chendgoura," has been used in traditional medicine to cure a variety of conditions, including gastrointestinal disorders, fever, toothache, rheumatism, high blood pressure, and renal and cardiovascular diseases [21]. The plant is also used in all North African countries, including Libya, Algeria, and Morocco, for the treatment of diabetes [21,22]. In that context, the antidiabetic activity of the *Ajuga iva* herb extract has been explored in several articles from plant species growing in Morocco and Algeria [22–26]. All these reports have proven the antidiabetic effect of the plant through different mechanisms. The phytochemical investigation of the plant proved the presence of phenolic acid and flavonoids at high concentrations [21]. In addition, phytoecdysteroids, tannins, terpenoids, fatty acids, and steroids have also been found in the plant [21,26]. The plant, *A. iva*, has acquired several biological properties, e.g., antioxidant, antimicrobial, and anticancer activities, by producing these bioactive compounds [23,26–30]. To the best of our knowledge, no studies have been reported on the chemical composition or antidiabetic capabilities of this Libyan plant, making our study the first to address these topics. The current research is designed to investigate the plant constituents and their role in plant bioactivities, especially their antidiabetic effect. The study includes *in vitro* and *in silico* investigations of the plant's activity as α-glucosidase and α-amylase inhibitors. The study also includes an *in vivo* investigation of the plant's antidiabetic effect using STZ-induced diabetic rats as an animal model.

# 2 Materials and methods

## 2.1 Plant material and extraction process

The materials, consisting of aerial parts of the plant, *Ajuga iva* (L.) Schreb, were gathered during the spring season of 2023 from Jebel Akhdar, East Libya. The plant was identified by taxonomists at the Botany Department, Faculty of Science, Benghazi University, where a specimen of the plant materials was saved at their herbarium. The plant materials were dried in the shade for 3 weeks. The dried reduced materials (500 g) were macerated in a 70% ethanol–water mixture till exhaustion. After filtration, the solvent was evaporated under reduced pressure at a temperature not higher than 50°C, yielding 5.5 g of the dried extract.

## 2.2 LC-MS analysis of the *Ajuga iva* extract

The solvents used in the LC-MS analysis were analytical-grade solvents obtained from commercial sources. The extract scanning was carried out using a Shimadzu ExionLC (Shimadzu, Kyoto, Japan) outfitted with a TurboIonSpray SCIEX X500R QTOF (SCIEX, Framingham, MA, USA). DMSO was used as a solvent for the plant, *A. iva*, extract to prepare a concentration of 0.5 mg/mL. The extract solution was centrifuged for 2 min at 5,000 rpm and filtered through a Milipore 0.2 m membrane. Then, 1 mL of the filtrated extract was placed in a vial and

transferred to the autosampler. The injection volume was adjusted to 3.0 µL. The capillary voltage of −4,000 V, nebulizer gas of 2.0 bar, nitrogen flow of 8 L/min, and dry temperature of 200°C were the modifications made to the instrument's specifications. The mass sensitivity was adjusted at 50,000 FSR, the mass precision was 1 ppm, and the TOF recurrence was adjusted at a rate up to 20 kHz. Chromatographic separation with the gradient elution method was applied using an RP-C18 column (2.1 mm I.D., 100 mm length, and 3 µm particle size) from GL-Science (Japan). The parameters of the separation were adjusted as follows: a rate of flow of 0.35 mL/min for 30 min of running. Formic acid (0.1%) and pure acetonitrile were used as the mobile phases A and B, respectively. The gradient system was composed of 0.1% formic acid in water (A) and acetonitrile (B); for the first 4 min, the system was composed of 96% A and 4% B. The ratio of acetonitrile (B) was increased to 6% in 10 min, 7% in 12 min, 8% in 15 min, 13% in 18 min, 15% in 23 min, 20% in 25 min, and 28% in 27 min, and maintained until 30 min. The annotation processes were based on several analysis outcomes, including the molecular weight of the compounds, their fragmentation pattern compared to the literature, the suggestions of the machine library, and the reported constituents of *A. iva* and other species of *Ajuga* growing in different locations.

## 2.3 *In vitro* antidiabetic assay

The reported technique was used to determine α-glycosidase activity [31]. The substrate was *p*-nitrophenyl D-glycopyranoside. In brief, the substrate and α-glucosidase enzyme (0.1 U/mL) were dissolved in $KH_2PO_4$ buffer (0.1 M, pH 6.7). The samples were subsequently mixed in DMSO at concentrations ranging from 0.1 to 50 mg/mL. After 10 min of 37°C incubation of the enzyme (100 µL) and samples in a 96-well microplate, 200 µL of the substrate was added to the mixture to prolong the enzymatic reaction for 30 min. To stop the reaction, 1 mL of $Na_2CO_3$ (1 M) was added, and the absorbance was measured at 405 nm. To calculate the $IC_{50}$ values, all sample concentrations were examined in duplicate. As a reference substance, acarbose was employed.

α-Amylase inhibition was assessed following the literature method [32]. The plant's inhibitory potential for α-amylase was determined by mixing various concentrations of the *A. iva* extract with the enzyme, α-amylase, and starch solutions. After mixing 250 µL of the sample solution with 250 µL of 0.02 M PBS (pH 6.9) solution, which contained 240 U/mL of the α-amylase enzyme, the mixture was stored at 37°C for 20 min. After that, the mixture was incubated with 1% starch in PBS (pH 6.9) for 20 min at 37°C. Then, the solution was incubated at 90°C for 10 min after the addition of 250 µL of dinitrosalicylic acid. After diluting the cooled reaction mixture with 1 mL of distilled water, the absorbance at 540 nm was determined. The $IC_{50}$ values were calculated, and the positive control used in this study was acarbose.

## 2.4 *In vivo* antidiabetic experiment

### 2.4.1 Experimental animals

Fifty adult male albino rats of the Sprague–Dawley strain (140–160 g) were utilized to test the antidiabetic efficacy of the extract. Benghazi University's Faculty of Pharmacy's Ethical Committee, Benghazi, Libya, approved the study procedure (Assalam# EA 2). Before beginning the research, the animals were housed in controlled laboratory settings for at least 1 week. A regular pellet meal, which contains a mixture of minerals, sucrose, vitamins, corn oil, cellulose, and starch, was used as food for the rats.

### 2.4.2 Experimental design

To induce diabetes, overnight-starved rats were injected with the newly produced STZ (50 mg/kg) dissolved in citrate buffer (0.1 M, pH 4.5). The animals were observed for effective diabetes induction by measuring FBG levels on days 2, 5, and 7 of STZ injection. Only those rats with FBG levels of 250 mg/dL were considered for the study. Five animal groups (ten in each) were employed, and the doses of the ethanolic extracts were exactly as reported for the plant extract by Saidi et al. [23].

The animals were divided into the following five groups: As the standard control group, Group 1 consisted of healthy (non-diabetic) rats that were given distilled water. Groups 2–5 were STZ-diabetic rats, wherein Group 2 was the untreated diabetic control group and was given just distilled water; Group 3 received plant extract (400 mg/kg dose); Group 4 was given 500 mg/kg of extract; and Group 5 was given glibenclamide (2.5 mg daily dose) as a standard treated group. The oral treatments with *A. iva* extract were given to the animals once daily for 1 month, beginning on the eighth day following diabetes induction. The improvement in glucose tolerance and the shift in FBG were recorded weekly. The FBG, insulin, AST, ALT, GSH, GSPx, SODs, and malonaldehyde (MDA) were assessed in the rat's blood.

## 2.5 Biochemical parameters

### 2.5.1 Blood glucose measurements

The blood glucose levels of the rats treated with the extract and the control groups were assessed according to the procedure of Muthuraman et al. [33]. To precipitate the proteins, 3.8 mL of isotonic $Na_2SO_4$–$CuSO_4$ solution and 0.5 mL of 10% $Na_2WO_4$ solution were mixed with 0.1 mL of blood sample. The materials were centrifuged for 10 min at 1,500 rpm in order to produce a protein-free solution. After that, 1 mL of alkaline tartarate was combined with the clear supernatant, and the mixture was heated to a boil for 10 min. Following cooling, 3 mL of water and 3 mL of phosphomolybdic acid were thoroughly mixed. The mixture was left to stand for 5 min to develop color. The produced color was measured at 630 nm and compared with a blank. The milligrams per deciliter are used to represent the values.

### 2.5.2 Blood insulin assay

The radioimmunoassay process was based on the rivalry between radio-iodinated (125I) insulin and unlabeled insulin in the blood for the few binding sites on a particular antibody. At the end of the incubation period, the second antibody-polyethylene glycol-assisted separation technique was used to separate the free insulin and that bound by antibodies [34]. The insulin levels in the blood were then determined by measuring the radioactivity linked to the samples' antibody-bound friction.

### 2.5.3 Determination of antioxidant enzymes

The concentrations of the antioxidant enzymes, i.e., GSPx, SODs, CAT, and the level of GSH, were measured using the manufacturer's instructions. The levels of MDA were also estimated using the manufacturer's instructions.

## 2.6 Histopathology

Rats were anaesthetized for the histopathological assays by intramuscular injection of xylazine (11 mg/kg) and ketamine hydrochloride (100 mg/kg) (Sigma-Aldrich; Merck KGaA). Following this, liver and pancreas tissue were taken and fixed in a 10% formalin solution for 3 h at room temperature before being immersed in paraffin. Sections of tissue (5 µm thick) were cut and dyed for 5 min at room temperature with hematoxylin and eosin [35]. Colored tissue sections were captured at ×40 magnification using a light microscope. The images were assessed by a pathologist using the R package CR Image.

## 2.7 Docking method

In this study, the compounds tentatively identified by LC-MS analysis were utilized for docking investigations and molecular interaction possibilities since the *A. iva* extract showed substantial *in vitro* and *in vivo* antidiabetic properties. Using Autodock4 software, the binding affinities of the tentatively identified phytochemical components of the *A. iva* extract to the α-glucosidase and α-amylase binding sites were determined. The RCSB data bank website provided the X-ray geometry of the targeted α-glucosidase and α-amylase, along with their original docked ligands, which were retrieved with PDB codes (1B2Y) and (5NN4), respectively [36,37]. The extracted receptor structure was supplemented with Kollman charges and polar hydrogen atoms after the water molecules were eliminated. The active sites were identified using the co-crystallized receptor–ligand complex structures of α-glucosidase and α-amylase. The re-docking of the original ligands into the binding active sites of α-glucosidase and α-amylase was replicated, with RMSD values of 0.93 and 0.88 Å, respectively. Using Merck molecular force field 94 level 44, the molecular geometries of the phytochemical elements of *A. iva* extract were reduced and saved as PDB files. The Lamarckian genetic method was used for the molecular docking study, and 500 binding site runs were made in total. A population of 150 individuals with 27,000 generations and 250,000 energy evaluations was used in each corresponding run [38]. The crossover, mutation, and elitism operator weights were set to 0.8, 0.02, and 1, respectively. For α-glucosidase and α-amylase, the grid box with dimensions of 40 × 46 × 40 and 44 × 40 × 40 points, with a spacing of 0.382 Å, was selected. It was centered at (0.067, −1.695, −22.993) and (17.388, 5.268, 46.733). Using Chimera X and the Discovery Studio Client, the binding interactions between the phytochemical components that were docked into the binding sites of α-glucosidase and α-amylase were visualized [39,40].

## 2.8 Statistical analysis

The IBM SPSS® program was employed to perform one-way ANOVA analysis and obtain the *p*-value.

# 3 Results and discussion

## 3.1 Phytochemical profiling of *A. iva* extract

The phytochemical analysis of the *A. iva* extract was performed using the LC-MS technique to profile the contents of the plant species growing in Libya. The plant, *A. iva*, growing in different locations in the world has been phytochemically investigated in several previous reports [21,23,25,29]. The results of these reports indicate the prevalence of phenolic acids, flavonoids, terpenoids, and steroidal constituents in the plant [21,23,25,29]. Several reports have also investigated *A. iva* essential oil constituents and documented the presence of simple volatile terpenoids and hydrocarbons in the plant [27,29,30,41]. Certain steroidal compounds (ecdysteroid), e.g., 20-hydroxyecdysone, cyasterone, makisterone A, and ajugasterone D, have been identified and considered as chemomakers for the plant *A. iva* and other plants of the genus *Ajuga*, such as *A. nipponensis, A. pyramidalis, A. multiflora, A. macrosperm, A. linearifolia, A. japonica, A. incisa, A. decumbens, A. chamaecistus, A. bracteosa,* and *A. australis* [42]. Furthermore, caffeic acid and its glycosides, e.g., caffeic acid and echinacoside, and quinic acid, are characteristic constituents of different Ajuga species, including *A. iva* [23,43–46]. In addition, specific iridoids, e.g., harpagide and harpagoside, have also been reported in several species of Ajuga [47–49]. All these constituents of *A. iva* primarily have a role and contribute to the plant's activities and therapeutic application in the treatment of diabetes mellitus. The results of the LC-MS analysis of *A. iva* species growing in Libya were consistent with the reported constituents of the plant and revealed the presence of ecdysteroid chemomarkers, phenolic acids, iridoids, and flavonoids (Table 1). Out of tens of peaks in the LC-chromatogram (Figure 1), 28 compounds were tentatively identified in *A. iva* extract. The compounds were defined based on their molecular weight, mass fragments, and NIST library identification. The reported data for the plant, *A. iva*, and *Ajuga* species were also used in the annotation of the plant constituents listed in Table 1. In that context, several atomic mass units (amu) were used as a guide in the annotation of the plant constituents. For example, the presence of 169 amu [M-gallic acid] in the mass spectra of

**Table 1:** *Ajuga iva* constituents tentatively identified by LC-MS analysis

| R.t | Names | [M-H] − (*m/z*) | M.W. | MS/MS | RP% |
|---|---|---|---|---|---|
| 2.50 | Myricetin* | 317.0980 | 318.1058 | 271, 153, 108 | 0.06 |
| 2.84 | (−)-Quinic acid* | 191.0821 | 192.0898 | 146, 127, 105 | 0.06 |
| 2.99 | DL-Malic acid | 133.0337 | 134.0414 | 115, 87 | 0.14 |
| 4.36 | Epigallocatechin gallate* | 457.1813 | 458.1891 | 169 | 0.01 |
| 10.26 | Harpagide* | 363.1788 | 364.1866 | 221, 169, 129 | 0.01 |
| 10.34 | Makisterone A* | 493.2198 | 494.2275 | 470, 282, 309, 237, 173, 153, 135 | 0.01 |
| 10.64 | Rhamnetin* | 315.1526 | 316.1604 | 153 | 0.01 |
| 10.75 | Quercetin* | 301.0970 | 302.1048 | 152, 123, 108 | 0.01 |
| 10.90 | 20-Hydroxyecdysone* | 479.1862 | 480.1940 | 423, 371, 281, 160, 134 | 0.01 |
| 11.13 | Pyrocatechol | 109.0456 | 110.0533 | 108, 91 | 0.74 |
| 11.21 | Campestanyl ferulate* | 577.2280 | 578.2358 | 563, 465, 443 | 0.02 |
| 11.51 | *p*-Coumaric acid* | 163.0639 | 164.0717 | 135, 119 | 0.03 |
| 11.93 | Eriodyctiol-7-hexoside | 449.1685 | 450.1763 | 287, 152 | 0.17 |
| 12.00 | 3,4-Dihydroxybenzaldehyde | 137.0446 | 138.0524 | 108 | 0.27 |
| 12.04 | Engeletin | 433.2674 | 434.2751 | 287, 259 | 0.28 |
| 12.11 | Echinacoside | 785.3484 | 786.3562 | 623, 421, 271, 160 | 0.01 |
| 12.19 | Quercetagetin-7-*O*-glucoside | 479.1487 | 480.1565 | 317 | 0.02 |
| 12.34 | Caffeic acid* | 179.0609 | 180.0687 | 134 | 0.03 |
| 12.68 | Ajugasterone D* | 477.1283 | 478.1361 | 429, 301, 249, 195, 167 | 0.02 |
| 12.87 | Naringenin-7-*O*-rutinoside* | 579.2113 | 580.2191 | 270, 269, 153 | 0.01 |
| 13.33 | 2″-Galloylhyperin | 615.2183 | 616.2260 | 301 | 0.01 |
| 13.82 | Harpagoside | 493.2927 | 494.3005 | 331, 277, 215 | 0.01 |
| 14.01 | Ellagic acid* | 301.1710 | 302.1788 | 257, 185, 116 | 0.01 |
| 14.54 | 24-dehydroprecyasterone* | 517.3484 | 518.3562 | 367,345, 271, 247 | 0.01 |
| 15.07 | 5,7-Dihydroxy-4-methylcoumarin | 191.0626 | 192.0703 | 147,121, 81 | 0.02 |
| 15.94 | Chrysoeriol* | 299.0972 | 300.1050 | 284, 227, 187 | 0.05 |
| 15.98 | 3′-Hydroxy-.alpha.-naphthoflavone | 287.2630 | 288.2708 | 255, 115 | 0.06 |
| 20.55 | 3-Hydroxymyristic acid | 243.2302 | 244.2380 | 194, 177 | 0.3 |

*Compounds have been reported in *A. iva* [23,29,50].

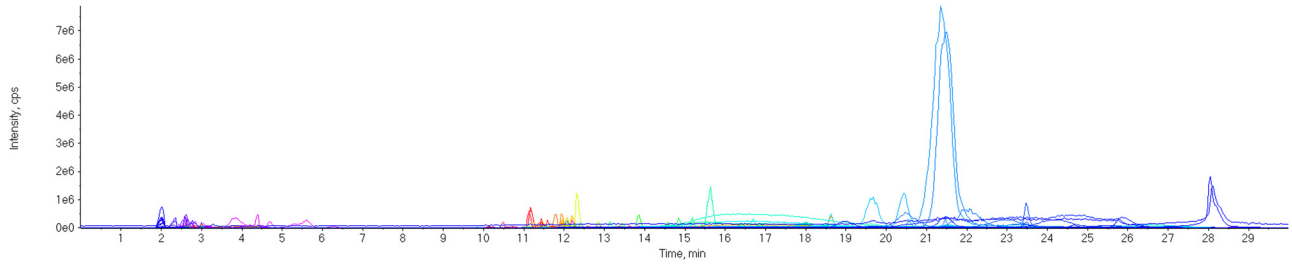


**Figure 1:** Total LC-chromatogram of the *Ajuga iva* extract.

epigallocatechin gallate (*m*/*z* 457.1813 [M-H]) was an indication for the gallic acid fragment of the compound; the presence of *m*/*z* 331 [M-glu] in the harpagoside (*m*/*z* 493.2927 [M-H]) was a sign for the removal of 162 amu for the glucose unit from the parent molecule; in the mass spectra of eriodyctiol-7-hexoside (*m*/*z* 449.1685 [M-H]), a mass fragment at *m*/*z* 287 was a sign to the removal of a glucose unit with 162 amu and indication for the presence of eriodyctiol flavonoid in the glycosylated form; and the presence of a mass unit at *m*/*z* 270 (M-glu.-Rham.-H) in the mass spectra of naringenin-7-*O*-rutinoside (*m*/*z* 579.2113 [M-H]) was an indication for the removal of rutinoside (glucose and rhamnose) moiety from the original compound and the presence of naringenin in the glycosylated form in the plant extract. The reported compounds from *A. iva* were also correlated with the data of LC-MS analysis, and out of the 28 tentatively identified compounds listed in Table 1, 16 compounds (assigned by the asterisk "*") have been reported from the plant. The tentatively identified compounds in Table 1 indicate the capability of the plant to biosynthesize various classes of natural products, including flavonoids, phenolic acids, iridoids, and steroids.

## 3.2 Antidiabetic activity of *A. iva*

The hypoglycemic effect of *A. iva* has been proven in several previous reports [22,23,25,51–53]. In addition, it is well known that *A. iva* aerial parts are used in the form of decoctions and infusions in folk medicine for the management of diabetes [21]. The current research provides an investigation of antidiabetic activity for *A. iva* growing in Libya. Reduction of the *in vitro* α-glucosidase and α-amylase enzyme activities and the *in vivo* evaluation of FBG and insulin levels were measured in a rat's animal model.

### 3.2.1 *In vitro* antidiabetic activity

The results in Table 2 demonstrated the effect of *A. iva* extract on the α-glucosidase and α-amylase enzymes in comparison to the standard enzyme inhibitor, acarbose. The enzymes α-glucosidase, and α-amylase are implicated in the metabolism of carbohydrase through their effect in the hydrolysis of polysaccharides, i.e., starch and glycogen, to disaccharides and further monosaccharides, glucose [54]. Because they delay the breakdown of carbohydrates, α-amylase and α-glucosidase inhibitors can help control hyperglycemia by lowering postprandial plasma glucose levels [54]. The current results indicate the plant's activity as a natural inhibitor of these enzymes and support the herbalist's claim for the antidiabetic effect of the plant. As shown in Table 2, *A. iva* extract induced inhibition of α-amylase and α-glucosidase at $IC_{50}$ values of 0.18 ± 0.002 and 0.12 ± 0.004 mg/mL compared to the $IC_{50}$s of the standard acarbose at 0.11 ± 0.01 and 0.09 ± 0.011 mg/mL, respectively. The results indicated a comparable effect of the plant extract on the standard antihiatic drug, acarbose. Comparable outcomes have been noted for the *A. iva* extract derived from plant species that grow outside of Libya. For example, similar findings have been reported for *A. iva* growing in Taza, Morocco, with $IC_{50}$ values of 0.172 and 0.130 mg/mL for the inhibition of α-amylase and α-glucosidase, respectively [22]. In addition, the α-amylase inhibition effect of *A. iva* growing in Masmouda, Morocco, has shown lower activity ($IC_{50}$ value of 1.52 mg/mL) as compared to the current results and reported results of the plant growing in Taza, Morocco [23]. On the other hand, the *A. iva* extract obtained from Algerian species has induced 70 % inhibition of α-amylase (55).

**Table 2:** α-Amylase and α-glucosidase inhibitory effects of the *A. iva* extract and acarbose antidiabetic standard drug

| Samples | $IC_{50}$ (mg/mL) | |
|---|---|---|
| | α-Glucosidase | α-Amylase |
| *A. iva* extract | 0.12 ± 0.004 | 0.18 ± 0.002 |
| Acarbose | 0.09 ± 0.011 | 0.11 ± 0.012 |

The results are calculated as the mean of three measurements with standard deviation.

**Table 3:** Effect of 400 and 500 mg/kg doses of the plant extract on the FBG levels

| FBG | Normal control | Diabetic | Diabetic + glibenclamide | Diabetic + 400 mg of the *A. iva* extract | Diabetic + 500 mg of the *A. iva* extract |
|---|---|---|---|---|---|
| Zero | 99.50 ± 2.50 | 367 ± 6.70 | 359 ± 3.60 | 360.70 ± 4.30*** | 367.70 ± 6.80*** |
| Week 2 | 99.46 ± 2.70 | 390.5 ± 4.40 | 180 ± 5.40 | 265.20 ± 3.50** | 200.60 ± 3.70** |
| Week 4 | 96.75 ± 2.30 | 453.6 ± 5.60 | 100.4 ± 2.70 | 140.50 ± 5.80* | 112.30 ± 5.70* |

Values are presented as mean ± SE ($n$ = 10) observations, *$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.001.

### 3.2.2 *In-vivo* antidiabetic activity

As presented in Table 3, both *A. iva* extract concentrations (400 and 500 mg/kg) have significantly reduced the levels of FBG in the STZ-induced diabetic rats. The lower dose (400 mg/kg) dropped the FBG from 360.7 to 140.5 mg/dL, while the higher dose (500 mg/kg) dropped the FBG from 367.7 to 112.3 mg/dL.

As shown in Table 4, oral treatment with plant extract (400 and 500 mg/kg) induced a major ($p$ < 0.01) increase in the insulin serum level (4.435 ± 0.5 and 4.985 ± 0.4, respectively) compared to the diabetic rats (1.543 ± 0.4). The spike in insulin levels generated by *A. iva* extracts was similar to that caused by the standard glibenclamide.

The study examined how the *A. iva* extract affected the biochemical parameters in the STZ-induced diabetes rats and measured the levels of AST and ALT to evaluate liver function. Serum levels of AST and ALT were significantly elevated in the STZ-treated animals compared to their values in control animals and diabetic rats treated with glibenclamide. Table 5 shows that the administration of *A. iva* extract considerably reduced blood levels of AST and ALT in diabetic rats as compared to the untreated group. Moreover, the ability of the *A. iva* extract to replenish several antioxidant defense systems like GSHPx, GSH, CAT, and SOD was measured. The low and high doses of the plant extract have significantly increased the levels of antioxidant enzymes. However, the high dose of the plant was extremely active in restoring the levels of these enzymes, *i.e.,* GSHPx, CAT, and SOD, compared to the standard antidiabetic drug, glibenclamide (Table 5). The *in vivo* antidiabetic results of *A. iva* growing in Libya are, in part, like the plant species growing in different locations [23,25]. The results in our study indicated the *in vivo* antioxidant activity of the plant extract, which plays a role in the plant's activity for the management of diabetes induced by STZ. The current effect of *A. iva* as an antidiabetic is mostly due to the plant constituents, including phenolic acids, flavonoids, and steroids.

The pancreatic and liver specimens from the treated and control Sprague–Dawley strain rats were subjected to

**Table 4:** Effect of 400 and 500 mg/kg doses of the plant extract on serum insulin levels

| Groups | Normal control | Diabetic | Diabetic + glibenclamide | Diabetic + 400 mg of the *A. iva* extract | Diabetic + 500 mg of the *A. iva* extract |
|---|---|---|---|---|---|
| Serum insulin levels (μU/mL) after 4 weeks | 5.53 ± 0.03 | 1.54 ± 0.40 | 4.95 ± 0.20* | 4.43 ± 0.50* | 4.89 ± 0.40* |

*Statistically significant from the diabetic control at $p$ < 0.01.

**Table 5:** Enzymes regulation activity of the *Ajuga iva* extract

| Enzymes | Control | Diabetic | Diab + glibenclamide | Diab + 400 mg of the *A. iva* extract | Diab + 500 mg of the *A. iva* extract |
|---|---|---|---|---|---|
| AST (U/L) | 24.60 ± 0.80 | 133.60 ± 4.90 | 29.78 ± 2.70 | 43.20 ± 5.40* | 29.40 ± 5.50* |
| ALT (U/L) | 28.30 ± 2.60 | 109.80 ± 2.70 | 29.75 ± 2.36 | 34.90 ± 6.60* | 30.90 ± 4.49* |
| GSH (U/g) | 58.80 ± 0.70 | 35.80 ± 7.90 | 56.20 ± 0.70 | 45.00 ± 0.80* | 57.90 ± 0.70* |
| GSHPx (U/g) | 15.90 ± 0.90 | 9.50 ± 0.50 | 13.90 ± 0.40 | 11.80 ± 0.90* | 13.80 ± 0.90* |
| CAT (U/g) | 25.40 ± 0.90 | 6.40 ± 0.43 | 14.20 ± 0.80 | 18.10 ± 0.40* | 22.85 ± 0.90* |
| SOD (U/mL) | 4.50 ± 0.140 | 1.65 ± 0.76 | 4.23 ± 0.65 | 3.98 ± 0.76* | 4.45 ± 0.65* |

Values are expressed as mean ± SE, $n$ = 10 for each group. *$p$ < 0.05, compared with the control.

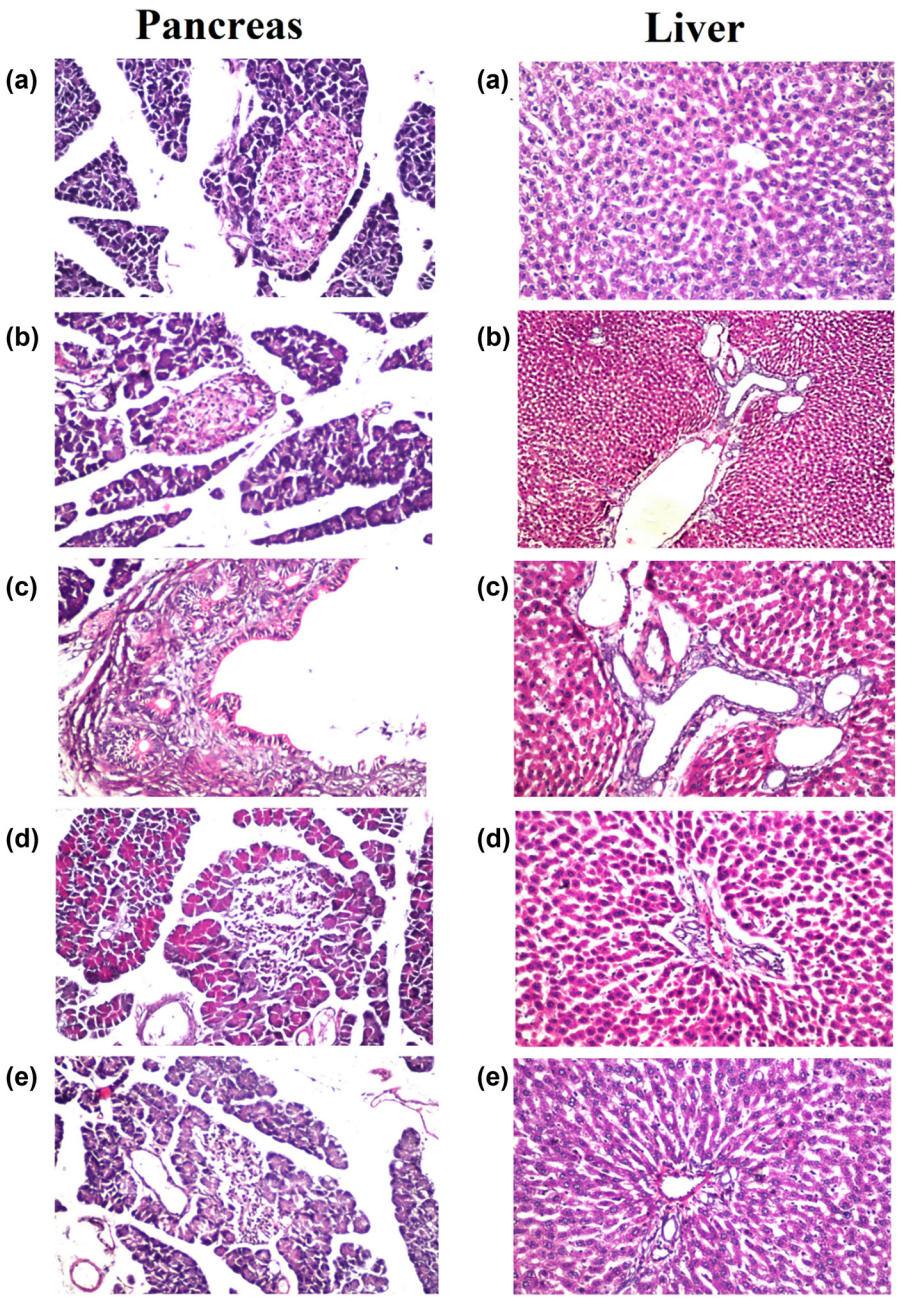


**Figure 2:** The alteration of pancreatic and liver tissue morphology in STZ-induced diabetic rats: (a) non-treated rats; (b and c) diabetic rats; (d) rats treated with 500 mg/kg of *Ajuga iva*; and (e) rats treated with glibenclamide.

histopathological examination. Microscopic analysis of the control rats indicated the standard appearance of islands of Langerhans cells as the endocrine portion, as well as the acini and ducts as exocrine (Figure 2a). When compared to the control group, the diabetic pancreas tissue samples showed a significant change in the cellular architecture. Atrophy was observed in the islands of Langerhans cells (Figure 2b) and associated with epithelial hyperplasia with newly formed ducts (Figure 2c). However, treatment with a 500 mg/kg dose of *A. iva* attenuated the atrophy and caused regeneration of islets of Langerhans cells, with no other histopathological alteration recorded (Figure 2d). Rats that were given glibenclamide had significantly improved cellular injury in their pancreas sections, as seen by the restoration of islet cells, decreased cell injury, increased number of symmetrical vacuoles, and more islet cells (Figure 2e).

The interlobular triad at the margins of lobules, the hepatic cord made up of hepatocytes extending outward

**Table 6:** Binding affinities, number of H bonds, and amino acid interactions of phytochemical *Ajuga iva* extract constituents docked into the binding pockets of α-glucosidase and α-amylase

| No. | Phytochemical constituents | α-Amylase | | | α-Glucosidase | | |
|---|---|---|---|---|---|---|---|
| | Ligands | Binding affinities (kcal/mol) | H bonds | Residues H-bonding | Binding affinities (kcal/mol) | H bonds | Residues H-bonding |
| 1. | Myricetin | −5.2 | 0 | | −4.7 | | |
| 2. | (−)-Quinic acid | −5 | 0 | | −4.8 | | |
| 3. | DL-Malic acid | −4.8 | 0 | | −4.8 | | |
| 4. | Epigallocatechin gallate | −6.9 | 0 | | −6 | 1 | GLU537 |
| 5. | Harpagide | −7 | 2 | ASN352<br>ASP317 | −7 | 3 | GLU801<br>LEU847<br>GLY908 |
| 6. | Makisterone A | −7.2 | 3 | ASP402<br>GLY403<br>GLN404 | −7 | 1 | ARG585 |
| 7. | Rhamnetin | −5.4 | 0 | | −6 | 0 | |
| 8. | Quercetin | −6.7 | 0 | | −6.1 | 0 | |
| 9. | 20-Hydroxyecdysone | −7 | 0 | | −7.2 | 1 | ARG585 |
| 10. | Pyrocatechol | −5.8 | 0 | | −6.1 | 0 | |
| 11. | Campestanyl ferulate | −7.1 | 1 | HIS201 | −7.5 | 1 | LEU868 |
| 12. | *p*-Coumaric acid | −6 | 0 | | −5.4 | 0 | |
| 13. | Eriodyctiol-7-hexoside | −9.4 | 4 | LYS200<br>ILE235<br>GLU233<br>ASP197 | −8.2 | 3 | ASN883<br>ASN883<br>ALA910 |
| 14. | 3,4-Dihydroxybenzaldehyde | −6.1 | 0 | | −6.4 | 0 | |
| 15. | Engeletin | −4.8 | 0 | | −4.2 | 0 | |
| 16. | Echinacoside | −8.8 | 6 | LYS200<br>GLY306<br>GLY304<br>HIS299<br>ASP300<br>ASP300 | −8.3 | 8 | GLY605<br>ARG585<br>PHE490<br>VAL193<br>LEU195<br>ARG608<br>GLU192<br>ARG585 |
| 17. | Quercetagetin-7-*O*-glucoside | −5.7 | 0 | | −5.5 | 0 | |
| 18. | Caffeic acid | −5.2 | 0 | | −6 | 0 | |
| 19. | Ajugasterone D | −5.8 | 0 | | −5.9 | 0 | |
| 20. | Naringenin-7-*O*-rutinoside | −5 | 0 | | −5.2 | 0 | |
| 21. | 2″-Galloylhyperin | −8.7 | 8 | ASP300<br>ASP300<br>GLU233<br>GLY306<br>LYS200<br>ASP197<br>GLU233<br>GLY306 | −7.7 | 4 | GLU801<br>THR771<br>LEU847<br>GLY908 |
| 22. | Harpagoside | −6.8 | 2 | GLU233<br>ASP300 | −7 | 3 | GLU196<br>ARG585<br>THR491 |
| 23. | Ellagic acid | −5.8 | 0 | | −5.9 | 0 | |
| 24. | 24-Dehydroprecyasterone | −5.1 | 0 | | −5.3 | 0 | |
| 25. | 5,7-Dihydroxy-4-methylcoumarin | −5.6 | 0 | | −5.8 | 0 | |
| 26. | Chrysoeriol | −5.8 | 0 | | −5.9 | 0 | |
| 27. | 3′-Hydroxy-.alpha.-naphthoflavone | −5.2 | 0 | | −5.7 | 0 | |
| 28. | 3-Hydroxymyristic acid | −5.5 | 0 | | −5.1 | 0 | |

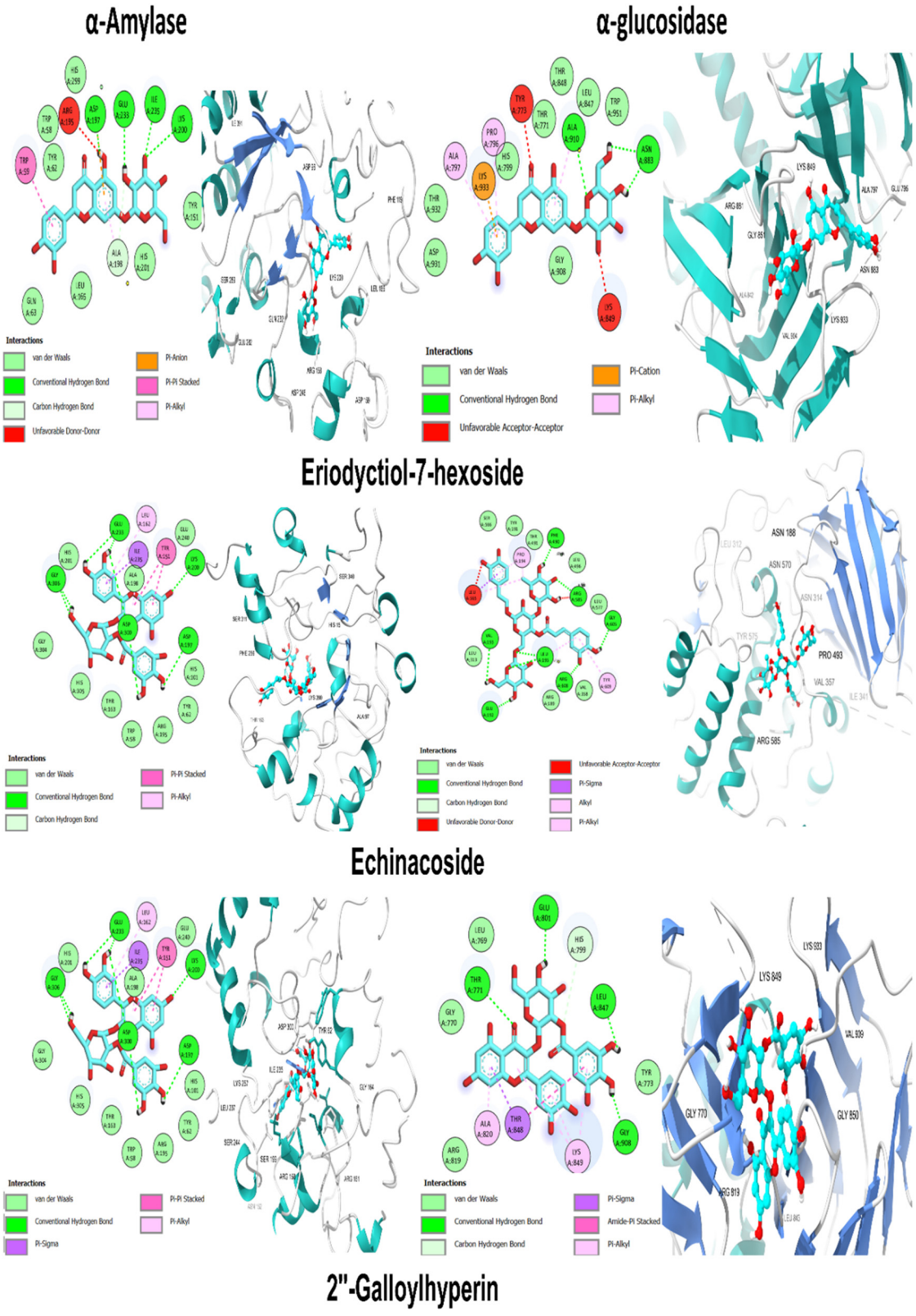


**Figure 3:** 2D and 3D interactions of α-amylase and α-glucosidase with ligands: eriodyctiol-7-hexoside, echinacoside, and 2″-galloylhyperin.

from the central vein, and the central veins near the center of the hepatic lobules were all visible under a microscope in liver sections from control rats (Figure 2a). Investigation of Figure 2b revealed that the diabetic liver had severe dilatation of the portal vein, associated with hyperplasia in the bile ducts, periductal fibrosis, vacuolated cytoplasm, and cellular infiltration. The somewhat normal liver cells and nuclei, as well as some pyknotic nuclei, were also recorded (Figure 2c). The damage to the liver cells was reversed in the treated groups (Figure 2d). On the other hand, glibenclamide-treated rats' liver sections displayed normal hepatocytes and microvasculature (Figure 2e). This study revealed that *A. iva* possesses significant antidiabetic activity, which supports its traditional use for the treatment of diabetes mellitus. This study found that *A. iva* has strong anti-diabetic action, lending credence to its traditional use in the treatment of diabetes mellitus. Therefore, phytochemicals derived from *A. iva* may be used as a possible medicinal agent for the treatment of diabetes. Because of its higher inductive rate and selectivity, STZ is most widely used to induce diabetes mellitus. It destroys pancreatic β-cells through DNA alkylation and strand breakage and then causes diabetes mellitus [56]. It has a structure that is comparable to glucose and hence interacts with glucose on transfer across the pancreatic beta cellular membrane transporter GLUT-2. It is administered to fasting research animals to overcome competition from glucose for entrance [57,58]. This medicinal herb's ability to lower blood sugar levels is associated with physiologically active molecules and secondary plant metabolites, including flavonoids, phenolic compounds, alkaloids, terpenoids, flavonoids, tannins, and sterols, which are present in the plant. As a result, these biologically active compounds have been shown to lower blood glucose levels [23,57]. *A. iva's* mechanism of action may involve insulin potentiation through various means, such as enhancing insulin release from pancreatic β-cells, augmenting peripheral tissues' glucose uptake, reducing hepatic gluconeogenesis, impeding the metabolic breakdown of carbohydrates, or averting oxidative stress [59].

### 3.3 Docking study for antidiabetic activity *A. iva*

Molecular docking studies can provide insights into the biological activity of plant constituents by predicting the interaction between a ligand and a protein. They also provide more details on interactions and possible methods of action at various proteins' binding sites [60]. In an attempt to explain the observed enzyme inhibition of *A. iva* extract constituents, molecular docking was utilized to determine the binding mechanisms between the identified constituents on the one hand, and the active residues of α-glucosidase and α-amylase on the other. Free-binding energy, H-bonds, C–H bonds, and van der Waals (VDW) interactions were the main areas of attention for the molecular docking investigation. The C–H bonds and pi–sigma interactions are linked to the stability of the ligands (selected molecules) and the docked receptor complex, while H-bonds and VDW are linked to binding interactions. Table 6 displays the docking binding energy scores between the binding sites of the target proteins, α-amylase and α-glucosidase. Furthermore, the H bonds, C–H bonds, and VDW interactions with the amino acids found in the binding sites of α-glucosidase and α-amylase were examined. Additionally, Table 6 displays the findings of the ligand assessment for their binding energy with various target proteins. Eriodyctiol-7-hexoside, echinacoside, and 2″-galloylhyperin showed the lowest binding energies with α-amylase (−9.4, −8.8, and −8.7 kcal/mol) and α-glucosidase (−8.2, −8.3, and −7.7 kcal/mol). The connection ofEriodyctiol-7-hexoside with α-amylase and α-glucosidase was mostly linked to 8 VDW interactions with each protein and related to 7 hydrogen bonds (LYS849, LEU847, TYR773, LYS933, ALA797, ARG195, TRP59, and ALA198, respectively) (Figure 3 and Table 6). Each protein's five VDW interactions with echinacoside were mostly linked to 14 hydrogen bonds in its association with α-amylase and α-glucosidase (ILE235, TRP58, TYR62, TRP59, HIS305, TYR609, PRO194, LEU313, ARG189, and THR491, respectively) (Figure 3 and Table 6). However, the connections of 2″-galloylhyperin with α-amylase and α-glucosidase indicated 12 hydrogen bonds (GLU801, LEU847, GLY908, THR771, LYS200, ASP197, ASP300, 3GLU233, and 2GLY306) and 10 VDW interactions (ARG819, GLY770, LEU769, HIS799, TYR773, GLU240, HIS201, HIS101, ARG195, and GLY304), as well as other non-covalent interactions (Figure 3 and Table 6).

## 4 Conclusion

The current research concerns first-time phytochemical and biological evaluations of *Ajuga iva* growing in Libya. The plant has a high reputation in traditional medicine as a hypoglycemic herb all over the world, and several previous articles have documented its antidiabetic activity. Compared to the plant growing in different areas, the Libyan species of *A. iva* had, in large part, similar phytochemical constituents and demonstrated similar biological activities, including *in vitro* and *in vivo* antidiabetic and antioxidant activities. The plant extract repaired the degenerative effect of STZ on the

pancreatic and liver tissues, as demonstrated by the histopathological examination. The docking studies have shown that eriodyctiol-7-hexoside, echinacoside, and 2″-galloylhyperin had the highest binding energies to the α-amylase and α-glucosidase enzyme target sites, suggesting the role of these compounds in the bioactivities of *A. iva* as an antidiabetic agent. Based on the available data, *A. iva* growing in Libya has phytochemical and biological characteristics comparable to species of plants growing in other regions, and their polyphenol levels contribute significantly to the plant's antidiabetic impact.

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**Ethical approval:** Benghazi University's Faculty of Pharmacy's Ethical Committee, Benghazi, Libya has approved the study procedure (Assalam# EA 2).

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