Research Article

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Anti-diabetic activity-guided isolation of α-amylase and α-glucosidase inhibitory terpenes from *Capsella bursa-pastoris* Linn.

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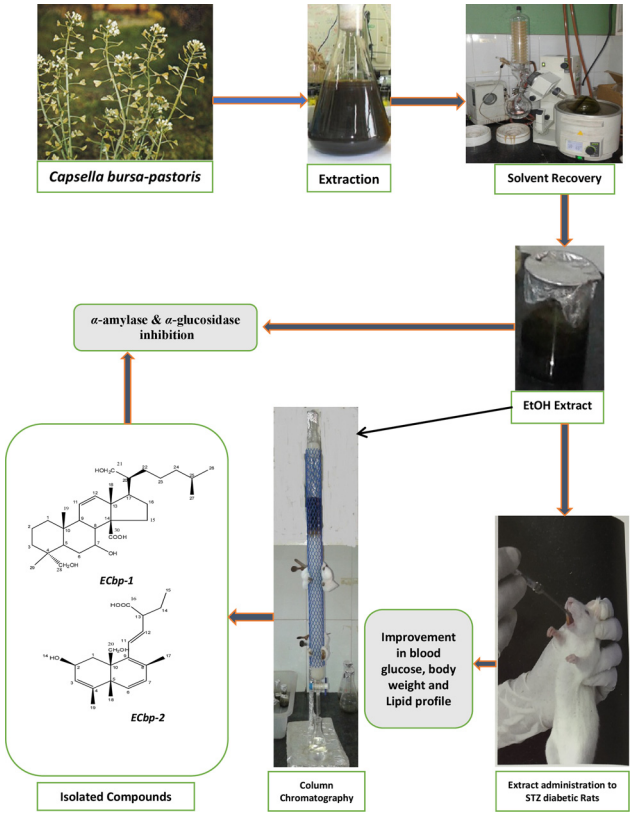
Abstract: The hypoglycaemic and hypolipidemic potential of ethanol extract of *C. bursa-pastoris* (ECbp) in streptozotocin (STZ)-provoked diabetic rats was evaluated, and compounds with their α-amylase and α-glucosidase inhibitory potential were isolated. Acute oral toxicity was evaluated in rats. Streptozotocin (STZ) (50 mg/kg body weight) was injected intraperitoneally into rats for diabetes induction. In diabetic rats, ECbp (0.2 g/kg b.w, p.o.) was administered orally for 21 days, and its outcome on blood glucose levels and body weight was observed on a weekly basis besides lipid profile. Compound isolation from ECbp was performed using column chromatography. Oral feeding of ECbp did not produce any toxic effects or death at a dose of 2,000 mg/kg body weight. A serum glucose reduction trend was observed in rats fed with glucose pre-treated with 200 mg/kg b.w. ECbp also appreciably ($p < 0.001$, $p < 0.01$, and $p < 0.05$) diminished raised blood glucose with decreased blood cholesterol levels and led to increased serum high-density lipoprotein levels in comparison to diabetic control rats. Body weight levels were considerably higher ($p < 0.05$) in diabetic rats treated with ECbp than in diabetic control rats. Isolation of two terpene derivatives (ECbp-1 and ECbp-2) was performed using ECbp, which exhibits significant α-amylase and α-glucosidase inhibition.



Graphical abstract

Keywords: *Capsella bursa-pastoris*, terpenes, antidiabetic, α-amylase, streptozotocin, α-glucosidase

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1 Introduction

Hyperglycaemia and altered lipids, carbohydrates, and protein metabolism are prominent symptoms of an endocrine

metabolic disorder, i.e. diabetes mellitus, as well as increased risk of cardiovascular disease complications [1]. The basic differences between type-1 and type-2 diabetes are the basic processes of their progress and physiological features such as connection with overweight, age, and insulin. The common features of both types of diabetes are hyperglycemia and macrovascular and microvascular complications. Moreover, in both forms of diabetes, metabolic alterations in lipoproteins are responsible for cardiovascular disease pathogenesis in the same manner [2]. Impaired antioxidant defences in the body, which give rise to free radical generation, lead to oxidative stress, which may also lead to the advancement and development of diabetes and associated complications [3]. In type-2 diabetes mellitus, postprandial serum glucose reduction is one of the chief target therapeutic approaches for its treatment, and, at present, only a few drugs are available that are causing such an effect for postprandial hyperglycemia control. These drugs inhibit the hydrolysis of oligosaccharides and disaccharides to simple absorbable monosaccharides [4]. These drugs control postprandial hyperglycemia by selectively inhibiting enzymes (α-amylase and α-glucosidase) found in the small intestine (brush border cells), and control the formation of absorbable monosaccharides by breaking down oligosaccharides and disaccharides [5–7]. Enzyme inhibition is caused by drugs such as acarbose/voglibose, which delay carbohydrate digestion and extend the overall digestion time of carbohydrates, which reduces the glucose absorption rate and ultimately leads to a reduction in postprandial plasma glucose levels [8]. It has a significant effect on the health, life expectancy, lifestyle, and associated problems of people with diabetes. Oral hypoglycaemic agents and insulin are currently available drug options for their management, but these are not free from adverse effects. Since then, various medicinal plants and their preparations have been used traditionally as alternatives for the management of diabetes worldwide [9]. These medicinal plants possess anti-diabetic phytoconstituents that are controlled by multiple mechanisms and modes. Hence, medicinal herbs may be used as effective alternatives and valid therapeutic tools for the management and treatment of diabetes and associated complications [10].

Capsella bursa-pastoris L. (Brassicaceae) is a yearly or biennial plant commonly known as Shepherd's purse, Shepherd's bag, or Shepherd's scrip, or Lady's purse [11]. It has been found worldwide in Cyprus, Europe, Pakistan, Saudi Arabia, Turkey, Iraq, Azerbaijan, India, Iran, China, and other Asian countries. *Capsella bursa-pastoris* is also distributed in North Africa, Central America, and Eastern Europe [12–14]. In China and Japan, the plant has been used for many centuries because of its various therapeutic potentials, such as its ability to halt blood loss of lesions, intensify the formation of urine, and regulate body temperature [15]. The herb as a whole was consumed to treat inflammation caused by disorders in the kidneys, painful micturition, boils and haemorrhoids, severe menstruation, the occurrence of chyle in urine, and hypertension [16]. In some countries, the leaves and roots of this herb are consumed as raw or cooked vegetables [17,18]. Tea made from this herb has antiscorbutics, protein precipitation, diuretics, decreased blood pressure, stimulants, vasoconstrictors, and vulnerability. The pharmacological activities include antimicrobial and anticancer activities, smooth muscle stimulatory effects on guinea pig small intestine, very powerful contraction of guinea pig uterus, infertility effect, negative chronotropic and cardiac inotropic effects on guinea pigs and rabbits, reduction of penetrability in the blood vessel, hepatoprotective, sedative effects, and acetylcholinesterase inhibitor activity. The various phytoconstituents isolated are fatty acids, phytosterols, phenolics, flavonoids, organic acids, peptides, and amino acids [19].

Terpenes are bioactive compounds that occur both as hydrocarbons and terpenoids (oxygen-containing compounds), present naturally in many herbs, and are known to possess significant hypoglycaemic properties [20]. Terpenes show antidiabetic potential by either of the mechanisms that include stimulation of insulin secretion [21–24], enhanced plasma BER (β-endorphin immunoreactivity) by activation of α1-ARs to improve the discharge of β-endorphin, which has the potential to excite opioid micro-receptors to diminish gluconeogenesis in the liver and boost glucose uptake in the soleus muscle [25,26], α-glucosidase inhibition [27–29], increasing glucose uptake and glycogen synthesis and blood insulin concentrations. It appears that the reason for this outcome was the facilitation of insulin secretagogue impact in β-cells of the pancreas [30] by appreciably augmenting the blood pyruvate concentration and liver glycogen level [31] by improving insulin discharge from β-cells of the pancreas, which is due to improved stimulation of β-cells and insulin synthesis [32–34], and by enhancing glucose utilization in striated muscles, which leads to lower plasma glucose levels and increases in insulin discharge from pancreatic β-cells [24,28,35,36]. This is actually a result of inhibiting glucose-6-phosphatase enzyme due to the formation of insulin despite the lack of glucose concentration, which is needed for stimulation [37].

2 Materials and methods

2.1 Plant material and extraction

C. bursa-pastoris was assembled in June–July 2013 from Narbal District Budgam of Jammu & Kashmir and was

authenticated by Centre for Biodiversity and Taxonomy, University of Kashmir, under voucher specimen No. 2078/2013 KASH Herbarium, dated 10/07/2013. The plant material was cleaned, reduced to small fragments, and then shade-dried at 25–30°C for a month. The dried plant material was pulverized into a coarse powder. The powdered plant material was weighed (3.7 kg) and stored in labelled air-tight bottles for further analysis. The powdered herb material was transferred to a 10 L glass percolator and then extracted by using absolute ethanol with occasional stirring and shaking. After 24 h, the extract was filtered using filter paper (Whatman No.1), and the filtrate was again extracted with equal volumes of solvent. After 48 and 72 h, the procedure was repeated. The pooled supernatants were dried under vacuum at 40°C using an IKA RV 10 (Rotary evaporator). The dried extract was weighed, poured into a labelled vial, and kept in a desiccator for future use.

2.2 Chemicals, reagents, and instruments

Solvents were purchased from Merck Mumbai and Rankem (New Delhi, India). The analytical grade reagents, chemicals, and solvents were used in the study and obtained from Himedia (A-516, Mumbai 400086, India). Biochemical parameters were assessed using commercially accessible diagnostic kits obtained from Primal Healthcare Limited (Mumbai, India). Melting points were established by using a melting apparatus (Perfit); Bruker spectrospin (400 MHz) was used for recording ^{1}H NMR spectra employing $CDCl_3$ and dimethyl sulphoxide (DMSO)-d_6 solvents and tetramethylsilane (TMS) as an internal standard. δ (ppm) was used to denote the chemical shift with TMS and coupling constant (J, in Hz). For spin-coupled patterns, the notations used throughout are as follows: s = singlet, d = doublet, dd = double doublet, ddd = double-double doublet, qd = quaternary doublet, t = triplet, m = multiplet, and br = unresolved broad signal. ^{13}CNMR spectra were recorded on Advance DRY 300, Bruker spectrospin 100 MHz in 5 mm spinning tubes at 27°C, and mass spectra (MS) were examined using Electron Impact ionization at 70 eV on a UPLC system coupled to a Q-TOF Synapt mass spectrometer (Waters, USA) fitted with direct inlet probe system.

2.3 *In vitro* α-amylase inhibition test

The α-amylase repressive potential was measured using the technique described by Dong et al. [38], with some modifications. In this assay, a solution of the sample (40 µL) with DMSO or acarbose (in a mixture of sodium phosphate buffer [20 mM], pH 6.9 and 0.006 M sodium chloride) was mixed with α-amylase (1 U/mL in phosphate buffer, pH 6.9; 200 µL solution) and incubated for 30 min at room temperature. To each tube, 400 µL of 0.25% starch solution in phosphate buffer (pH 6.9) was added to initiate the reaction. The reaction was carried out at 37°C for 5 min. The reaction was terminated by adding 1.0 mL of the DNS reagent (12% sodium potassium tartrate in 0.4 M sodium hydroxide and 1% 3,5-dinitrosalicylic acid). The tubes containing the above contents were then kept in a water bath (boiling) for 10 min and then cooled to 25°C. The volume was made to 10 mL in each test tube by distilled water and absorbance (A) was recorded at λ_{max} of 540 nm. The control test tube incubation, representing 100% enzyme activity, was performed in the same manner by replacing the extract with a buffer solution. To measure the absorbance of the test samples, only the blank absorbance of the buffer solution (blank incubation) was determined. The α-amylase repressive action was defined as % inhibition and was estimated using the following formula:

$$\% \text{ Inhibition} = \frac{A_c - (A_t - A_b)}{A_c} \times 100,$$

where A_c is absorbance of 100% enzyme activity, A_t is the absorbance of the test sample with enzyme, and A_b is the absorbance of the test sample without enzyme. The sample potency is expressed by IC_{50}, which is the amount of extract required to inhibit 50% of enzyme activity.

2.4 α-Glucosidase inhibition assay

Dong et al.'s [38] method with slight modifications was used for evaluating α-glucosidase inhibition efficacy. In this assay, the test sample in DMSO (60 µL) or acarbose in DMSO and 50 µL of α-glucosidase mixture (0.2 U/mL in 0.1 M phosphate buffer, pH 6.8) was mixed and maintained at 37°C for 20 min in 96-well plate. About 50 µL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside in 0.1 M phosphate buffer solution (pH 6.8) was added to each well, followed by 20 min incubation at 37°C. To each well, 160 µL of 0.2 M sodium carbonate was added to halt the reaction. A microplate reader was used to record the absorbance (A) at 405 nm, which was matched with the control (60 µL of buffer instead of the test sample). The blank contained only the buffer, and the absorbance was measured. Percent inhibition, which indicated α-glucosidase inhibitory potential, was expressed as

$$\%\text{Inhibition} = \frac{A_c - (A_t - A_b)}{A_c} \times 100,$$

where A_c is the absorbance of 100% enzyme activity, A_t is the absorbance of the test sample with enzyme, and A_b is the absorbance of the test sample deprived of the enzyme. The sample efficacy is given by the IC_{50}, which is the amount necessary to stop 50% of the enzyme action.

2.5 Experimental animals

Male and female Wistar albino rats (150–200 g) were obtained from the Indian Institute of Integrative Medicine Jammu, J&K, and housed under restricted conditions (12-h light/12-h dark-cycle), temperature of 22°C ± 2°C and humidity of 45% ± 5%). The experimental rats were adapted to the environment before and during the experiment. Rats were fed rat feed (Ashirwad Industries, Mohali, Punjab, India), and water was provided *ad libitum*. The approval for this experimental study was granted under approval (No. F-IAEC (Pharm. Sc.) APPROVAL/2013/20, dated 19-09-2013), Institutional Animal Ethics Committee (IAEC), Department of Pharmaceutical Sciences, University of Kashmir, Hazratbal, Srinagar, India. The ethanol extract of *C. bursa-pastoris* (ECbp) and the standard drug were orally administered.

2.6 Acute toxicity testing

Oral acute toxicity assessment was conducted in compliance with the Organization for Economic Cooperation and Development Guidelines 423 (acute toxicity classic method) [39]. Rats were scrutinized separately at least once within the first half an hour after oral administration of ECbp (2,000 mg/kg), regularly in the initial 24 h, with particular care provided during the initial 4 h, and daily afterwards for 14 days to determine toxicity.

2.7 Glucose tolerance test

Fasting overnight Wistar albino rats (150–200 g) were chosen for the evaluation of glucose tolerance. The rats were divided arbitrarily into IV groups, with six rats (n = 6 per group). Group I was marked as the normal control, which was fed 1 mL/kg b.w. of vehicle (0.5% CMC in distilled water as a suspending agent). Group II served as the glucose-challenged control that received glucose (2 g/kg b.w.). Group III was fed glibenclamide (Glib) (5 mg/kg b.w.) as a standard drug [40]. Group IV was given 0.2 g/kg b.w. of ECbp. Experimental groups III and IV were given glucose after 20 min of administration of Glib and ECbp, respectively. Blood was collected from the tail vein for glucose estimation at time intervals of 0, 30, 60, and 120 min after glucose administration [41]. A one-touch glucometer (MyLife Pura, Switzerland) was used to measure the blood glucose levels. Blood glucose concentrations and the area under the curve (AUC) were calculated. The trapezoidal method was used to calculate AUC [42].

AUC was calculated using the following formula:

$$\text{AUC(mM/L/h)} = \frac{\text{BG0} + \text{BG30}}{2} \times 0.5 + \frac{\text{BG30} + \text{BG60}}{2} \times 0.5 + \frac{\text{BG60} + \text{BG120}}{2} \times 1,$$

where BG represents the blood glucose concentration recorded at 0, 30, 60, and 120-min time intervals.

2.8 Experimental design for *in vivo* anti-diabetic activity

Streptozotocin (50 mg/kg), freshly prepared in cold citrate buffer 0.1 M (pH 4.5), was administered by intraperitoneal injection to rats fasted overnight [43,44]. To avoid streptozotocin (STZ) provoked deadly hypoglycaemia, for the next 24 h, a 5% dextrose solution was given to rats after 6 h of streptozotocin (STZ) injection. STZ causes fatal hypoglycaemia due to huge pancreatic insulin discharge [45]. About 72 h after the injection of STZ, diabetes in rats was established by determining plasma glucose *via* the tail vein with a digital glucometer (MyLife Pura, Switzerland) by glucose oxidase-peroxidase method using the strip method. To stabilize plasma glucose levels, diabetic rats were maintained under optimal laboratory conditions for 2 weeks [43]. After 2 weeks of diabetes induction, plasma glucose level was once more ascertained, and rats with equal or more than 200 mg/dL blood glucose levels were chosen for further study. The rats in this study were arbitrarily divided into four groups, with six rats in each group (n = 6).

Group I: Normal control (1 mL/kg. b.w., 0.5% carboxymethyl cellulose in water, orally)

Group II: STZ (Toxic control given 50 mg/kg, b.w. orally)

Group III: streptozotocin (STZ) + Glib (5 mg/kg, b.w. orally) [46]

Group IV: streptozotocin (STZ) + ECbp (200 mg/kg, b.w. orally)

Glib, ECbp extract, and blank vehicle were fed orally to their respective rat groups for 21 days at 10.00 a.m. daily. Initially and every week (0, 7, 14, and 21 days), fasting plasma glucose levels and any change in the body weight of rats were estimated. At the termination of the third week, that is on 21st day, vehicle, ECbp, and Glib were given to the night-long fasted

Table 1: α-Amylase and α-glucosidase % inhibition by standard acarbose and ECbp

Conc. (mg/mL)	Acarbose		ECbp	
	α-Amylase	α-Glucosidase	α-Amylase	α-Glucosidase
0.150	31.29 ± 4.35	16.81 ± 2.76	7.20 ± 1	9.72 ± 1.92
0.310	46.74 ± 4.18	28.94 ± 2.67	11.31 ± 2.1	14.97 ± 1.99
0.620	54.51 ± 2.61	39.44 ± 3.35	14.35 ± 1.34	21.55 ± 5.01
1.250	61.64 ± 4.21	51.24 ± 2.49	26.44 ± 4.87	35.44 ± 6.22
2.500	74.27 ± 3.02	63.82 ± 5.88	32.96 ± 6.4	49.32 ± 3.42
5.000	81.33 ± 2.31	76.82 ± 4.49	56.02 ± 4.52	62.92 ± 6.51
IC_{50}	0.46 ± 0.02	1.98 ± 0.08	4.28 ± 0.81	2.57 ± 0.89

rats, and 1-h after treatment, all rats were anaesthetized with chloroform. Plasma samples were drawn through retro-orbital plexus penetration for biochemical analyses.

2.9 Biochemical studies

Serum glucose levels were estimated using a glucometer. Commercially available diagnostic kits were used to estimate serum lipid profiles, including triglyceride (TG), high-density lipoprotein (HDL), and total cholesterol (TC). Friedewald's formula was used to determine the serum very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) concentrations as follows:

$$\text{V-LDL} = \text{Triglycerides}/5,$$

$$\text{LDL} = \text{TC} - (\text{HDL} + \text{VLDL}).$$

A semi-autoanalyzer (Photometer 5010V5^{+}, Germany) was used to determine all the biochemical parameters.

2.10 Isolation of compounds

ECbp (135 g) was subjected to column chromatography (52″:2.5″) over a silica gel (200–400 mesh, 200 g) bed using hexane as the eluent with increasing amounts of ethyl acetate. Individual 100 mL fractions were collected, and similar fractions were pooled after monitoring the TLC by using anisaldehyde-sulphuric acid reagent as a visualizing agent, which was prepared by adding 0.5 mL of anisaldehyde and 10 mL of glacial acetic acid to 85 mL of methanol and 5 ml of concentrated sulphuric acid, in that order. The development and elution of the column were performed with successive sequences of solvents in many combinations, hexane–ethyl acetate ([95:05] three fractions each of 100 mL, 95:05 [7 frs.], 95:05 [6 frs.], 90:10 [6 frs.] v/v), which yielded 22 fractions (Frs. A-V). The first three fractions (A-C) were pooled after monitoring by TLC, which showed a prominent single spot and was labelled as compound ECbp-1. Similarly, on the basis of TLC, fractions Q–V were pooled and showed similar single spot and was labelled as ECbp-2.

2.11 Statistical assessment

The data were expressed as mean ± SD, and were assessed by one-way analysis of variance. For multiple comparisons, Dunnett's test was applied using Graphpad Prism version 5.0, and p values < 0.05 were considered statistically significant.

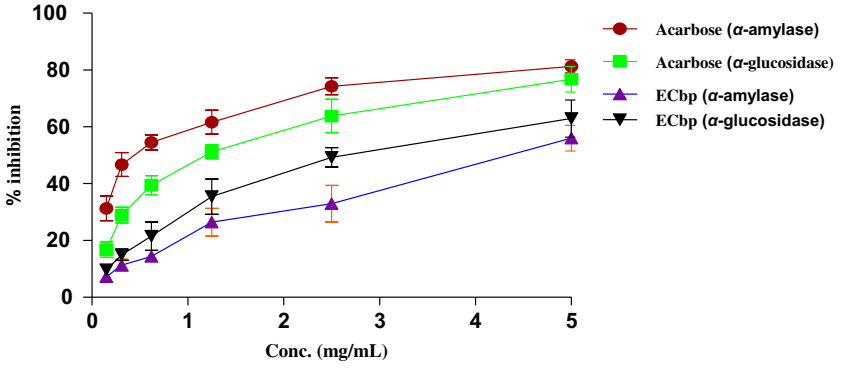


Figure 1: α-Amylase and α-glucosidase enzyme inhibition ability of acarbose and ECbp.

3 Results and discussion

The percentage yield of ECbp extract was 9.4% (348.6 g). ECbp and acarbose were tested in the concentration range of 0.15–5 mg/mL for *in vitro* inhibition assay of α-amylase and α-glucosidase. The IC_{50} values, which represent the concentration required to inhibit 50% of enzyme activity for α-amylase and α-glucosidase in acarbose were 0.46 ± 0.02 and 1.98 ± 0.08, respectively. For ECbp, they were 4.28 ± 0.81 and 2.57 ± 0.89 for α-amylase and α-glucosidase, respectively (Table 1 and Figure 1).

3.1 Acute toxicity study

ECbp at a dose of 2,000 mg/kg in rats after oral administration did not produce any signs of toxicity, and no fatality was observed for up to 14 days. The results of this toxicity test revealed that up to an oral dose of 2,000 mg/kg b.w. ECbp was nontoxic. Therefore, 1/10th of 2,000 mg/kg b.w., which is equal to 200 mg/kg b.w. dose was selected for determining the activity.

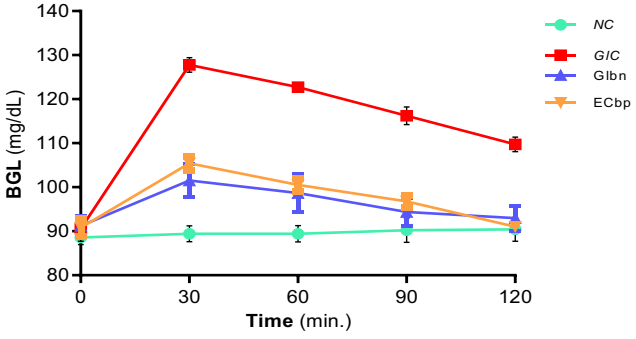


Figure 2: Effect of ECbp extract on blood glucose levels in glucose-challenged rats. Data represented as mean ± SD, *n* = 6; BGL: blood glucose level; NC: normal control; GlC: glucose control; Glib: glibenclamide; ECbp: ethanol extract of *C. bursa-pastoris*.

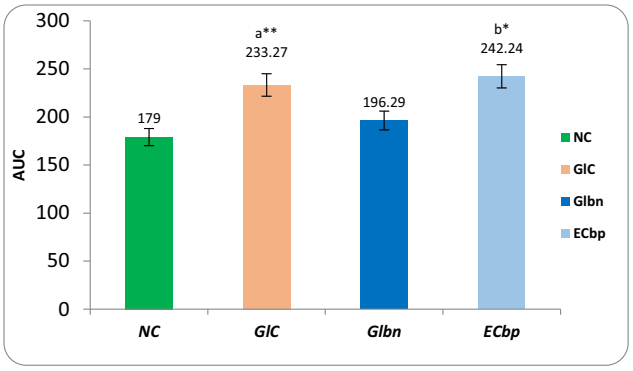


Figure 3: Effect of ECbp on AUC of blood glucose levels of glucose-challenged diabetic rats. Data represented as mean ± SD; NC: normal control; GlC; glucose control; Acar: acarbose; Glbn: glibenclamide; ECbp: ethanol extract of *C. bursa-pastoris*. [a]Glucose-challenged control GlC vs normal control; [b]treated group vs glucose-challenged control; *$p < 0.05$, **$p < 0.01$.

3.2 Effect of ECbp on plasma glucose of glucose-challenged rats

A significant ($p < 0.001$) increase in blood glucose levels was observed after STZ administration in rats compared with that in normal control rats. ECbp at a dose of 200 mg/kg b.w. and Glib (5 mg/kg b.w.) via oral treatment led to a significant decrease in blood glucose level ($p < 0.001$) in the diabetic rats compared to those in the diabetic control group rats (Figure 2).

At a dose of 200 mg/kg b.w., ECbp extract treatment exhibited a reduction trend in the plasma glucose levels from 83, 105.4, 100.5, 96.8, and 91.03 mg/dL at 0, 30, 60, 90, and 120 min, respectively, while as standard Glib (5 mg/kg, b.w.) exhibited a significant reduction trend in plasma glucose levels from 85.9, 112.5, 110,104.8, and 100.6 mg/dL after 0, 30, 60, 90, and 120 min, respectively. The toxic group showed 90.6, 127.8, 122.7, 116.2, and 109.4 mg/dL after 0, 30, 60, 90, and 120 min, respectively (Figure 2).

The percentage reduction in AUC by ECbp (200 mg/kg, b.w) in glucose-fed rats was found to be 9.76% compared to

Table 2: Effect of 21 days treatment of ECbp (200 mg/kg b.w.) on plasma glucose levels in diabetic rats

Treatment groups	Fasting plasma glucose level (mg/dL)				% Increase in FBG vs NC	% Reduction in FBG vs toxic
	0 day	7 day	14 day	21 day		
NC	89.4 ± 3.64	91.6 ± 3.78	92.0 ± 4.84	91.4± 3.13	100	—
DC (STZ)	217 ± 4.52^{a**}	221.8 ± 5.1^{a**}	219.8 ± 6.18^{a**}	217.6 ± 9.02^{a**}	138.07	100
STZ + Glbn	211.4 ± 5.94	179.2 ± 2.27^{b*}	146.2 ± 4.54^{b**}	100.3 ± 3.16^{b**}	9.74	53.91
STZ + ECbp	212.6 ± 6	188.2 ± 6.48^{b*}	159.1 ± 3.55^{b*}	124.6 ± 3.56^{b**}	36.32	42.74

Data as presented as mean ± SD, *n* = 6; FPG: fasting plasma glucose: STZ: streptozotocin; Glbn: glibenclamide; ECbp: ethanol extract of *C. bursa-pastoris*; [a]Diabetic control versus normal control, [b]Treated group versus diabetic control, *$p < 0.05$, **$p < 0.01$.

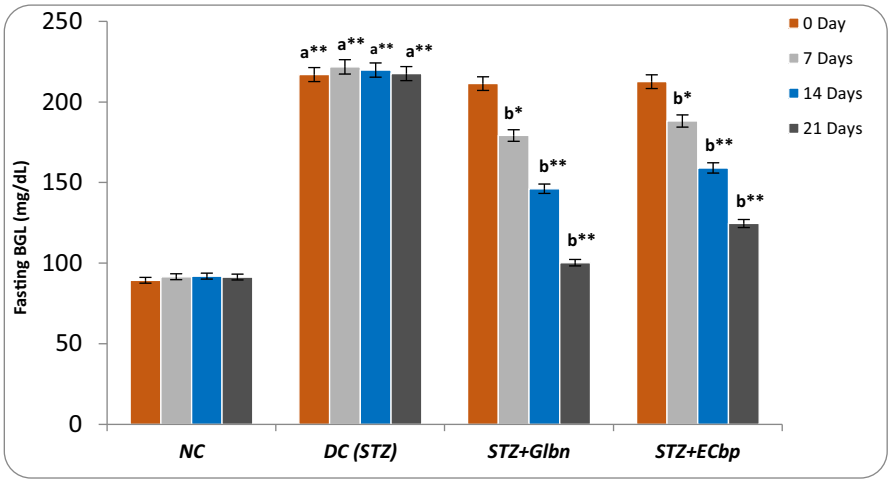


Figure 4: Effect of 21 days treatment with ECbp (200 mg/kg b.w.) on plasma glucose in STZ-induced diabetic rats. *C. bursa-pastoris* (ECbp) ethanol extract at 0.2 g/kg, b.w, dose for 3 weeks in rats prevented the weight loss of rats ($p < 0.05$) compared to that in diabetic control. A notable ($p < 0.001$) reduction in the average body weight with feeding STZ was noticed in diabetic rats compared to that in normal control rats (Figure 5). In diabetic rats, treatment with ECbp and Glib significantly ($p < 0.01$ and $p < 0.01$) increased body weight compared to diabetic control rats.

the standard Glib (32.73%). ECbp leads to decreases in AUC and was comparable to that of Glib (Figure 3).

3.3 Effect on plasma glucose levels and body weight

The effect of 21 days of ECbp treatment on fasting blood glucose (FBG) in diabetic rats was studied (Table 2, Figure 4). STZ (50 mg/kg, b.w., i.p.) successfully induced diabetes and significantly ($p < 0.01$) increased fasting blood glucose (FBG) compared with normal control rats. Treatment with ECbp (200 mg/kg, b.w., p.o) showed a significant ($p < 0.05$) decrease in the FBG levels on the 21st day compared to that in diabetic control rats (Figure 4). The decrease in FBG was significantly ($p < 0.01$) enhanced by ECbp (42.74%) after 21 days treatment compared to the toxic control (STZ), while Glib (5 mg/kg, b.w) showed 53.91% reduction in FBG compared to toxic control after 21 days treatment.

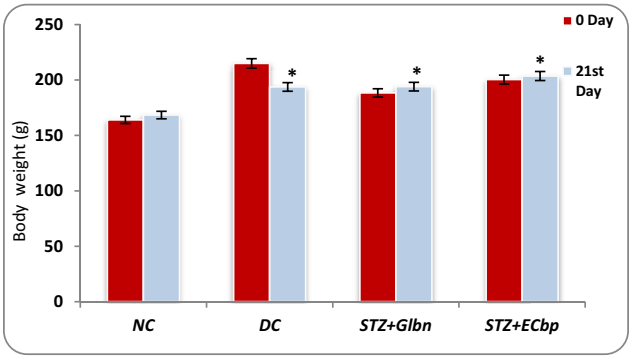


Figure 5: Effect of ECbp on average body weight of diabetic rats. Data are presented as mean ± SD, $n = 6$, NC: normal control (1 mL/kg b.w. of 0.5% CMC in distilled water); DC: diabetic control (STZ, 50 mg/kg, b.w., *i.p.*); Glbn: glibenclamide (5 mg/kg, b.w); ECbp: ethanol extract of *C. bursa-pastoris*; *$p < 0.05$.

3.4 Effect on serum lipid profile

Treatment with the ECbp extract for 21 days at a dose of 0.2 g/kg, b.w. notably reduced the elevated levels of all cholesterols, viz., TC, LDL-C, VLDL-C, and TGs, significantly ($p < 0.05$) compared to diabetic control. However, the HDL-cholesterol levels were significantly ($p < 0.01$) increased compared to diabetic control rats. In the Glib-treated rat group, significantly ($p < 0.01$) reduced elevated levels of all these cholesterol levels were observed (Figure 6). Additionally, a significant reduction in atherogenic index was also found ($p < 0.01$) in the ECbp treatment group and produced a pronounced normalization of lipid profile significantly ($p < 0.05$) compared to the diabetic control. Also, the HDL-cholesterol levels were significantly increased ($p < 0.01$) compared to the diabetic control, besides improvement in the atherogenic index (Figure 7).

3.5 Isolated compounds from ECbp

ECbp-1 (109 mg) was obtained as a white crystalline mass, melting point 192 ± 5°C from hexane–ethyl acetate (95:5 v/v) eluent.

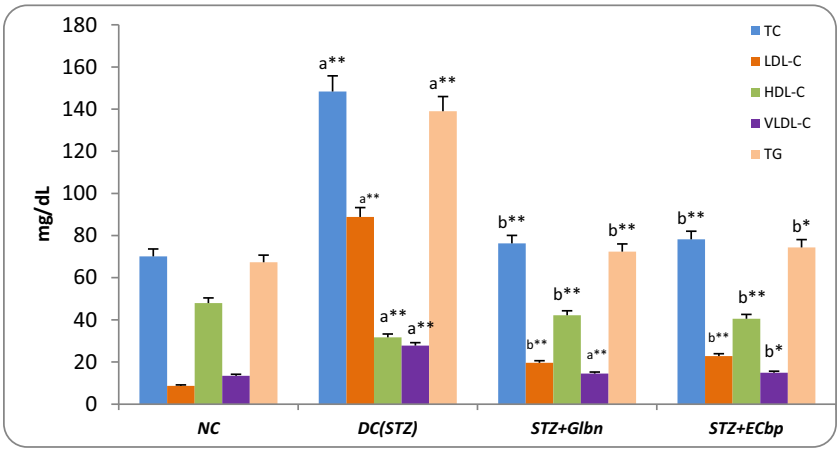


Figure 6: Effect of 3 weeks treatment of ECbp (0.2 g/kg b.w) on the lipid profile of diabetic rats. Data are presented as mean ± SD, n = 6. NC: normal control; DC: diabetic control; Glbn: glibenclamide; ECbp: ethanol extract of *C. bursa-pastoris*. [a]Diabetic control vs Blank control, [b]treated group vs diabetic control, $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$.

^{1}H NMR: ($CDCl_3$, 300 MHz): δ_H 5.68 (1H, dd, J = 11.2, 3.9 Hz, H = 11), 5.33 (1H, d, J = 4.8, H-12), 4.15 (2H, d, J = 7.2 Hz, H-28), 4.08 (2H, d, J = 6.9 H-21), 3.45 (1H, s, OH), 2.82 (1H, dd, J = 4.8, 11.1 Hz, H-7), 1.27 (3H, s, H-29), 1.18 (3H, s, H-19), 0.97 (3H, s, H-18), 0.87 (3H, d, J = 6.6 Hz, H-27), 0.83 (3H, d, J = 6.6 Hz, H-26)

^{13}C NMR: ($CDCl_3$, 75 MHz): δ_C 33.9 (C-1), 29.6 (C-2), 34.0 (C-3), 40.5 (C-4), 50.3 (C-5), 22.5 (C-6), 68.8 (C-7), 26.8 (C-8), 41.2 (C-9), 34.2 (C-10), 130.0 (C-11), 127.6 (C-12), 34.4 (C-13), 36.0 (C-14), 20.7 (C-15), 22.4 (C-16), 34.5 (C-17), 11.2 (C-18), 18.5 (C-19), 33.7 (C-20), 60.2 (C-21), 31.4 (C-22), 29.3 (C-23), 29.2 (C-24), 28.9 (C-25),13.9 (C-26), 14.0 (C-27), 62.0 (C-28), 20.7 (C-29), 170.9 (C-30)

+ve ESI-MS m/z (rel. int.): 490 $[M]^+$ $C_{30}H_{50}O_5$ (29), 489 $[M\text{-}H]^+$ (100), 445 $[M\text{-}CO_2]^+$ (3)

Compound ECbp-2 (73 mg) was obtained as an orange-coloured shining waxy mass with a melting point 171 ± 5°C from the ethanol fraction using hexane:ethylacetate (85:15 v/v) as an eluent. On the basis of ^{13}C NMR and MS, the molecular mass of ECbp-2 was established at m/z 332. Its ESI-MS spectrum displayed a pseudomolecular ion peak at m/z 333, corresponding to the molecular formula $C_{20}H_{29}O_4$ $[M + H]^+$ of a diterpenoic acid. The formula indicated the presence of seven double bond equivalents, four of which were attributed to four vinylic linkages, two to a bicyclic carbon framework of a clerodane moiety, and the remaining one to a carboxylic group.

^{1}H NMR: ($CDCl_3$, 300 MHz): δ_H 6.20 (1H, d, J = 6.5 Hz, H-11), 5.41 (4H, *brm*, H-3, H-6, H-7, H-12), 3.63 (1H, *dt*, J = 6.0, 6.9 Hz, H-2), 4.14 (2H, *brs*, H-20), 2.80 (1H, *m*, H-13), 1.63 (2H, *m*, H-1), 1.33 (6H, *s*, H-17, H-19), 1.03 (2H, *m*, H-14), 1.25 (3H, *s*, H-18), 0.87 (3H, *t*, J = 6.5 Hz, H-15).

^{13}C NMR: ($CDCl_3$, 125 MHz): δ_C 39.8 (C-1), 62.3 (C-2), 127.1 (C-3), 131.9 (C-4), 39.3 (C-5), 130.2 (C-6), 128.3 (C-7), 128.2 (C-8), 145.2 (C-9), 37.3 (C-10), 127.7 (C-11), 127.1 (C-12), 50.6 (C-13) 23.1 (C-14), 14.1 (C-15), 179.0 (C-16), 20.5 (C-17), 22.6 (C-18), 19.4 (C-19), 65.0 (C-20).

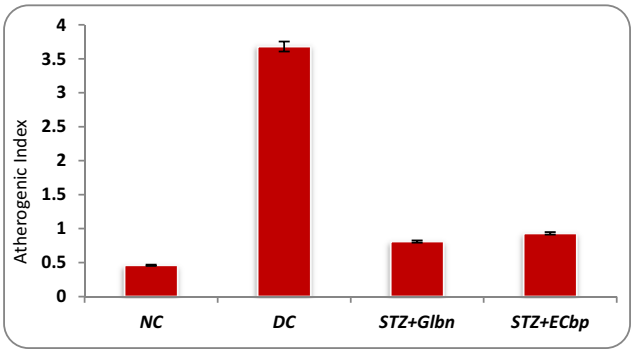


Figure 7: Effect of 3 weeks treatment of ECbp (0.2 g/kg b.w) on the atherogenic index. Data are expressed as mean ± SD, n = 6. NC: normal control; DC: diabetic control; Glbn: glibenclamide; ECbp: ethanol extract of *C. bursa-pastoris.*

ESI-MS *m/z* (*rel. int.*): 333 $[M + H]^+$ $C_{20}H_{29}O_4$(10), 275(8) 7,21,28-trihydroxy-lanosta-11-en-30-oic acid 2,20-dihydroxy-cleroda-3, 6, 8, 11-tetraen-16-oic acid.

3.6 α-Amylase and α-glucosidase inhibition assays

In vitro α-amylase and α-glucosidase inhibition assays were carried out on ECbp-1, ECbp-2, and acarbose in the concentration range of 0.15 to 5 mg/mL. The compounds showed strong inhibition of both the carbohydrate-digesting enzymes in a concentration-dependent manner (Figure 8).

The use of herbal remedies by humans has been well documented for thousands of years. Two ethnic systems of alternative medicine, i.e. Ayurveda and Unani, document various crude drug formulations for the management of numerous ailments. These remedies contain a variety of extracts from different herbs [43], and various herbs have been part of traditional remedies in several cultures as a remedial choice against diabetes mellitus worldwide [47]. Although several oral and systemic anti-diabetic treatment options are available in the market, the need for natural anti-diabetic drug options is increasing [48,49]. The main action of α-amylase and α-glucosidase is the hydrolysis of carbohydrates into monosaccharide glucose. α-Amylase controls the hydrolysis of polysaccharide-starch, which leads to glucose prior to systemic entry [50]. Inhibition of α-amylase can cause a decline in postprandial high glucose levels. The presence of α-glucosidase in the small intestine is responsible for the production of glucose via disaccharide hydrolysis. Numerous phytoconstituents exist in the plant kingdom and possess α-glucosidase inhibition potential [37]. ECbp is a generous example with both α-amylase and α-glucosidase inhibitory potential, which leads to the minimum absorption of monosaccharide-glucose. The

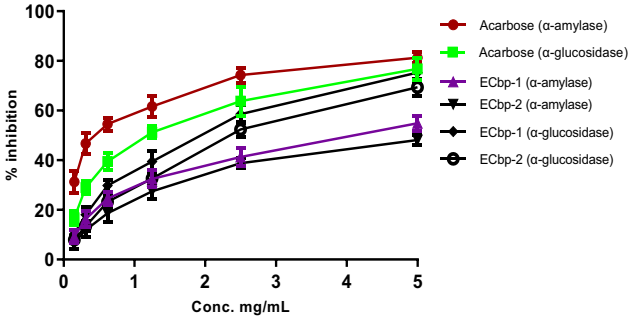
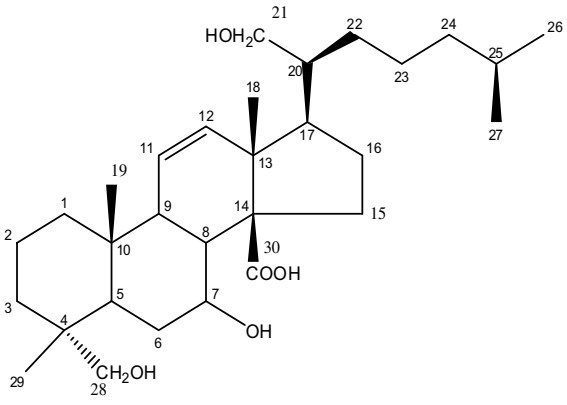


Figure 8: α-Amylase and α-glucosidase inhibition ability of acarbose, ECbp-1, and ECbp-2.

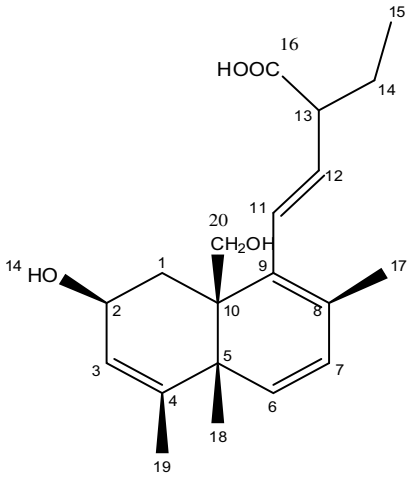
inhibition of both of these enzymes avoids the abrupt upsurge in postprandial hyperglycaemia and plays a dominant role in the management of DM; thus, targeting α-glucosidase and α-amylase inhibition may be a challenging goal to control the sudden rise in hyperglycaemia [51]. The results obtained in this study agree with those of a number of other studies [52,53]. Numerous medicinal plants and their different parts can reduce the plasma glucose concentration. This ability is due to the tannins, terpenoids, and flavonoids present in these herbs, which have been evaluated for their potential to inhibit α-amylase and α-glucosidase [54]. The α-amylase and α-glucosidase inhibitory actions of terpenoids, tannins, and flavonoids from plants have also been well documented. The α-amylase suppressing capacity of tannins was attributed to their ability to combine with carbohydrates and proteins [55]. Some examples of terpenes possessing action against DM are stevioside and its aglucon Steviol from *Stevia rebaudiana,* bassic acid, from *Bumelia sartorum*, trans-dehydrocrotonin from *Croton cajucara,* D-limonene from citrus fruits, rebaudioside from *Stevia rebaudiana* lupeol from mango, alpha-amyrin from *Ficus bengalensis,* Betulin from *Euclea undulata,* tinosporaside from *Tinospora cordifolia,* lactucain A from *Lactuca indica,* urosolic acid from *Rosmarinus officinalis,* and palbinone from *Moutan cortex* [30].

In this study, inhibition of both enzymes was concentration-dependent by both ECbp and acarbose (Table 1). This was also evidenced by comparing their IC_{50} values (Figure 1). ECbp inhibited α-glucosidase (IC_{50} = 2.57 ± 0.89) more effectively than α-amylase (IC_{50} = 4.28 ± 0.81) as compared to the standard drug acarbose with α-amylase (IC_{50} = 0.46 ± 0.02) and α-glucosidase (IC_{50} = 1.98 ± 0.08). The research proposes that rats treated with streptozotocin target damage to insulin-secreting β-cells of the pancreas, thereby producing a diabetic condition. Insufficient levels of insulin further result in the inability of cells to use glucose, resulting in the formation of reactive oxygen species [56]. Additionally, these experimental rats demonstrate various diabetic malfunctions, such as cardiomyopathy, retinopathy, and nephropathy, which mainly develop through oxidative stress-induced mechanisms [3]. Body weight drop due to the disproportion of metabolic pathways is normally linked to DM [57]. In the current study, diabetic rats treated with ECbp gained significant weight, most likely due to repealing glycogenolysis and gluconeogenesis, thereby helping to restore normal metabolic pathways [58]. ECbp at a dose of 0.2 g/kg, b.w for 21 days in rats prevented the weight loss ($p < 0.05$) compared to the diabetic control. In this study, a significant ($p < 0.001$) reduction in body weight was observed in diabetic rats (STZ) compared with that in normal control rats (Figure 5). In diabetic rats, ECbp



ECbp-1

7, 21, 28- trihydroxy-lanosta-11-en-30-oic acid



ECbp-2

2,20-dihydroxy-cleroda-3, 6, 8, 11-tetraen-16-oic acid

Figure 9: Structures of ECbp-1 and ECbp-2.

treatment and Glib significantly ($p < 0.01$ and $p < 0.01$) showed increased body weight compared to the diabetic control rats. In contrast, an abnormal lipid profile was observed in diabetic rats compared to the normal group. This may be due to an imbalance in the various metabolic and regulatory pathways that have developed, which is mainly due to the deficiency of insulin [59]. Treatment with the ECbp at 0.2 g/kg, b.w. decreased the elevated levels of all cholesterols (TC, LDL-C, and VLDL-C) and TGs significantly ($p < 0.05$), whereas the HDL-cholesterol levels were significantly ($p < 0.01$) increased compared to diabetic control. On the other hand, Glib treatment also significantly ($p < 0.01$) reduced elevated levels of all TC, LDL-C, and VLDL-C and TGs (Figure 6). An improvement in the atherogenic index was also observed after treatment with ECbp (Figure 7).

Based on ^{13}C NMR and MS details, the molecular weight of ECbp-1 was found to be *m/z* 490, consistent with the molecular formula $C_{30}H_{50}O_5$. It showed the presence of six double-bond equivalents, four of which were present in the tetracyclic carbon framework of lanostane and one each in a carboxylic group and vinylic linkage. The ^1H NMR band of ECbp-1 exhibited two downfield signals at δ values of 5.68 (dd, J = 11.2, 3.9 Hz) and 5.33 (d, J = 4.8 Hz) ascribed to H-11 and H-12 vinylic protons, respectively. Two doublets at δ 4.15 (J = 7.2 Hz) and 4.08 (J = 6.9 Hz), each assimilating for two protons, were attributed to H-26 and H-21 hydroxymethyl protons, respectively. A double doublet at δ 2.82 (J = 4.8, 11.1 Hz) was assigned to the H-7 carbinol proton. The methyl protons appearing at δ 1.27 (s, H-29), 1.23 (s, H-28), 1.18 (s, H-19), 0.97 (s, H-18), and 0.87 (d, J = 6.6 Hz, H-27) supported the presence of lanostane nucleus. This was further confirmed by the presence of 30 signals in the ^{13}C NMR spectrum. Prominent signals appeared for carboxylic carbon at δ 170.9 (C-30); vinylic carbon at δ 130.0 (C-11) and 127.6 (C-12); hydroxymethyl carbons at δ 60.2 (C-21) and 62.0 (C-26); and carbinol carbon at δ 68.8 (C-7). The NMR data were compared to those in earlier reports [60]. On the basis of the above discussion, the structure of ECbp-1 was elucidated as 7,21,28-trihydroxy-lanosta-11-en-30-oic acid (Figure 9).

The ^1H NMR band of ECbp-2 showed two downfield signals at δ 6.20 (1H, *d*, J = 6.5 Hz, H-11) and 5.41 (4H, m, H-3, H-6, H-7, H-12) for vinylic protons. A double-triplet at δ 3.63 (J = 6.0, 6.9 Hz) was assigned to the H-2 carbinol proton. A two-proton broad singlet at δ 4.14 was assigned to hydroxymethyl protons (H-20). Two singlets at δ 1.33 (6H), 1.25 (3H), and a triplet at δ 0.87 (3H), attributable to H-17, H-19, H-18, and H-15 methyl protons, supported the presence of clerodane moiety. Further evidence supporting the proposed structure is derived from the ^{13}C NMR spectrum. ECbp-2 ^{13}C NMR band exhibited signals for carboxylic carbon at δ 179.0 (C-16); vinylic carbons at δ 127.1–131.9; and hydroxylated carbons at δ 62.3 (C-2) and 65.0 (C-20). The positions of the vinylic linkages, hydroxyl groups, and carboxylic function were established on the basis of the HMBC experiment. It displays key interactions between C-16 and H-13/H-15; C-3 and H-2/H-19; and C-8 and H-17/H-12. The NMR data of ECbp-2 were compared with the reported data for clerodane derivatives and found to be in good agreement [61]. Based on this analysis, the most likely structure of ECbp-2 was confirmed to be 2,20-dihydroxy-cleroda-3,6,8,11-tetraen-16-oic acid (Figure 9).

4 Conclusions

As indicated by the WHO, more than 176 million individuals worldwide experience diabetes, and this number is rising very fast; it is estimated that by the year 2030, this number will be twofold. Traditionally used herbs should be thoroughly studied because of the lack of side effects, as suggested by the WHO Expert Committee on Diabetes. Our study on *C. bursa-pastoris* ethanol extract showed promising results in improving serum glucose and lipid levels in STZ-induced diabetic rats, in addition to significant *in vitro* inhibition of carbohydrate digesting enzymes (α-amylase and α-glucosidase) responsible for postprandial hyperglycaemia in diabetic rats. Also, two potent compounds, a triterpene 7,21,28-trihydroxy-lanosta-11-en-30-oic acid (ECbp-1) and a diterpene 2,20-dihydroxy-cleroda-3,6,8,11-tetraen-16-oic acid (ECbp-2), showing promising inhibitory potential towards *α*-amylase and *α*-glucosidase enzymes were isolated from ECbp. Hence, it may be concluded that ECbp-1 and ECbp-2 are responsible for the anti-diabetic effect of the ECbp. In the future, an *in vivo* study will be conducted to prove and support the *in vitro* results of the isolated ECbp-1 and ECbp-2 terpenes.

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Conflict of interest: The authors state no conflict of interest.

Ethical approval: Under approval (No. F-IAEC (Pharm. Sc.) APPROVAL/2013/20), Institutional Animal Ethics Committee (IAEC), Department of Pharmaceutical Sciences, University of Kashmir, Hazratbal, Srinagar, India granted approval for this experimental study.

Data availability statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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