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Calamintha incana (Sm.) Helder: A New Phytoextract with In Vitro Antioxidant and Antidiabetic Action

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Abstract: This study aimed to assess the antioxidant and antidiabetic properties of ethanolic phytoextracts of *Calamintha incana* (Sm.) Helder leaves. Initially, the chemical characterization of the phytocomplex was performed using high-performance liquid chromatography (HPLC)/mass spectrometry (MS). The cytotoxicity of the ethanolic extract was assessed using an MTT assay in HepG2 cells. Subsequently, antioxidant activity was evaluated using a DPPH test. Finally, enzymatic tests with α-amylase, α-glucosidase, pancreatic lipase, and dipeptidyl peptidase IV (DPP-IV) were performed to evaluate their effects on glucose metabolism. The chemical composition of the extract is p-linolenic acid (13.2%), myristic acid (12.1%), and p-cymene (10.5%). The extract demonstrated low toxicity, with none of the tested concentrations inducing 50% cell death. Furthermore, the ethanolic extract revealed potent antioxidant activity using DPPH (IC_{50} was 35.9 ± 0.7 µg/mL) and reducing power capacity (IC_{50} was 90.3 ± 0.8 µg/mL). Regarding the antidiabetic activity, the extract caused a significant inhibition of α-amylase, α-glucosidase (IC_{50} 46.3 ± 0.2, 56.8 ± 0.1 µg/mL, respectively), weak inhibition of pancreatic lipase and no notable inhibition of dipeptidyl peptidase IV. In conclusion, *C. incana* has antioxidant and antidiabetic properties and appears to exert insulin-independent hypoglycemic action.

Keywords: *Calamintha incana*; antidiabetic activity; α-amylase; α-glucosidase; pancreatic lipase; dipeptidyl peptidase IV (DPP-IV)

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1. Introduction

Medicinal herbs have been used for millennia and are regarded as the roots of modern medicine. Plant-derived compounds are abundant sources of secondary metabolites for drugs [1], each with a unique physiological impact on the body [2]. Several secondary metabolites offer various pharmacological uses, including antioxidant, anticancer, antibacterial, antiviral, and antiprotozoal activities [3–6].

Owing to their numerous health advantages, including anti-inflammatory and anti-aging effects, antioxidants have recently gained significant scientific attention [7]. Free radicals are considered to be pathophysiological agents. Consequently, consuming antioxidants protects against oxidative stress by limiting the production of reactive species [8]. Additionally, experimental data show that high antioxidant levels may help reduce numerous forms of free-radical damage linked to diabetes mellitus (DM) development [8].

DM is a complicated multifactorial condition characterized by hyperglycemia (high blood sugar) and glucose intolerance, either due to a relative lack of insulin production or diminished capacity to effectively stimulate glucose absorption. Furthermore, as the disease progresses, it causes harmful changes in the body, such as nephropathy, retinopathy, and cardiovascular issues [9]. Diabetes is divided into two subtypes: type I and type II. While insulin replacement is often used to treat type I diabetes, oral hypoglycemic medications are used to treat type II diabetes [10].

Furthermore, many conventional diabetes treatments do not include a single dose, because most drugs involve recurrent injections, frequently throughout the life of patients with diabetes. Many standard treatments are ineffective and have adverse side effects [10].

Because of these constraints, management strategies for integrating medicinal plants have been researched [11]. These plants have been claimed to be less expensive antidiabetic drugs with fewer adverse effects. However, the ability of most conventional plants to cure diabetes has not yet been clinically verified.

Several studies have reported that phytochemicals, such as phenols, flavonoids, and terpenoids, may possess curative benefits in the treatment of diabetes and obesity complications, as well as inhibitory effects on α-amylase, α-glucosidase, lipase, and dipeptidyl peptidase IV (DPP-IV) [12–18]. Medicinal plants help maintain low blood glucose levels, prevent high blood pressure, boost the body's antioxidant system and insulin regulation, and are a safer option for managing obesity and diabetes [19].

Calamintha incana is an indigenous herb that has long been used in Jordan and other countries [20]. No scientific studies have been conducted to establish the effectiveness of this plant in the treatment of diabetes mellitus (DM). Thus, verifying its efficacy is critical, as this plant has the potential to significantly improve diabetes management. *Calamintha incana* (Sm.) Helder (family Lamiaceae) is known as 'Zataeman' in Jordan. The plant is distributed in Eastern Mediterranean, Central Asia, Europe, North Africa, and North America [21]. It is an aromatic plant used as a spice and herbal tea in Jordan and Levant [22]. In traditional medicine, the plant is used as an expectorant, antispasmodic, diaphoretic, and for the treatment of general weakness [23]. Furthermore, antibacterial [24], antioxidant [20,25], cytotoxic [20], and enzyme (tyrosinase, α-glucosidase, and α-amylase) inhibitory activity has been reported for *Calamintha incana* essential oil [25]. This study aimed to determine the chemical profile of *C. incana* leaves and examine their antioxidant and antidiabetic activities.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals, analytical solvents, and enzymes were of analytical grade and purchased from Sigma Chemical Co., St. Louis, MO, USA. DPP-IV inhibition assay kit (Cayman Chemical Kit, Item number: 700210, Ann Arbor, MI, USA). HepG2 liver cells (ATCC® HB-8065™) were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). RPMI1640 medium, penicillin–streptomycin, L-glutamine, and phosphate-buffered saline PBS were purchased from Euroclone, Italy. Fetal bovine serum (FBS) (Biowest, Nuaillé, France) and trypsin/ethylenediaminetetraacetic acid (EDTA) (PAN-Biotech, Aidenbach, Germany) were also obtained. The 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*(-tetrazolium bromide) (MTT) was purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Collection of C. incana

C. incana leaves were collected in the spring of 2022 from Ajloun, Jordan. The botanical identity of the plant was obtained from the Biological Sciences Department at the University of Jordan. CI-UJ-15 is a voucher specimen attributed to *C. incana* in the herbarium of the Department of Biological Sciences.

2.3. Preparation of C. incana Ethanolic Extract

For six weeks, the leaves were air-dried at room temperature in the dark before being processed into a powder with an electric blender. Powdered plants (50 g) were extracted in 500 mL of 100% ethanol (1:10 weight/volume ratio). The extract was maintained at 20 °C for three days with constant stirring, before filtering using a Buchner funnel and Whatman No. (1) filter paper. Under low pressure, a rotary evaporator was used to remove the solvents efficiently and gently from the samples. The crude extract was prepared and

stored in an airtight container at 20 °C until further examination [26]. The ethanolic extract content was 0.17%.

2.4. Identification of Chemical Compounds

The plant extracts were chromatographically analyzed using high-performance liquid chromatography (HPLC)/mass spectrometry (Kyoto, Japan), CBM-20A (CONTROL BUS MODULE), CTO-30A (COLUMN OVEN), LC-30AD (LIQUID CHROMATOGRAPH), SIL-30AC (AUTO SAMPLER), and LCMS-8030 (LIQUID CHROMATOGRAPH MASS SPECTROMETER) (Triple Quad Ms). Briefly, 25 mg of the dry crude extract was dissolved in 1 mL of methanol, and 10 µL of the sample solution was injected. Separations on an ACE 5 C18 (250 mm × 4.6 mm; 5 mm) column were accomplished with a flow rate of 0.8 mL/min at 30 °C. Mobile phases A:B:C (80:12:8) water with acetic acid (0.1%) (A), methanol with 0.1% acetic acid (B), and acetonitrile with 0.1% acetic acid (C), were used for the analysis (C). The elution program continued at 80:12:8 (A:B:C) for 8 min. The polarity was gradually increased by 25:60:15 at 40–45 min, then returned to the mobile phase (80:12:8) to recondition the column for 5 min. Extract samples and solvents for mobile phases were filtered using a 0.22 mm filtration system before processing HPLC injections (Millipore Corp., Billerica, MA, USA). Each sample was examined in triplicate. The chemical components of the plant samples were detected by comparing their retention indices (RI) and mass spectra (MS) with those in the NIST and WILEY mass spectral libraries.

2.5. Cell Culture

The HepG2 cells were grown in RPMI1640 media complemented with 10% (*v*/*v*) fetal bovine serum (FBS), 1% (*v*/*v*) penicillin–streptomycin, and 1% (*v*/*v*) L-glutamine, and incubated at 37 °C in a humidified atmosphere with 5% CO_2. When the cell culture flask reached 90% confluence, the old medium was removed and the surface was washed with 1× phosphate buffer saline (PBS). After 5 min of incubation, the cells were detached from the flask surface using trypsin/EDTA. Trypsin was deactivated by neutralizing it in an FBS-supplemented medium. The cell suspension was then transferred to fresh culture flasks and separated for further investigation [20].

Cytotoxicity Assay

The cytotoxicity of *C. incana* ethanolic extract against HepG2 cells was evaluated according to a previously published procedure [20]. The HepG2 liver cells were seeded into 96-well plates at a density of 1×10^4 cells/well in a volume of 100 mL, and maintained at 37 °C in a 5% CO_2 humidified incubator for 24 h, before being treated with 100 mL of plant extract at various doses (25, 50, 100, and 200 µg/mL) to the specific well. After 72 h, 20 mL of (5 mg/mL) MTT (freshly prepared by dissolving 50 mg MTT in 1 mL culture media) was added and incubated at 37 °C for 3 h. After aspirating the medium, MTT crystals (purple formazan) were dissolved in dimethyl sulfoxide (DMSO; 200 mg mL/well) to dissolve the formazan crystals generated in the cells. The absorbance was measured at 540 nm using a microplate reader (ELISA), the cytotoxicity of the *C. incana* ethanolic extract was determined as a percentage (%) of the control (medium alone), which was set to zero, and the IC_{50} values were determined. Doxorubicin (0.085–2.25 µg/mL) was used as the positive control.

$$\text{Percentage (\%) of cell death} = 1 - (\text{absorbance of treated cells/absorbance of untreated cells}) \quad (1)$$

2.6. Antioxidant Activity

2.6.1. DPPH Assay

The antiradical capacity of the ethanolic extract of *C. incana* was evaluated as defined by Chen et al. [27]. Briefly, 1 mL of an ethanolic extract of *C. incana* (10–100 µg/mL) was combined with 1 mL of a (0.1 mM) DPPH solution (dissolved in ethanol). Ascorbic acid (10–100 µg/mL) was used as the positive control. The mixture was then incubated for

30 min in the dark. Absorbance was measured against a blank (ethanol) at 517 nm. The DPPH radical scavenging activity of the extract and positive control was estimated using the formula (2). The assay was performed in triplicate, and the IC_{50} values of the extract and positive control value were assessed.

$$\text{DPPH scavenging capacity (\%)} = (\text{abs}_{\text{blank}} - \text{abs}_{\text{sample}}/\text{abs}_{\text{blank}}) \times 100 \quad (2)$$

abs: absorbance.

2.6.2. Reducing Power Assay

The reducing power of *C. incana* ethanolic extract was evaluated using the procedure described by Ekin et al. [28]. Briefly, 1 mL of ethanolic extract (10–100 µg/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The reaction mixture was incubated for 20 min at 50 °C, then quickly cooled, mixed with 2.5 mL of trichloroacetic acid (TCA) (10%), and centrifuged at 3000 rpm for 10 min. Of that, 2.5 mL of supernatant was collected and thoroughly mixed with 2.5 mL of distilled water and ferric chloride (0.1 mL), before allowing it to stand for 10 min. The absorbance of the mixture was measured at 700 nm against the blank (phosphate buffer at pH 6.6). Additionally, the reducing power of the *C. incana* ethanolic extract was evaluated using ascorbic acid as a positive control. The assay was performed in triplicate, and the IC_{50} values of the extract and positive control were determined.

2.7. Antidiabetic Activity

2.7.1. α-Amylase Inhibition Assay

Sagbo's method [29] was used to assess α-amylase activity. Fifteen (15) microliters of the extract at different doses (20, 40, 60, 80, and 100 mg/mL, diluted in phosphate buffer) or the positive control (acarbose: antidiabetic drug) were added to 5 mL of porcine pancreatic lipase solution in a 96-well plate. After 10 min of incubation at 37 °C, 20 mL of the starch solution was added, followed by another 30 min of incubation at 37 °C. The reaction was then paused by adding 75 mL of iodine reagent and 10 mL of 1 M HCl to each well. The blank phosphate buffer (pH 6.9) was prepared instead of the extract and the positive control. For each test sample, no enzyme or starch controls were used. The % inhibitory activity was estimated using Equation (3), after determining the absorbance at 580 nm. The assay was conducted in triplicate.

$$\text{\% Inhibition} = (1 - \text{absorbance of the untreated (control)/absorbance of the test well}) \times 100 \quad (3)$$

2.7.2. α-Glucosidase Inhibition Assay

Alpha-glucosidase inhibition tests were performed as described by Sancheti et al. [30]. Briefly, 20 mL of 50 mg/mL α-glucosidase solution was added to 5 mL of the plant extract (20–100 mg/mL) in 96-well plates. Next, 60 mL of potassium phosphate buffer (pH 6.8) containing 67 mM potassium phosphate was added. After 5 min of incubation, 10 mL of 10 mM p-nitrophenyl-α-d-glucopyranoside solution was added and incubated for an additional 20 min at 37 °C. Subsequently, 25 mL of 100 mM sodium carbonate (Na_2CO_3) solution was added. Blanks were prepared by adding 5 mL of deionized water instead of the plant extract, and 20 mL of deionized water instead of the enzyme. Epigallocatechin gallate (20–100 mg/mL) was used as a positive control. The percentage of inhibition was calculated using Equation (4), after measuring the absorbance at 405 nm. The assay was conducted in triplicate.

$$\text{\% Inhibition} = (1 - \text{abs of the test well/abs of the untreated (control)}) \times 100 \quad (4)$$

abs: absorbance.

2.7.3. Pancreatic Lipase Inhibition Test

The assay was performed using a previously termed procedure [31]. Briefly, 96-well plates were filled with 10 mL of ethanolic extract (20–100 mg/mL), positive control (reference drug: orlistat; 20–100 mg/mL), or negative control (distilled water). Next, 40 mL of porcine pancreatic solution (10 mg/mL) in 50 mM Tris-HCl buffer was prepared (pH 8.0). After 15 min, 150 mL of substrate solution (20 mg p-nitrophenyl phosphate (pNPP) in 2 mL isopropanol was added to 18 mL 50 mM Tris-HCl buffer (pH 8.0), and incubated for 25 min at 37 °C. The absorbance was measured at 405 nm. Equation (5) was used to calculate the percentage of inhibition. The test was performed in triplicate.

$$\% \text{ Inhibition} = (1 - \text{abs sample/abs untreated (control)}) \times 100 \quad (5)$$

abs: absorbance.

2.7.4. Dipeptidyl Peptidase IV (DPP-IV) Inhibition Test

The potential inhibitory effect of ethanolic extracts of *C. incana* on the dipeptidyl peptidase IV (DPP-IV) was assessed using an assay kit (Cayman chemical kit, item number: 700210, Ann Arbor, MI, USA). The chromogenic substrate (gly-pro p-nitroanilide) was cleared by the serine protease DPP-IV, resulting in the release of 4-p-nitroaniline (pNA), a yellow product. Briefly, in a 96-well plate, 35 µL of the ethanolic extract (20–100 µg/mL) or positive control (Sitagliptin; 20–100 µg/mL) was added to 15 µL of dipeptidyl peptidase IV (DPP-IV) solution, 0.1 M Tris-HCL buffer (pH 8.0). The mixture was incubated for 5 min at 37 °C. Then, 50 µL of 20 mM of the substrate (gly-pro p-nitroanilide) was dissolved in Tris buffer to commence the reaction, and incubated further for 30 min at 37 °C. Subsequently, 25 µL of 25% acetate buffer (pH 4.5) was added to stop the reaction. Absorbance was measured at 405 nm using a microplate reader. Instead of the plant extract, a blank was prepared using 35 mL buffer. [32]. DPP-IV enzyme activity decreased when the synthesis of the pNA yellow product was reduced, owing to enzyme inhibition. The assay was conducted in triplicate. Equation (6) was employed to compute the % inhibition:

$$\% \text{ Inhibition} = (1 - \text{abs of the test well/abs of the untreated (control)}) \times 100 \quad (6)$$

abs: absorbance.

2.8. Statistical Analysis

All assays were performed in triplicate. Data are expressed as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for statistical analysis using GraphPad Prism 9.0.2 (GraphPad Software, San Diego, CA, USA). Differences between samples were determined using a Tukey's post hoc test. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Phytochemical Analysis of C. incana Ethanolic Extract

The chemical compositions of the constituents of the *C. incana* ethanolic extract, along with their percentage content, order of elution, and retention time, are listed in Table 1. Chemical analysis of the extract revealed 27 components, accounting for 91.5% of the overall composition. The HPLC/MS chromatogram of the extract is shown in Figure 1.

The ethanolic extract of *C. incana* was characterized by high levels of linolenic acid (13.2%), myristic acid (12.1%), and p-cymene (10.5%). Fatty acids constituted the highest class of identified compounds (31.3%), followed by sesquiterpenes (23.9%), and monoterpenes (20.7%). In contrast, ketones constituted the lowest percentage class in the extract (0.2%).

Table 1. Chemical composition of *C. incana* ethanolic extract.

Number	RT (min)	Compounds	Peak Area%
1	2.0	Azulene	0.2
2	3.5	3-Octanol	4.3
3	5.5	p-cymene	10.5
4	7.0	Limonene	2.1
5	9.5	3-nonanone	0.2
6	10.2	Unknown	1.2
7	11.5	Camphor	3.5
8	12.1	1,8-Cineole	0.2
9	12.5	Unknown	3.7
10	15.3	p-Coumaric acid	0.6
11	16.0	Jasmone	2.8
12	17.0	Piperitone oxide	4.3
13	19.5	Unknown	2.3
14	20.2	Gallic acid	1.1
15	20.5	Caffeic acid	0.8
16	21.8	beta-Damascenone	0.1
17	23.0	Calamenene	0.7
18	25.0	Caryophyllene	2.2
19	26.8	δ-cadinene	6.1
20	29.8	Spathulenol	7.4
21	30.0	α-bisabolol	4.7
22	30.8	Myristic acid	12.1
23	32.5	Palmitic acid	6.0
24	34.0	Linolenic acid	13.2
25	35.5	Catechin	0.4
26	38.0	Quercetin	2.5
27	40.0	Chlorogenic acid	5.5
Total identified compounds %			91.5%
RT: Retention Time			
Fatty acids			31.3%
Sesquiterpenes			23.9%
Monoterpenes			20.7%
Phenols			7.2%
Alcohol			4.3%
Flavonoids			2.9%
Aromatic acid			0.8%
Non-benzenoid aromatic hydrocarbon			0.2%
Ketone			0.2%

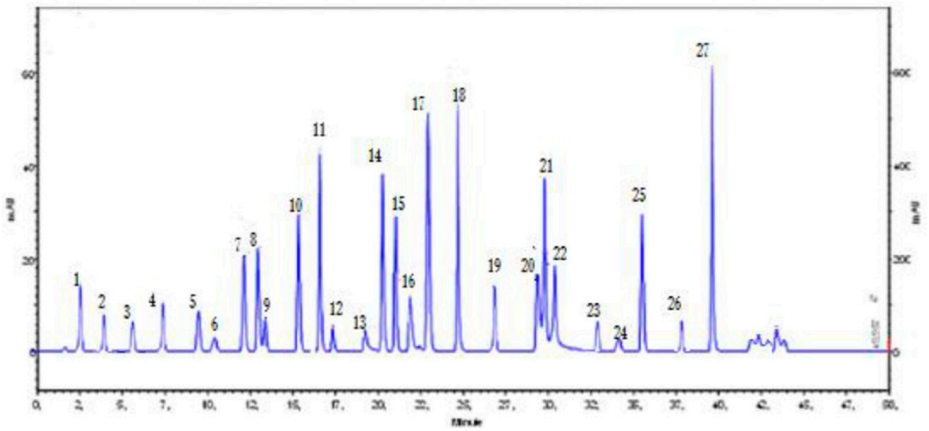

Figure 1. HPLC/MS chromatogram of ethanolic extract of *C. incana*.

3.2. Cytotoxicity

The MTT assay was used to assess the in vitro cytotoxic activity of *C. incana* extract against HepG2 liver cells at various dosages. The cytotoxicity results showed that the ethanolic extract of *C. incana* had low toxicity in HepG2 cells at all concentrations tested, in a dose-dependent manner (Figure 2). Nevertheless, at the maximum dose (200 mg/mL), the extract induced less than 50% cell death, but the IC_{50} value (concentration that may induce 50% cell death) was determined to be 260.20 ± 0.1 mg/mL. Furthermore, doxorubicin showed a significant cytotoxic effect on HepG2 cells (IC_{50} = 0.23 ± 0.2 mg/mL).

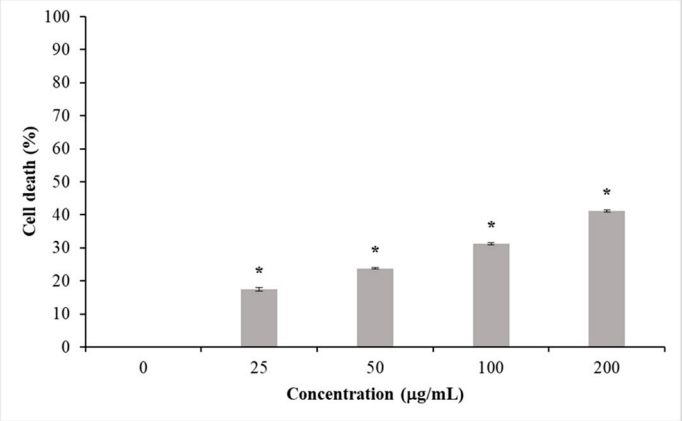

Figure 2. The cytotoxic effect of the ethanolic extract of *C. incana* in HepG2 liver cells using an MTT assay. Data were presented as the mean ± SD (N = 3). * $p < 0.05$ vs. untreated cells (control) (ANOVA, Tukey's post hoc test).

3.3. Antioxidant Activity

3.3.1. DPPH Assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to evaluate the free radical scavenging activity of the ethanolic extract of *C. incana*. Antioxidant constituents donate hydrogen atoms to DPPH to produce hydrogenated DPPH (DPPH-H). The change in color from deep purple to light yellow was read for absorbance at 517 nm.

The results are expressed as the mean ± SD of the IC_{50} (µg/mL). Overall, the ethanolic extract had lower antiradical activity than ascorbic acid (positive control) (IC_{50} values were 35.9 ± 0.1 and 19.6 ± 0.2 µg/mL, respectively) (Figure 3A).

3.3.2. Reducing Power Assay

The reducing power test technique is based on the premise that compounds with reduction potential react with yellow potassium ferricyanide (Fe^{3+}) to create green–blue potassium ferrocyanide (Fe^{2+}). The ferric–ferrous complex is then formed after potassium ferrocyanide reacts with ferric chloride, with an absorption maximum of 700 nm.

The results indicated that the ethanolic extract's reducing power ability was significantly lower than that of ascorbic acid (positive control) (IC_{50} 90.3 ± 0.5 µg/mL vs. ascorbic acid 26.4 ± 0.2 µg/mL) (Figure 3B).

3.4. Antidiabetic Activity

3.4.1. α-Amylase Inhibition Assay

The results of the present study indicate that the ethanolic extract of *C. incana* had a substantial effect on α-amylase at all concentrations (Figure 4). At the highest concentration investigated (100 µg/mL), the extract exhibited a noticeable effect on α-amylase by 88.5%. Nevertheless, the percentage (%) inhibition of α-amylase was lower than that of the positive control. Inhibition by the ethanolic extract and acarbose was dose-dependent. However, acarbose (positive control) was more robust (IC_{50} 33.5 ± 0.1 mg/mL) compared to the extract (IC_{50} 46.3 ± 0.2 mg/mL) (Table 2).

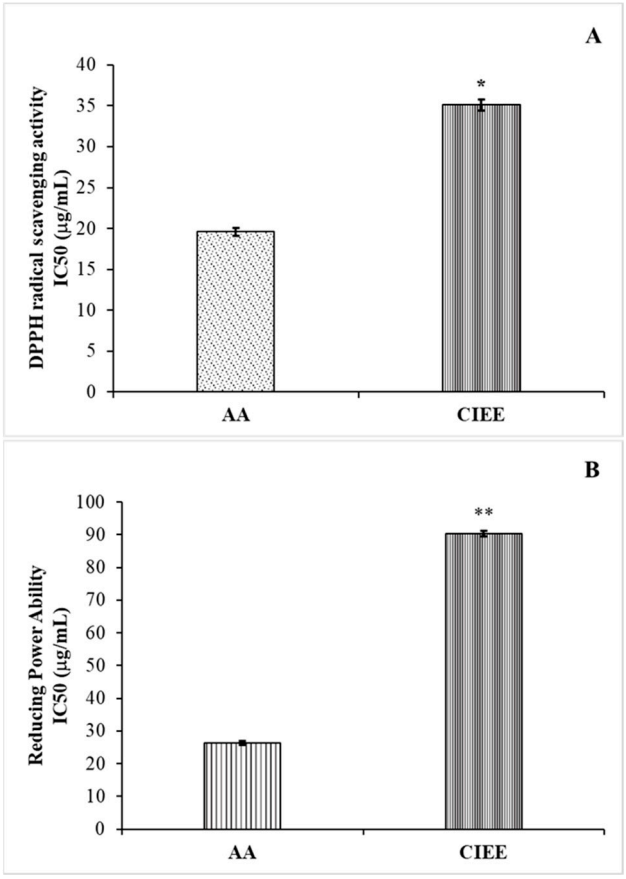

Figure 3. The antioxidant activity of *C. incana* ethanolic extract (CIEE), and ascorbic acid (AA) (positive control). (**A**) DPPH radical scavenging activity; (**B**) reducing power ability. Data are presented as the mean (IC_{50} µg/mL) ± SD of a triplicate measurement. * $p < 0.05$, ** $p < 0.01$.

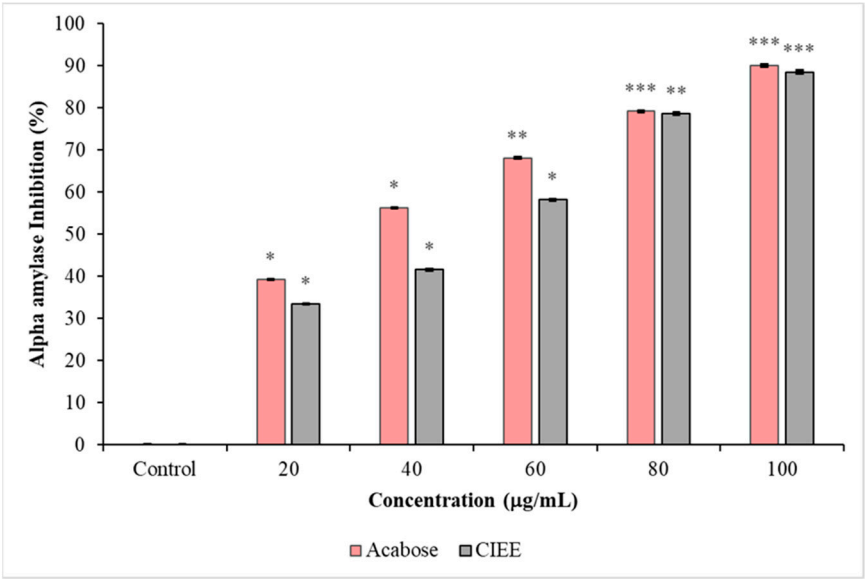

Figure 4. The effect of ethanolic extract of *C. incana* on α-amylase activity. CIEE: *Calamintha incana* ethanolic extract, Acarbose: positive control. Data represented as the mean ± SD (N = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated enzyme (control) (ANOVA, Tukey's post hoc test).

Table 2. α-amylase, α-glucosidase, pancreatic lipase, and DPP-IV inhibitory effects of the ethanolic extract of *C. incana*, and corresponding positive control IC_{50} values (µg/mL).

	Ethanolic Extract of *C. incana*	Positive Control (Acarbose)
IC_{50} values of α-amylase (µg/mL)	46.3 ± 0.2	33.5 ± 0.1
	Ethanolic extract of *C. incana*	Positive control (Epigallocatechin gallate)
IC_{50} values of α- glucosidase (µg/mL)	56.8 ± 0.1	37.1 ± 0.2
	Ethanolic extract of *C. incana*	Positive control (Orlistat)
IC_{50} values of pancreatic lipase (µg/mL)	639.9 ± 0.1	19.1 ± 0.2
	Ethanolic extract of *C. incana*	Positive control (Sitagliptin)
IC_{50} values of DPP-IV (µg/mL)	>600	18.6 ± 0.3

3.4.2. α-Glucosidase Inhibition Assay

Regarding the influence of the extract on α-glucosidase activity, the findings indicated that the *C. incana* ethanolic extract exerted a significant dose-dependent effect on α-glucosidase (Figure 5). The extract showed a noticeable effect on α-glucosidase activity at the highest concentration (100 µg/mL) of 70.5%. Furthermore, epigallocatechin gallate (positive control) caused more inhibition to the α-glucosidase rather than the extract (IC_{50} 37.1 ± 0.2 mg/mL and 56.8 ± 0.1 mg/mL, respectively) (Table 2).

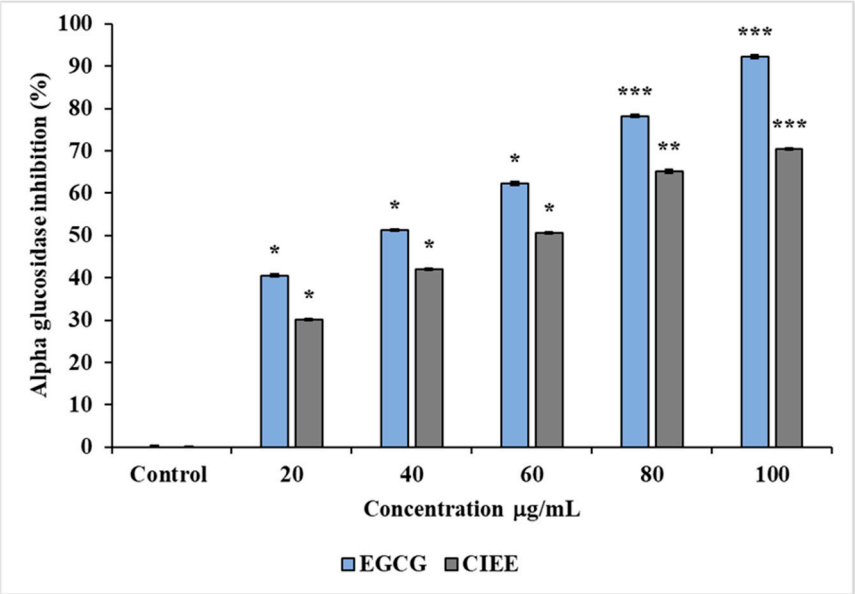

Figure 5. The effect of ethanolic extract of *C. incana* on α-glucosidase activity. CIEE: *Calamintha incana* ethanolic extract, EGCG: epigallocatechin gallate (positive control). Data denoted as the mean ± SD (N = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated enzyme (control) (ANOVA, Tukey's post hoc test).

3.4.3. Pancreatic Lipase Inhibition Assay

At all tested doses, the ethanolic extract of *C. incana* inhibited pancreatic lipase very weakly (Figure 6). At the same dose (100 mg/mL), the greatest level of inhibition of *C. incana* extract was 8.1%, which was much lower than that of the positive control, orlistat (92.2%). This implies that the ethanolic extract of *C. incana* may have a weak antidiabetic activity by inhibiting this enzyme.

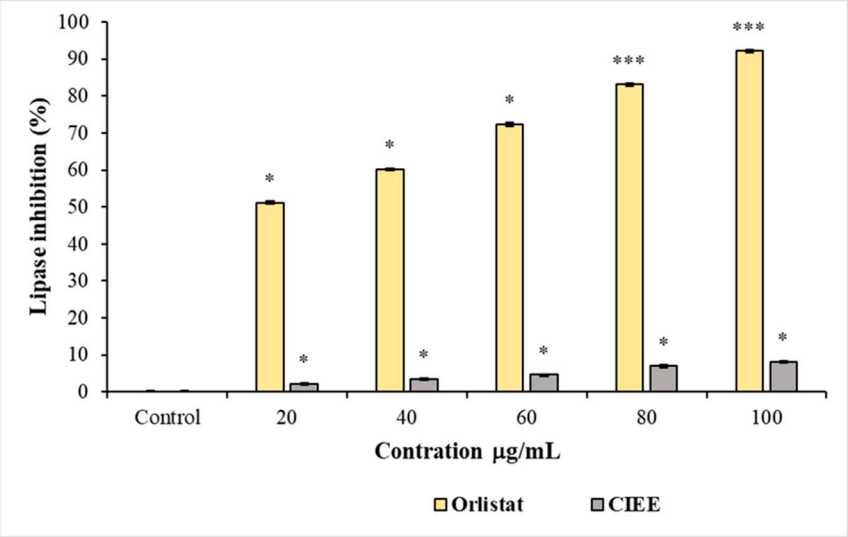

Figure 6. The effect of ethanolic extract of *C. incana* on pancreatic lipase activity. CIEE: *Calamintha incana* ethanolic extract, Orlistat: positive control. Data presented as the mean ± SD (N = 3). * $p < 0.05$, *** $p < 0.001$ vs. untreated enzyme (control) (ANOVA, Tukey's post hoc test).

3.4.4. Dipeptidyl Peptidase-IV (DPP-IV) Inhibition Assay

Figure 7 shows that the ethanolic extract of *C. incana* did not exert any considerable inhibition at any of the assessed concentrations compared to the positive control (sitagliptin), which displayed 95.2% inhibition of DPP-IV activity at the highest concentration (100 mg/mL).

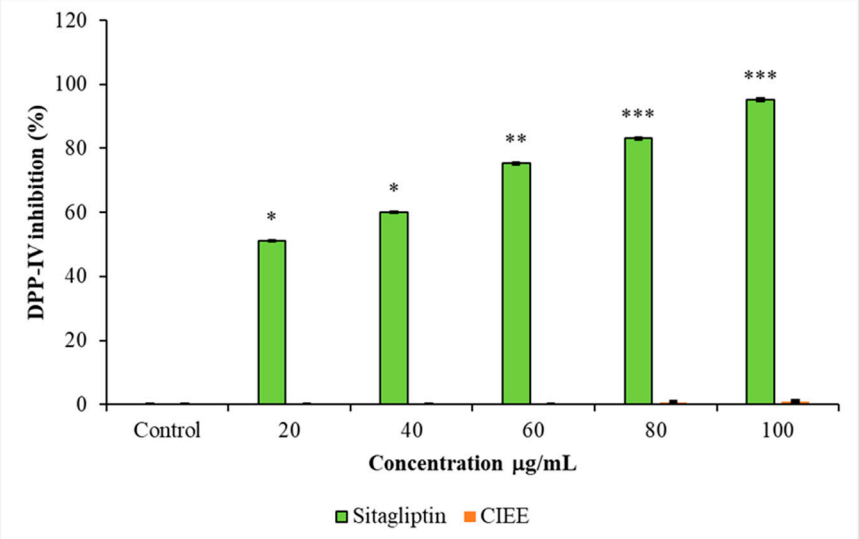

Figure 7. The effect of ethanolic extract of *C. incana* on DPP-IV activity. CIEE: *Calamintha incana* ethanolic extract, sitagliptin: positive control. Data stated as the mean ± SD (N = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated enzyme (control) (ANOVA, Tukey's post hoc test).

4. Discussion

4.1. Phytochemical Analysis of C. incana Ethanolic Extract

An essential tool for the development of active substances from plant tissues is to examine their phytochemical content. In the present study, several naturally occurring compounds from the investigated plant were identified, which may be the cause of their varied biological activities.

Linolenic acid (13.2%), myristic acid (12.1%), and p-cymene (10.5%) were the predominant compounds in the *C. incana*. Minor compounds, such as caffeic acid (0.8%) and

3-nonanone (0.2%), were also found. Fatty acids represented the highest extract components (31.3%), followed by sesquiterpenes (23.9%), and monoterpenes (20.7%). In contrast, ketones accounted for 0.2%.

Compared to the study conducted by Althaher et al. [20] on the same species of Calamintha, benzenamine,4-methyl-3-nitro, (2*S*,4*R*)-p-Mentha-6,8-diene 2-hydroperoxide, cis-piperitone oxide, menthone, and azulene (34.1%, 31.4%, 7.7%, 4.1%, 3.6%), respectively, were identified as the main components of the oil of *C. incana*. Most essential oil components (78.76%) were non-terpenoid aromatic compounds. Monoterpenes comprised 10.88%, whereas sesquiterpenes accounted for 6.01% [20]. The biological characteristics and chemical composition of *C. incana* vary significantly. Even within the same species, plants may differ in their phytochemical composition. These differences are linked to several parameters that might significantly affect the production, composition, and biological activities of the extract, including genetic makeup, ecological factors, habitat, developmental stage, harvesting time, and extraction procedures [20,26].

4.2. Cytotoxicity

Herbal medicines have considerable safety concerns. Natural plant products must be standardized, and preliminary studies must be conducted to determine hazards such as undesirable side effects, overdose, and toxicity [20]. Even at the highest concentration (200 mg/mL), the extract triggered less than 50% cell death in the HepG2 cells. The ethanolic extract of *C. incana* was less toxic than the essential oil extract from the same plant employed in a previous investigation, which revealed substantial toxicity against MCF-7, CaCo-2, and MRC-5 cell lines at various dosages (IC_{50} were 51.57 ± 1.67, 33.05 ± 3.45, and 58.48 ± 6.54 mg/mL, respectively) [20]. The comparatively low toxicity of *C. incana* extract suggests that the ethanolic extract of *C. incana* might be safe for users.

4.3. Antioxidant Activity

As increasing oxidative stress has been identified as a significant element in the development and progression of various life-threatening illnesses, including neurological and cardiovascular disorders, the antioxidant capability of plants has received much attention. Supplementing with exogenous antioxidants or increasing the body's natural antioxidant defenses is a potential method for addressing the detrimental consequences of oxidative stress [33]. In this study, we investigated the antioxidant activity of the ethanolic extract of *C. incana*. The results revealed that the extract had a significant antioxidant effect compared with the positive control (ascorbic acid) (Figure 1).

In comparison to the study conducted by Althaher et al. [20], the essential oil of *C. incana* also showed inhibition in the DPPH radical scavenging assay (IC_{50} = 15.38 µg/mL) and reducing power ability (IC_{50} = 9787.5 µg/mL). In addition, the total antioxidant capacity of *C. incana* essential oil was investigated in Turkey using a DPPH radical scavenging test with an IC_{50} of 19.28 ± 0.74 mg Trolox equivalents/g oil, and a FRAP assay with an IC_{50} of 53.63 ± 0.10 mg Trolox equivalents/g oil [25].

4.4. Antidiabetic Activity

Two carbohydrate-hydrolyzing enzymes (α-amylase and α-glucosidase) are primarily responsible for postprandial hyperglycemia. α-Amylase initiates carbohydrate digestion by hydrolyzing polysaccharide (1,4-glycosidic linkages) into disaccharides. Postprandial hyperglycemia is caused by α-glucosidase, which catalyzes the conversion of disaccharides to monosaccharides [34]. As a result, α-amylase and α-glucosidase inhibitors assist in hyperglycemia management by slowing carbohydrate digestion, thus reducing postprandial plasma glucose levels [35,36]. Insulin promotes glucose reduction by increasing the transfer of glucose from the circulation into skeletal muscle cells. Insulin-stimulated glucose uptake is well known to be diminished in individuals with type II diabetes [37]. Therefore, identifying compounds that might increase glucose absorption is an essential first step in the development of novel strategies for treating insulin resistance [38].

As reported above, the gas chromatographic analysis highlighted the predominance of fatty acids, sesquiterpenes, and monoterpenes. Linolenic acid and myristic acid were the predominant fatty acids. p-Cymene and spathulenol predominate among the monoterpenes and sesquiterpenes, respectively. Fatty acids can contribute to the regulation of metabolic syndrome by acting on lipid and glucose metabolism, as previously reported [39–41]. For example, chronic administration of myristic acid reduces insulin resistance and body weight in animal models [42]. Linolenic acid can also contribute to the regulation of glucose metabolism, as recently reported [43]. Amylases and glucosidases can also interact with terpenoids. Alqahtani et al. observed that treatment with terpenoids can improve insulin resistance by acting on amylase and glucosidase [44]. As with the extracts of many plant species, the entourage effect of other components cannot be excluded [45]. Indeed, when evaluating the therapeutic effect of a plant extract, it is necessary to consider the entire phytocomplex [46]. Therefore, the other components present in *C. incana* can influence carbohydrate metabolism.

Furthermore, type II diabetes can be induced by insulin-producing pancreatic cell malfunction, which can be initiated by an excessive lipid build-up in the pancreas [47]. Pancreatic lipase is the primary enzyme responsible for dietary fat absorption in the gastrointestinal system via breakdown of triacylglycerols (TGs) into free fatty acids (FFAs) and monoacylglycerols (MAGs) [48]. Recently, researchers have been interested in pancreatic lipase inhibitors because of their anti-obesity effects, which act by delaying lipolytic processes. This action minimizes fat absorption, protects the pancreas, and restores normal cellular insulin generation [48]. Terpenoids may have an anti-obesity effect by acting as inhibitors of pancreatic lipase, as previously reported [49]. Furthermore, linolenic and myristic acids have lipase-mediated anti-obesity action.

Dipeptidyl peptidase IV (DPP-IV) is a ubiquitous enzyme involved in the rapid metabolism of incretin hormones, primarily glucagon-like peptide-1(GLP-1) and gastric inhibitory peptide (GIP), which maintain glucose homeostasis by increasing insulin secretion and reducing glucagon secretion [50]. Thus, DPP-IV inhibitors can prolong the action of cretins. Since incretins stimulate glucose-dependent insulin secretion from the pancreas, inhibiting DPP-IV is a unique therapeutic strategy for treating type II diabetes [18,51–55].

Therefore, the results of this study showed that the ethanolic extract of *C. incana* inhibited α-amylase, α-glucosidase, and pancreatic lipase, but not DPP-IV, compared with the respective positive controls. This indicates that the ethanolic extract of *C. incana* possesses an antidiabetic mechanism involving the inhibition of these enzymes. On the other hand, several medicinal plants improve the body's antioxidant system and insulin control, making them a safer choice for treating obesity and diabetes [19,52–55]. The antioxidant activity of the plant extracts was confirmed in this study. Moreover, the extract contains several phytochemicals, such as phenols, terpenes, and fatty acids, which may be responsible for its antiradical and antidiabetic properties.

5. Conclusions

Currently, various antidiabetic medications are used to treat or control diabetes, and their mechanisms of action are well known. These include the inhibition of α-amylase, α-glucosidase, lipase, and DPP-IV enzymes. This study provides the first pharmacological overview of the antioxidant and antidiabetic effects of *Calamintha incana* leaves. Regarding the cytotoxic effect indicated at the maximum dose (200 mg/mL), the extract showed less than 50% cell death in HepG2 cells. The ethanolic extracts of the selected plants showed high antioxidant capacity.

There is a growing trend in developing countries to use medicinal plants to treat diabetes, primarily because conventional medicines are expensive for most people. However, medicinal plants may serve as an alternative source of antidiabetic agents because of their high content of bioactive constituents, such as flavonoids, saponins, carotenoids, terpenoids, alkaloids, and glycosides, which may have antidiabetic effects. Many medicinal herbs have antidiabetic characteristics that limit carbohydrate digestion, increase insulin

production from pancreatic beta cells, restrict glucose release from the liver, activate insulin receptors, and enhance glucose absorption in peripheral tissues. The correct approach for the pharmacological characterization of plant extracts is the characterization of the phytocomplex in its entirety. Indeed, even if *C. incana* extract has a predominance of fatty acids and terpenoids, we cannot exclude a synergistic action with all the components of the extract (entourage effect).

To corroborate the findings of the present study, the antioxidant and antidiabetic effects of *C. incana* need to be investigated further using in vivo experimental models. Recent results will aid future studies on the use of traditional medicinal herbs to create nutraceuticals and medicines.

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