



## Research paper

## *Carlina curetum* plant phytoconstituents, enzymes inhibitory and cytotoxic activity on cervical epithelial carcinoma and colon cancer cell lines

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## ABSTRACT

**Introduction:** Plant products are a rich source of pharmacologically active molecules and are considered an important and attractive field of scientific investigation for the development of new drugs. The present study aimed to assess the  $\alpha$ -amylase,  $\alpha$ -glucosidase and lipase enzymes inhibitory activities and the cytotoxic effects of *Carlina curetum* four solvents fractions in order to better understand the anti-obesity, hypoglycemic and anticancer effects of this plant.

**Methods:** Several qualitative and quantitative phytochemical tests were performed on *Carlina curetum* solvent fractions utilizing standard phytochemical procedures, followed by an investigation into their ability to inhibit the enzymes  $\alpha$ -amylase,  $\alpha$ -glucosidase, and lipase and an assessment of cytotoxic activity against HeLa and Colo-205 cells using standard biochemical and biotechnological methods.

**Results:** The results revealed that the aqueous and methanol fractions had the highest  $\alpha$ -amylase enzyme inhibitory activity with IC<sub>50</sub> values of  $21.37 \pm 0.31$  and  $30.2 \pm 0.42$   $\mu$ g/mL, respectively, in comparison with Acarbose, which had an IC<sub>50</sub> value of  $28.18 \pm 0.42$   $\mu$ g/mL. The methanol fraction showed potent  $\alpha$ -glucosidase inhibitory activity with an IC<sub>50</sub> value of  $27.54 \pm 4.28$   $\mu$ g/mL; the  $\alpha$ -glucosidase inhibitory activity Acarbose was  $37.15 \pm 0.33$   $\mu$ g/mL. The hexane fraction had greater anti-lipase activity than Orlistat. In addition, 0.5 mg/mL of the *C. curetum* acetone and hexane fractions had pronounced cytotoxic effects on the Colo-205 cancer cell line, and 0.625 mg/ml of the *C. curetum* hexane fraction had potential cytotoxic effects against the cervical epithelial carcinoma (HeLa) cell line.

**Conclusion:** This study revealed that *C. curetum* has potential  $\alpha$ -amylase,  $\alpha$ -glucosidase, porcine pancreatic lipase enzyme inhibitory activity and cytotoxic activity against the HeLa and Colo-205 cancer cell lines, which indicates the presence of biologically active and cytotoxic compounds in this plant species. This may be considered a challenge for developing bioactive compounds in diabetes, obesity and cancer management.

### 1. Introduction

For centuries, plants, animals and other natural products have been considered an endless and valuable source of therapeutic activities. Certainly, a vast number of drugs have been isolated from plants or their semisynthetic molecules [1,2]. However, investigations into natural products and their isolated derivatives are associated with some basic difficulties, such that most pharmaceutical companies shifted their fundamental focus toward purified synthetic or semisynthetic chemical compounds [3]. Unfortunately, chemical medications do not meet the expectations of patients, pharmaceutical companies or global

health organizations, as evidenced by decreasing numbers of novel medications reaching the pharmaceutical market and unexpected harmful side effects, contraindications and drug-drug interactions [4–6]. For these reasons, pharmaceutical company interests have moved again toward natural product-based drugs, and many broad interdisciplinary and multidisciplinary investigations into natural products have been carried out [7]. A commonly used approach is to start pharmacological investigations with crude plant extracts and thereafter isolate and characterize the phytoconstituents responsible for the pharmacological effect [8–11].

Recently, it has been clearly shown that cancer, diabetes, and

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obesity are major crises and challenges to global health care organizations and pharmaceutical research centers in the 21<sup>st</sup> century [12]. There is a scientifically supported relationship between cancer, obesity and diabetes mellitus, such that many obese patients may also have diabetes cancer [13].

The value of herbal preparations can be explained in five major ways. The first is closely related to foodstuffs and the use of plants due to their nutritional value, secondly for manufacturing food supplements or nutraceuticals, thirdly to utilize plants or their products as traditional therapeutic agents, fourthly to use plants or their products such as essential oils in cosmetics and finally the exploration of evidence-based therapeutic uses for plant constituents and manufacturing conventional medications from these plants [14–16].

It has been estimated that about 420,000 plants species exist around the globe. Unfortunately, for most of these species, very limited information about their constituents or their therapeutic or nutritional benefits is available in the literature [17].

*Carlina curretum* Heldr. ex Halácsy (family Compositae) is commonly known as carline thistle. It is a perennial edible herb reaching 25–50 cm tall, sparsely cobwebbed to glabrescent, and rarely tomentose. The leaves are leathery, pinnatifid with spiny lobes, while the radical leaves are rosulate, tapering to a petiole; the cauline leaves are distant, narrow-lanceolate, pinnatisect into distant long and strong spiny lobes, canaliculate, spreading to recurved, sessile, with small auricles. *C. curretum* originated in the Mediterranean basin, especially on the Palestinian plains [10,18–20]. In Palestinian ethnomedicine, the leaves and stems of *C. curretum* are utilized to decrease blood glucose levels, to burn body fat and for the prophylaxis of cancer [21]. In fact, various species of *Carlina* plant have been utilized as herbal remedies in European traditional medicine. For example, *C. acaulis* root is still utilized therapeutically as a diaphoretic, diuretic and anthelmintic. Moreover, the decoction of its roots is used for the treatment of toothache, catarrh, skin lesions and rashes [22,23].

To the best of our knowledge, *C. curretum* is an unstudied plant species and nothing in the literature is known regarding its chemical constituents or biological/pharmacological uses. However, the current plant species not studied previously its chemical constituents and our study is the first study made phytochemical screenings. Therefore, the present study aimed to qualitatively and quantitatively evaluate the chemical constituents of *C. curretum* in various fractions and to assess the  $\alpha$ -amylase,  $\alpha$ -glucosidase and lipase enzyme inhibitory activities and the cytotoxic effects of these fractions against HeLa and Colo-205 cells in order to better understand the anti-obesity, hypoglycemic and anticancer effects of this plant.

## 2. Material and methods

### 2.1. Collection and preparation of plant material

The selected *C. curretum* aerial parts were harvested randomly from Nablus region of Palestine in May 2018. Characterization was established by the Pharmacognosist and Herbal Products Specialist Dr. Nidal Jaradat, in the Laboratory of Pharmacognosy at An-Najah National University and the given code for the voucher specimen was Pharm-PCT-521. The plant aerial parts were well cleaned and dried in the shade at ordinary temperature 25 °C and finally, the dried parts were grounded coarsely by the mechanical grinder and then kept in an airtight container with suitable labeling for future use.

### 2.2. Four solvents exhaustive fractionation

The powder made from the dried aerial parts of *C. curretum* was exhaustively extracted by a fractionation method by adding solvents in a sequential manner, depending on their polarities, beginning with the totally non-polar solvents hexane and acetone (polar aprotic organic solvents), then methanol (a polar alcohol) and finally distilled water (a

polar protic solvent). For the preparation of each extract fraction, about 25 g of the ground dried *C. curretum* plant parts was first placed in 0.5 L of hexane (Loba/Chemie, India) and soaked for 72 h in a shaker at 100 rotations per minute at 25 °C. The hexane was then replaced with 0.5 L of acetone (Alfa-Aesar, UK) in the same manner, then methanol (Loba/Chemie, India) and finally water. Each organic fraction was filtered and concentrated under vacuum on a rotary evaporator (Heidolph OB 2000-VV2000, Germany), while the aqueous fraction was dried using a freeze dryer (Mill Rock Technology, model BT85, China). Finally, all crude fractions were stored at 4 °C in the refrigerator until further use [24,25].

The yield of each plant fraction was calculated using the following formula:

$$\text{Yield\%} = (\text{weight of } C. \text{ curretum extract/dry } C. \text{ curretum weight}) \times 100\%$$

Accordingly, the obtained yields percentages were 5.04%, 1.72%, 3.2% and 5.04% for the hexane, acetone, methanol, and aqueous fractions, respectively.

### 2.3. Preliminary qualitative phytochemical examinations

A preliminary phytochemical determination of the four plant fractions was carried out to reveal the presence of secondary or primary metabolic products in each of the fractions. These examinations were carried out according to the Evans method. The qualitative results are expressed as (+) for the presence and (–) for the absence of these phytochemical classes [26].

### 2.4. Quantitative phytochemical determination of phenols, flavonoids and tannins

#### 2.4.1. Determination of the total phenolic content (TPC)

The following steps were followed to assess the total phenol content (TPC), adopting the procedure mentioned in a previous study by Cheung [27]. TPC is usually expressed in milligrams of Gallic acid equivalents per gram of dry plant fraction (mg GAE/g of plant extract). Briefly, the first step was started by preparing a standard reference solution containing Gallic acid (Sigma-Aldrich, Germany), which was produced by dissolving 100 mg of Gallic acid in 100 mL of distilled water. Serial dilutions were then prepared to obtain concentrations of 10, 40, 50, 70 and 100  $\mu\text{g/mL}$ . Then, stock solutions of the plant four fractions were prepared by dissolving 100 mg of each of plant fraction in 100 mL of distilled water. To prepare the reaction mixture, in each of the working test tubes, 0.5 mL of each plant fraction solution was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent (Sigma Aldrich, USA) dissolved in water and 2.5 mL of 7.5% of sodium bicarbonate solution (Alfa-Aesar, UK). The sample tubes were incubated for 45 min at 45 °C. The absorbance was measured on a UV-vis spectrophotometer (Jenway-Keison, UK) at a wavelength of 765 nm. All the working samples were prepared in triplicate for each analytic trial to calculate the mean and standard deviation. The TPC is expressed as mg GAE/g of plant extract.

#### 2.4.2. Colorimetric assay for the determination of the flavonoid content (TFC)

The total flavonoid content (TFC) in the four *C. curretum* fractions was assessed using a calibration curve of Rutin (standard reference compound) and expressed as milligrams of Rutin equivalents per gram of dry *C. curretum* fraction (mg RUE/g of dry *C. curretum* fraction). The experimental procedure is described by Chang et al. [28]. The calibration curve for Rutin was established using serial dilutions, starting with the preparation of a stock solution at 100  $\mu\text{g/mL}$ , using 10 mg of Rutin (Sigma-Aldrich, USA) dissolved in 10 mL of distilled water and diluted to 100 mL. Subsequently, the stock solution was diluted to provide a series of concentrations (10, 30, 40, 50, 70, 100  $\mu\text{g/mL}$ ). In

addition, to prepare working solutions, 0.5 mL of each solution was mixed with 3 mL of methanol, 0.2 mL of 10%  $\text{AlCl}_3$ , 0.2 mL of 1 M potassium acetate (Riede-de-haen, Germany) and 5 mL of distilled water, and then incubated at room temperature for 30 min. The previous steps were repeated for each plant fraction and finally, the absorbance was recorded using a UV-vis spectrometer at a wavelength of 415 nm; distilled water, methanol, 10%  $\text{AlCl}_3$  and potassium acetate were used as the blank solution. The samples were prepared in triplicate for each analytic trial to obtain the mean and standard deviation.

#### 2.4.3. Catechin assay for the determination of the total tannin content (TTC)

For the determination of TTC, the Sun et al. protocol was followed with minor modification [29]. Catechin (Sigma, USA) was used as the reference compound to construct the calibration curve for the required calculations in which a 100  $\mu\text{g}/\text{mL}$  stock methanolic solution was prepared, then serial dilutions were obtained (10, 30, 50, 70 and 100  $\mu\text{g}/\text{mL}$ ). Then, a 4% methanolic vanillin (Alfa-Aesar, UK) solution was freshly prepared and a 100  $\mu\text{g}/\text{mL}$  stock solution was made from each plant fraction using methanol as the solvent. For the working solution, each test tube contained 0.5 mL of each fraction mixed with 3 mL of vanillin solution and 1.5 mL of concentrated HCl (SDFCL, India). The obtained mixture was allowed to stand for 15 min, and then the absorption was measured at 500 nm against methanolic vanillin as a blank. All the working samples were analyzed in triplicate. The TTC in each fraction is expressed as Catechin equivalents (mg of CAE/g of the dry plant fraction).

#### 2.5. Porcine pancreatic lipase inhibitory assay

A porcine pancreatic lipase inhibition assay was carried out to assess the activity for each of *C. curetum* fraction to estimate their anti-obesity activity. Orlistat, a commercially available anti-obesity, and an anti-lipase therapeutic agent, was used as a reference control. The porcine pancreatic lipase inhibitory method was performed according to the protocol of Zheng et al. with some modifications [30]. A 500  $\mu\text{g}/\text{mL}$  stock solution from each plant fraction was dissolved in 10% dimethyl sulfoxide (DMSO), from which five different dilutions were prepared (50, 100, 200, 300 and 400  $\mu\text{g}/\text{mL}$ ). Then, a 1 mg/mL stock solution of porcine pancreatic lipase was freshly prepared before use, which was dispersed in Tris-HCl buffer. The substrate used was p-nitrophenyl butyrate (PNPB) (Sigma-Aldrich, Germany), prepared by dissolving 20.9 mg in 2 mL of acetonitrile. In addition, for each working test tube, 0.1 mL of porcine pancreatic lipase (1 mg/mL) was mixed with 0.2 mL of the plant fraction from each diluted solution series for each plant fraction. The resulting mixture then completed to 1 mL by adding Tri-HCl solution and incubated at 37 °C for 15 min. After this incubation period, 0.1 mL of p-nitrophenyl butyrate solution was added to each test tube. The mixture was then incubated for 30 min at 37 °C. Pancreatic lipase activity determined by measuring the hydrolysis of PNPB into p-nitrophenolate ions at 410 nm using a UV-vis spectrophotometer; the same procedure was repeated for Orlistat (Sigma-Aldrich, Germany). The inhibitory percentage of the anti-lipase activity was calculated using the following equation:

$$\text{Lipase inhibition\%} = (\text{AB}-\text{A}_{\text{ts}})/\text{AB} \times 100\%$$

where AB is the recorded absorbance of the blank solution and  $\text{A}_{\text{ts}}$  is the recorded absorbance of the tested sample solution.

#### 2.6. $\alpha$ -amylase inhibition assay

The  $\alpha$ -amylase inhibitory activity of each solvent fraction was assessed according to the standard method of Nyambe-Silavwe et al. with minor modifications [31]. Briefly, each extract fraction was dissolved in a few milliliters of 10% DMSO (Riedel-de-haen, Germany) and then

further dissolved in a buffer ( $(\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  (0.02 M), NaCl (0.006 M) at pH 6.9) to a concentration of 1000  $\mu\text{g}/\text{mL}$ . A dilution series was prepared (10, 50, 70, 100, 500  $\mu\text{g}/\text{mL}$ ). A volume of 0.2 mL of porcine pancreatic  $\alpha$ -amylase enzyme (Sigma-Aldrich, USA) solution with a concentration of 2 units/mL was mixed with 0.2 mL of the plant fraction, then incubated for 10 min at 30 °C. Thereafter, 0.2 mL a freshly prepared starch solution (1%) was added and the mixture was incubated for at least 3 min. The reaction was stopped by the addition of 0.2 mL dinitrosalicylic acid (DNSA) (Alfa-Aesar, UK), then the mixture was diluted with 5 mL of distilled water and heated for 10 min in a water bath at 90 °C. The mixture was left to cool down to room temperature, and then the absorbance was measured at 540 nm. A blank was prepared following the same procedure by replacing the plant fraction with 0.2 mL of the buffer.

Acarbose (Sigma-Aldrich, USA) was used as a positive control and prepared in the same procedure. The  $\alpha$ -amylase inhibitory activity was calculated using the following equation:

$$\% \text{ of } \alpha\text{-amylase inhibition} = (\text{A}_b - \text{A}_s)/\text{A}_b * 100\%$$

where  $\text{A}_b$  is the absorbance of the blank and  $\text{A}_s$  is the absorbance of the tested sample or control.

#### 2.7. $\alpha$ -glucosidase inhibitory activity assay

The  $\alpha$ -glucosidase inhibitory activity of each plant fraction was carried out according to a standard protocol with some modification [32]. In each test tube, a reaction mixture was prepared containing 50  $\mu\text{L}$  of phosphate buffer (100 mM, pH 6.8), 10  $\mu\text{L}$   $\alpha$ -glucosidase (1 U/mL) (Sigma-Aldrich, USA) and 20  $\mu\text{L}$  of varying concentrations of extract fractions (100, 200, 300, 400 and 500 mg/ml), which was then incubated at 37 °C for 15 min. Then, 20  $\mu\text{L}$  of pre-incubated 5 mM PNPG (Sigma-Aldrich, USA) was added as the substrate of the reaction and the samples were incubated at 37 °C for a further 20 min. The reaction was terminated by adding 50  $\mu\text{L}$  of  $\text{Na}_2\text{CO}_3$  (0.1 M). The absorbance of the released p-nitrophenol was measured by a UV/Vis spectrophotometer at 405 nm. Acarbose at similar concentrations as the plant fractions was used as a positive control.

The inhibition percentage was calculated using the following equation:

$$\% \text{ of } \alpha\text{-glucosidase inhibition} = (\text{A}_b - \text{A}_s)/\text{A}_b * 100\%$$

Where;  $\text{A}_b$  is the absorbance of the blank and  $\text{A}_s$  is the absorbance of the tested sample or control.

#### 2.8. Cell culture and cytotoxicity assay

HeLa (cervical adenocarcinoma) and Colo-205 (colon) cancer cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine. Cells were grown in a humidified atmosphere with 5%  $\text{CO}_2$  at 37 °C. Cells were seeded at  $2.6 \times 10^4$  cells/well in a 96-well plate. After 48 h, cells were incubated with various concentrations of the tested compounds for 24 h. Cell viability was assessed by the CellTiter 96® Aqueous One Solution Cell Proliferation (MTS) assay according to the manufacturer's instructions (Promega Corporation, Madison, WI). Briefly, at the end of the treatment, 20  $\mu\text{L}$  of MTS solution per 100  $\mu\text{L}$  of media was added to each well and incubated at 37 °C for 2 h. Absorbance was measured at 490 nm [33].

#### 2.9. Statistical analysis

The determination of TPC, TFC and TTC and the inhibitory effects of the plant fractions on  $\alpha$ -amylase,  $\alpha$ -glucosidase and porcine pancreatic enzymes were conducted in triplicate for each sample. The cytotoxic test was carried out in duplicate for each sample. The results are

**Table 1**  
Phytochemical screening assessment of four solvent fractions of *C. curetum*.

Phytochemical Classes	Aqueous fraction	Methanol fraction	Acetone fraction	Hexane fraction
Cardiac glycosides	+	-	+	+
Saponin glycoside	+	-	-	-
Alkaloids	+	+	+	+
Protein	+	-	-	-
Starch	+	-	-	-
Phenols	+	+	-	-
Terpenoids	-	+	+	+
Tannins	+	+	-	-
Steroids	-	-	+	+
Reducing sugar	+	+	-	-
Monosaccharide	-	-	-	-
Flavonoids	+	+	-	-

presented as means ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism software version 6.01. Comparisons between three or more groups were performed by one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test.

### 3. Results

#### 3.1. Qualitative assessment

The aerial parts of the four *C. curetum* solvent fractions contained a wide range of secondary metabolites, as shown in Table 1. The results show that the aqueous fraction was rich in secondary and primary metabolites including flavonoids, tannins, phenols, alkaloids, saponins and cardiac glycosides, protein, starch and reducing sugars. In addition, the methanol fraction contained flavonoids, terpenoids, tannins, phenols, alkaloids and reducing sugars. The acetone and hexane fractions contained only cardiac glycosides, alkaloids, terpenoids and steroids.

The qualitative results are expressed as (+) for the presence and (-) for the absence of these phytochemical classes

#### 3.2. Quantitative analysis of TP, TF and TT

From the gallic acid calibration curve of (Fig. 1), the following equation was obtained and used to estimate the total phenol contents in the four *C. curetum* fractions.

$$y = 0.0098x + 0.0215, R^2 = 0.9929$$

where y is absorbance at 765 nm and x is the total phenol content of the plant fraction.

According to the standard calibration curve of Rutin, as presented in

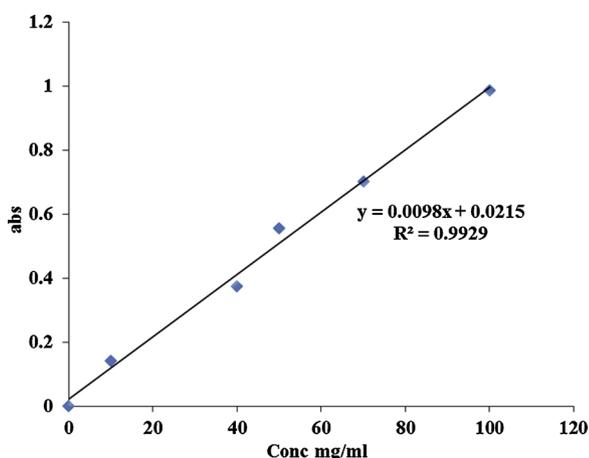


Fig. 1. Standard calibration curve of Gallic acid.

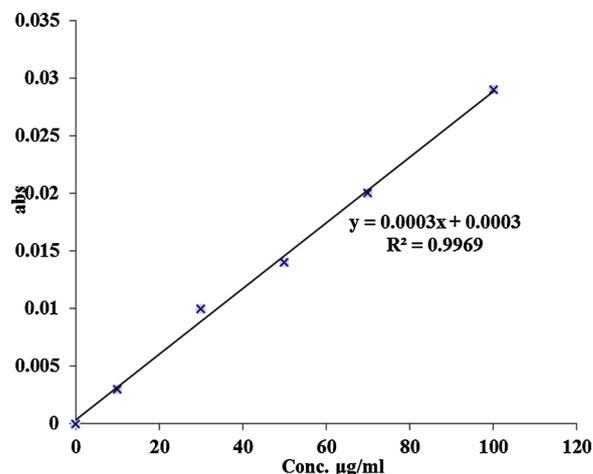


Fig. 2. Standard calibration curve of Rutin.

Fig. 2, the equation  $y = 0.0003x + 0.0003, R^2 = 0.9969$  was utilized to estimate the total flavonoid contents in the four plant fractions, where Y is the absorbance at 510 nm and X is the total flavonoid of the studied plant fraction.

According to the standard calibration curve of Catechin, as shown in Fig. 3, the equation  $y = 0.0011x + 0.0023, R^2 = 0.991$  was used to estimate total tannins contents in the four fractions, where Y is the absorbance at 500 nm and X is the total tannin content in the plant fraction.

In summary, Table 2 shows the quantitative test results of the *C. curetum* hexane, acetone, methanol and aqueous fractions.

#### 3.3. α-amylase inhibitory activity

Table 3 and Fig. 4 show the α-amylase inhibitory activities of the *C. curetum* hexane, acetone, methanol and aqueous fractions. The aqueous and methanol fractions showed strong anti-amylase activity compared with the reference anti-diabetic drug Acarbose. The IC<sub>50</sub> values were  $21.37 \pm 0.31 \mu\text{g/mL}$  and  $30.2 \pm 0.42 \mu\text{g/mL}$ , respectively, while the IC<sub>50</sub> of the anti-amylase compound Acarbose was  $28.18 \pm 0.42 \mu\text{g/mL}$ .

#### 3.4. α-glucosidase inhibitory activity

Table 4 and Fig. 5 depict the α-glucosidase inhibitory activity and IC<sub>50</sub> values of the *C. curetum* hexane, acetone, methanol and aqueous fractions in addition to the antidiabetic drug Acarbose. The results show that the *C. curetum* methanolic fraction had potent α-glucosidase inhibitory activity with an IC<sub>50</sub> value of  $27.54 \pm 4.28 \mu\text{g/mL}$ .

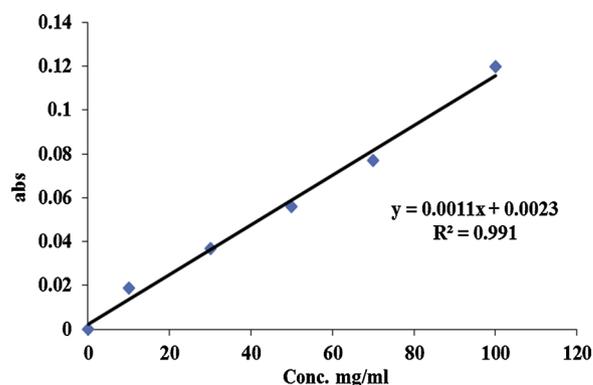


Fig. 3. Standard calibration curve of Catechin.

**Table 2**  
Quantitative phenol, tannin and flavonoid contents.

Fractions	Total flavonoids contents, mg of QUE/g of plant fraction, $\pm$ SD	Total phenol contents, mg of GAE/g of plant fraction, $\pm$ SD	Total Tannin contents, mg of CAE/g of plant fraction, $\pm$ SD
Hexane	–	–	–
Acetone	–	–	–
Methanol	22.33 $\pm$ 4.7	32.6 $\pm$ 2.3	5.18 $\pm$ 1.28
Aqueous	40.66 $\pm$ 11.78	57.05 $\pm$ 3.9	9.27 $\pm$ 3.2

### 3.5. Anti-lipase potential

Porcine pancreatic lipase inhibitory activity assay was utilised to evaluate the anti-lipase activity of the *C. curetum* hexane, acetone, methanol and aqueous fractions in addition to the reference anti-lipase drug Orlistat, which is used as an anti-obesity agent. The porcine pancreatic lipase inhibitory activities and the IC<sub>50</sub> values are shown in Table 5 and Fig. 6. The results indicate that the hexane fraction had the highest anti-lipase activity compared with Orlistat and had an IC<sub>50</sub> value of 154.8  $\pm$  1.86  $\mu$ g/mL. Orlistat had an IC<sub>50</sub> value of 12.3  $\pm$  0.35  $\mu$ g/mL, while the other fractions did not show any anti-lipase activity.

### 3.6. Cytotoxic effect of *C. Curetum* fractions

As shown in Fig. 7, the treatment of Colo-205 cells with 1, 0.5 and 0.25 mg/mL of the acetone and hexane fractions significantly induced cytotoxicity ( $p \leq 0.0001$ ) by approximately 90%, 90% and 55%, respectively, while 0.125 and 0.0625 mg/mL of both fractions did not have any significant effect. Treatment of Colo 205 cells with 10, 5 and 2.5 mg/mL of the aqueous fraction significantly induced cytotoxicity ( $p \leq 0.0001$  and  $p \leq 0.01$ ) by approximately 90%, 90% and 40%, respectively, while 1.25 and 0.625 mg/mL did not have a significant effect. 10 and 5 mg/ml of the methanol fraction significantly induced cytotoxicity in Colo 205 cells ( $p \leq 0.0001$  and  $p \leq 0.01$ ) by approximately 90% and 70%, respectively, but 2.5, 1.25 and 0.625 mg/mL did not have a significant effect.

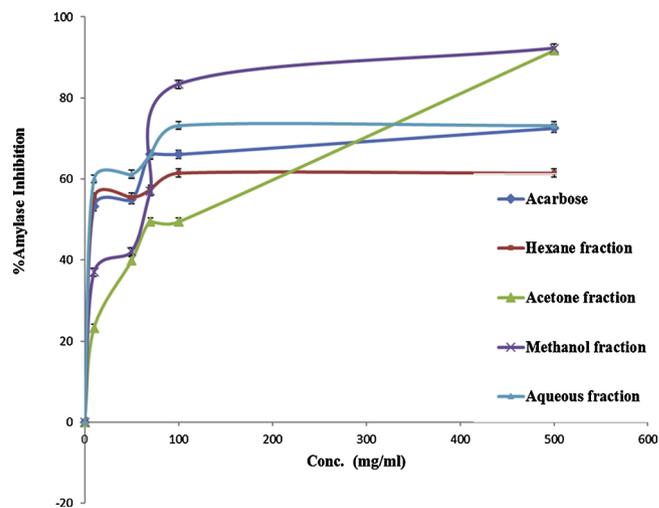
As demonstrated in Fig. 8, treatment of HeLa cells with 1, 0.5, 0.25, and 0.125 mg/mL of the acetone fraction significantly induced cytotoxicity ( $p \leq 0.0001$ ) by approximately 90%, while 0.0625 mg/mL did not have a significant effect. Treatment of HeLa cells with 2.5, 1.25 and 0.625 mg/mL of the hexane fraction significantly induced cytotoxicity ( $p \leq 0.0001$ ) by approximately 95%, 95% and 80%, respectively, while 0.1875 mg/mL did not have a significant effect. In addition, 10 and 5 mg/mL of the aqueous fraction significantly induced cytotoxicity in HeLa cells ( $p \leq 0.0001$ ) by approximately 90% and 75%, respectively, while 2.5 mg/mL did not have a significant effect. 10 mg/mL of the methanol fraction significantly induced cytotoxicity in HeLa cells ( $p \leq 0.0001$ ) by approximately 85%, while 5 and 2.5 mg/mL did not have a significant effect.

## 4. Discussion

Herbal products have been utilized for therapeutic purposes for time

**Table 3**  
 $\alpha$ -amylase inhibitory activity and IC<sub>50</sub> values of the *C. curetum* hexane, acetone, methanol and aqueous fractions in addition to the reference drug Acarbose.

Conc.	Acarbose	Hexane fraction	Acetone fraction	Methanol fraction	Aqueous fraction
0	0	0	0	0	0
10	53.22 $\pm$ 1.2	55.47 $\pm$ 0.53	23.21 $\pm$ 0.44	36.98 $\pm$ 0.53	59.99 $\pm$ 0.53
50	54.91 $\pm$ 0.58	55.47 $\pm$ 0.53	39.84 $\pm$ 1.86	42.07 $\pm$ 0.8	61.13 $\pm$ 0.53
70	66.1 $\pm$ 1.34	57.35 $\pm$ 0.53	49.4 $\pm$ 0.41	56.98 $\pm$ 1.06	65.84 $\pm$ 0.26
100	66.1 $\pm$ 1.62	61.5 $\pm$ 0.53	49.4 $\pm$ 0.41	83.39 $\pm$ 0.53	73.2 $\pm$ 0.01
500	72.54 $\pm$ 1.37	61.5 $\pm$ 0.41	91.69 $\pm$ 1.06	92.26 $\pm$ 0.26	73.2 $\pm$ 0.21
$\alpha$ -amylase inhibitory activity IC <sub>50</sub> ( $\mu$ g/ml)	28.18 $\pm$ 0.42	39.81 $\pm$ 0.42	60.26 $\pm$ 0.84	30.2 $\pm$ 0.42	21.37 $\pm$ 0.31



**Fig. 4.**  $\alpha$ -amylase inhibitory activity of the *C. curetum* hexane, acetone, methanol and aqueous fractions in addition to Acarbose.

immemorial, and to this day, many important and familiar medications originate from plants [34]. Throughout the phytochemical screening outcomes, it was observed that the polar fractions (methanol and aqueous) were rich in polyphenols. On the other hand, the hexane and acetone fractions contained steroidal metabolites. Moreover, the quantitative phytochemical tests revealed the absence of phenolic, flavonoid and tannin contents in the hexane and acetone fractions, while the aqueous fraction contained the highest contents of flavonoids, tannins, and phenols.

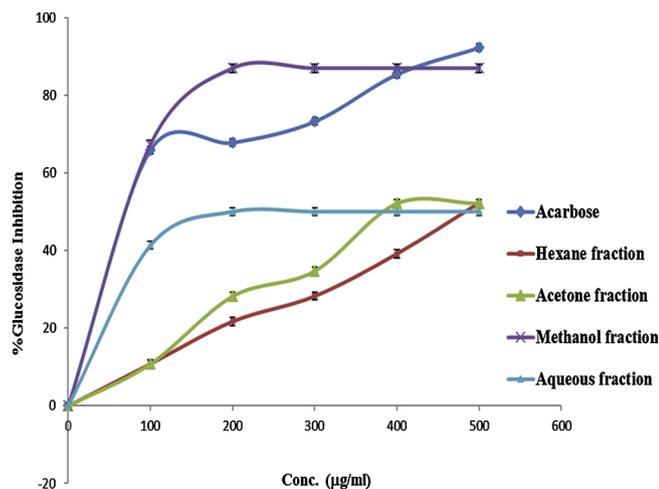
Diabetes mellitus is a chronic disorder that becomes a dangerous global health problem and considered one of the main risks of neuropathy, stroke, nephropathy, retinopathy, and cataracts. Some natural and synthetic products may be used as potent inhibitors of carbohydrate degrading enzymes such as  $\alpha$ -amylase and  $\alpha$ -glucosidase to decrease the risk of diabetes, especially type 2 diabetes and its serious complications.

In fact,  $\alpha$ -amylase and  $\alpha$ -glucosidase are key enzymes that have been major drug targets for the development of therapies to treat diabetes and to reduce the severity of complications. Thus, the current investigation assessed the  $\alpha$ -amylase and  $\alpha$ -glucosidase activities of the four fractions derived from *C. curetum* aerial parts using standard biochemical laboratory methods.

The results showed that the aqueous and methanol fractions had

**Table 4**The  $\alpha$ -glucosidase inhibitory activity and IC<sub>50</sub> values of the *C. curetum* hexane, acetone, methanol and aqueous fractions in addition to Acarbose.

Conc.	Acarbose	Hexane fraction	Acetone fraction	Methanol fraction	Aqueous fraction
0	0	0	0	0	0
100	65.8 ± 0.42	10.8 ± 3.07	10.8 ± 3.07	67.3 ± 9.2	41.3 ± 9.2
200	67.75 ± 0.35	21.7 ± 9.22	28.2 ± 9.22	86.9 ± 6.1	50 ± 9.2
300	73.2 ± 0.42	28.2 ± 6.1	34.7 ± 6.1	86.9 ± 6.1	50 ± 9.2
400	85.35 ± 0.35	39.1 ± 6.1	52.1 ± 6.1	86.9 ± 0	50 ±
500	92.22 ± 0.106	52.17 ± 0.3	52.1 ± 6.1	86.9 ± 0	50 ±
Anti-glucosidase activity IC <sub>50</sub> (µg/ml)	37.15 ± 0.33	4365 ± 4.96	1479 ± 6.12	27.54 ± 4.28	323.59 ± 5.52

**Fig. 5.**  $\alpha$ -glucosidase inhibitory activity of the *C. curetum* hexane, acetone, methanol and aqueous fractions in addition to Acarbose.

strong  $\alpha$ -amylase enzyme inhibitory activity in comparison with the reference anti-diabetic drug Acarbose with IC<sub>50</sub> values of  $21.37 \pm 0.31$  and  $30.2 \pm 0.42$   $\mu\text{g}/\text{mL}$ , respectively; the Acarbose anti-amylase IC<sub>50</sub> value was  $28.18 \pm 0.42$   $\mu\text{g}/\text{mL}$ . These results indicate that the aqueous fraction of *C. curetum* has powerful  $\alpha$ -amylase enzyme inhibitory activity, even more so than the commercially available antidiabetic drug Acarbose, while the methanolic fraction had nearly the same antidiabetic activity as Acarbose. However, assessing the  $\alpha$ -glucosidase inhibitory activity of the four *C. curetum* fractions revealed that the methanolic fraction had potent antiglucosidase property, greater than that of Acarbose with an IC<sub>50</sub> value of  $27.54 \pm 4.28$   $\mu\text{g}/\text{mL}$ ; the Acarbose  $\alpha$ -glucosidase IC<sub>50</sub> value was  $37.15 \pm 0.33$   $\mu\text{g}/\text{mL}$ .

The antidiabetic activity of *C. curetum* aqueous and methanolic fractions indicated that the phenolic, flavonoid and tannins contents may inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase. A study conducted by Kalita et al. showed that the phenolic content in potato inhibits the activity of  $\alpha$ -amylase and  $\alpha$ -glucosidase [35]. In another study performed by Salehi et al., there was a positive correlation between a plant's polyphenolic contents and  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activity [36].

Lipase is a key enzyme and a major drug target for developing

**Table 5**Porcine pancreatic lipase inhibitory activity and the IC<sub>50</sub> values of the *C. curetum* hexane, acetone, methanol and aqueous fractions in addition to the reference anti-obesity drug Orlistat.

Conc.	Orlistat	Hexane fraction	Acetone fraction	Methanol fraction	Aqueous fraction
0	0	0	0	0	0
50	91.05 ± 0.77	26.41 ± 0.78	9.48 ± 2.81	13.05 ± 6.6	8.72 ± 7.54
100	93.1 ± 0.42	35.13 ± 3.8	14.45 ± 0.741	13.49 ± 7.3	23.01 ± 1.6
200	94.3 ± 0.42	44.43 ± 4.4	27.99 ± 0.974	25.45 ± 5.2	32.07 ± 4.2
300	97.4 ± 0.12	70.15 ± 0.16	48.79 ± 9.81	61.57 ± 0	38.1 ± 3.73
400	97.5 ± 0	70.15 ± 0.16	61.79 ± 0.452	79.83 ± 0	46.04 ± 6.39
Anti-lipase activity IC <sub>50</sub> (µg/ml)	12.3 ± 0.35	154.8 ± 1.86	1023 ± 2.96	389 ± 3.82	2511 ± 4.69

medications used to treat obesity, overweight, hyperlipidemia and other metabolic disorders related to increased blood triglyceride levels. Clinical investigations have shown that decreased lipid hydrolysis by the inhibition of lipase may decrease the lethal complications of these metabolic disorders.

The porcine pancreatic lipase inhibitory activity assay was utilized to evaluate the anti-lipase activity of the *C. curetum* hexane, acetone, methanol, and aqueous fractions, with the reference anti-lipase drug Orlistat, which is used as an anti-lipase and anti-obesity agent. The porcine pancreatic lipase inhibitory activity results indicate that the hexane fraction had the greatest anti-lipase activity compared with Orlistat, with an IC<sub>50</sub> value of  $154.8 \pm 1.86$   $\mu\text{g}/\text{mL}$  versus the Orlistat IC<sub>50</sub> value of  $12.3 \pm 0.35$   $\mu\text{g}/\text{mL}$ . The other fractions did not show any anti-lipase activity. Variations between plant extracts in their contents of active phytochemicals can affect their inhibitory properties against porcine pancreatic lipase,  $\alpha$ -amylase, and  $\alpha$ -glucosidase.

Despite the extensive global application of herbal products, there is a serious lack of information concerning their impact on cancer cells. Conventional chemotherapeutic medications can induce cell death in both cancer cells and normal cells. Natural plant extracts or isolated bioactive compounds may exhibit apoptotic and cell cycle modulating properties and at the same time show limited toxicity in normal cells. In addition, it has been shown in ethnomedicine that herbs and other natural products have been intensively used for the treatment of different types of cancer in various civilizations since ancient times [37]. In this study, the crude *C. curetum* hexane, acetone, methanol, and aqueous fractions were screened in vitro for cytotoxic activity in the HeLa (human cervical carcinoma) and Colo-205 (colon cancer) cell lines. The results show that Colo-205 cell viability was reduced by approximately 90% with the 1 and 0.5 mg/mL *C. curetum* acetone and hexane fractions ( $p \leq 0.0001$ ), while the 10 and 5 mg/mL aqueous fractions significantly induced cytotoxicity ( $p \leq 0.0001$  and  $p \leq 0.01$ ) by approximately 90% and 90%, respectively. Moreover, the 10 mg/mL methanol fraction significantly induced cytotoxicity ( $p \leq 0.0001$  and  $p \leq 0.01$ ) by approximately 90%. In a study conducted by Arora et al., a 0.2 mg/mL *Achyranthes aspera* root extract reduced human colon cancer (Colo-205) cell viability by 60% [37].

The cytotoxicity of *C. curetum* on the HeLa cell line was significantly induced by 0.125 mg/mL of the *C. curetum* acetone fraction ( $p \leq 0.0001$ ) by approximately 90%, while 10 mg/mL of the *C. curetum* aqueous fraction significantly induced cytotoxicity ( $p \leq 0.0001$ ) by

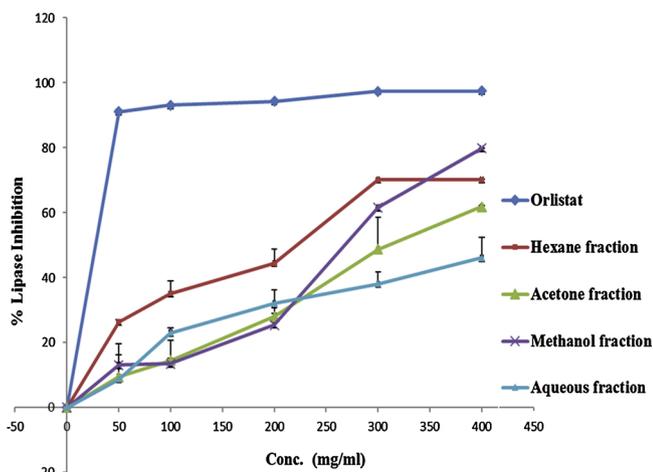


Fig. 6. Porcine pancreatic lipase inhibitory activity of the four *C. curetum* fractions and Orlistat.

approximately 90%. Moreover, 10 mg/ml of the methanol fraction significantly induced cytotoxicity ( $p \leq 0.0001$ ) by approximately 85%. In addition, the highest cytotoxic reactivity on HeLa cells was obtained with the 0.625 mg/mL *C. curetum* hexane fraction, which significantly induced cytotoxicity in cancer cells by ( $p \leq 0.0001$ ) approximately 95%.

In a study by Patel et al., the methanolic extract of *Solanum nigrum* at 10 mg/mL reduced viability by 65% in HeLa cancer cell line while at 0.0196 mg/mL led to only a 38% reduction [38]. Moreover, another study carried out by Nemati et al. showed that 0.5 mg/mL of *Consolida orientalis*, *Ferula assa-foetida*, *Coronilla varia*, and *Orobancha orientalis* ethanolic extracts exhibited cytotoxic effects against HeLa cancer by 7.59%, 25.30%, 54.42%, and 42.66%, respectively [39].

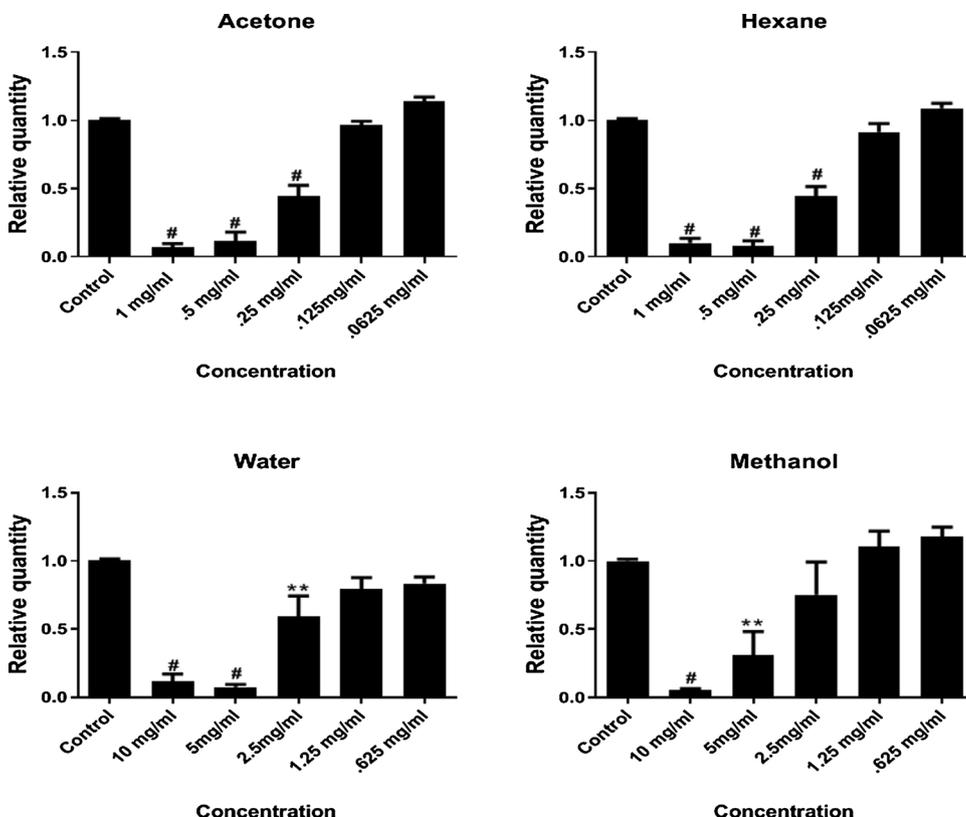


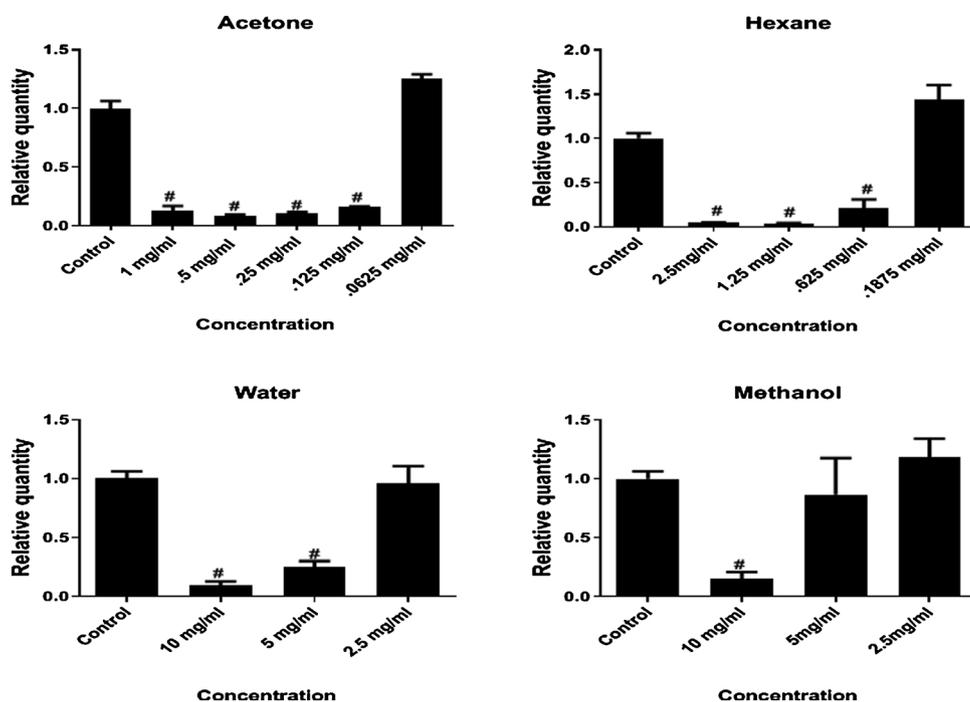
Fig. 7. The cytotoxic effects of various extracts derived from *C. curetum* on Colo-205 cells. Colo-205 cells were treated with various concentrations of the acetone, hexane, water and methanol extracts obtained from *C. curetum* and incubated for 24 h. Cytotoxicity was determined by the MTS assay. Results are depicted as relative quantities (RQs) compared to the control (with only media; C). # $P < 0.0001$  and \*\* $P < 0.01$ . Error bars represent SD.

In summary, only the 0.5 mg/mL of the *C. curetum* acetone and hexane fractions exhibited pronounced cytotoxic effects on the Colo-205 cancer cell line. Moreover, 0.625 mg/ml of the *C. curetum* hexane fraction exhibited potential cytotoxic effects against the cervical epithelial carcinoma (HeLa) cell line.

However, the exact mechanism of action that is responsible for the initiation of cytotoxicity and  $\alpha$ -amylase,  $\alpha$ -glucosidase, porcine pancreatic lipase enzymes inhibitory activity needs to be addressed. Thus, further studies on *C. curetum* aqueous, acetone and hexane fractions as a novel therapeutic agent having antidiabetic, anti-obesity and anticancer effects, originating from plants, are warranted. In addition, further in vivo clinical studies should be carried out to evaluate their effectiveness on humans.

5. Conclusion

These results show that the *C. curetum* aqueous fraction contains the highest contents of flavonoids, tannins, and phenols, while the aqueous and methanol fractions have the highest  $\alpha$ -amylase enzyme inhibitory activity in comparison with the reference anti-diabetic drug Acarbose. Moreover, the methanolic fraction has the best anti-glucosidase activity, even more than Acarbose. In addition, the hexane fraction has the highest anti-lipase activity compared with Orlistat. Additionally, the *C. curetum* acetone and hexane fractions showed the best cytotoxic effects against the Colo-205 cancer cell line and the hexane fraction induced the highest cytotoxic effect against HeLa cancer cells. Overall, this study revealed that *C. curetum* has potential  $\alpha$ -amylase,  $\alpha$ -glucosidase, porcine pancreatic lipase inhibitory and cytotoxic activity on HeLa and Colo-205 cancer cell lines, indicating the presence of biologically active and cytotoxic compounds in this plant species. This study provides only basic data, so further studies are necessary for the isolation and identification of biologically active substances in the *C. curetum* aqueous, methanol, acetone and hexane fractions.



**Fig. 8.** The cytotoxic effect of various extracts derived from *C. curetum* on HeLa cells. HeLa cells were treated with various concentrations of the acetone, hexane, water and methanol extracts obtained from *C. curetum* and incubated for 24 h. Cytotoxicity was determined by the MTS assay. Results are depicted as relative quantities (RQs) compared to the control (with only media; C). # $P < 0.0001$  and \*\* $P < 0.01$ . Error bars represent SD.

#### Authors' contributions

All research done by the authors

#### Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Yes.

#### Ethical approval

N/A.

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