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Cytotoxic Activity of Methanol Extract of *Cynanchum acutum* L. Seeds on Human Cancer Cell Lines

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***SUMMARY. Cynanchum acutum* L., Asclepiadaceae, has been used in the Egyptian folk medicine. HPLC analysis of the extract allowed the identification of 17 phenolic acids and 19 flavonoids. Quantitative analysis of some phenolic acids and flavonoids revealed the presence of vanillic acid (0.781 mg/100 g), benzoic acid (0.590 mg/100 g), pyrogallol (0.325 mg/100 g), catechin (0.32 mg/100 g), acacetin-neorutinoside (1.04 mg/100 g), hesperidin (3.48 mg/100 g), and kaempferol-3-glucoside-2"-p-coumaroyl (5.12 mg/100 g) as major compounds in the extract. The cytotoxic effect of the extract was investigated against colon cancer (Caco-2), breast cancer (MCF-7), hepatocellular carcinaoma (HepG-2), lung cancer (A549), prostate cancer (PC3), human epithelioma cells (Hep-2) and cervical carcinoma (Hela) cell lines. Cell viability was quantitated by MTT assay and IC_{50} was estimated. The IC_{50} values against Caco-2, MCF-7, HepG-2, A549, PC3, Hep-2 and Hela cell lines were determined 3.2, 2.7, 2.3, 2.5, 2.7, 2.6, and 6.6 mg/mL, respectively. Therefore, *Cynanchum acutum* L. seeds could be considered as a potential chemotherapeutic agent in cancer treatment in future.**

RESUMEN. Cynanchum acutum L., Asclepiadaceae, se ha utilizado en la medicina popular egipcia. El análisis por HPLC del extracto permitió la identificación de 17 ácidos fenólicos y 19 flavonoides. El análisis cuantitativo de algunos ácidos fenólicos y flavonoides reveló la presencia de ácido vainíllico (0,781 mg/ 100 g), ácido benzoico (0,590 mg/ 100 g), pirogalol (0,325 mg/ 100 g), catequina (0,32 mg/ 100 g), acancetinarutinósido (1,04 mg/ 100 g), hesperidina (3,48 mg/ 100 g) y kaempferol-3-glucósido-2"-p-cumaroil (5,12 mg/ 100 g) como principales compuestos en el extracto. El efecto citotóxico del extracto se investigó contra el cáncer de colon (Caco-2), cáncer de mama (MCF-7), carcinoma hepatocelular (HepG-2), cáncer de pulmón (A549), cáncer de próstata (PC3), células de epitelioma humano (Hep-2) y carcinoma de cuello uterino (Hela) La viabilidad celular se cuantificó mediante el ensayo MTT y se calculó la CI50. Se determinaron los valores de IC_{50} contra las líneas celulares Caco-2, MCF-7, HepG-2, A549, PC3, Hep-2 y Hela de 3.2, 2.7, 2.3, 2.5, 2.7, 2.6 y 6.6 mg/mL, respectivamente. Por lo tanto, las semillas de *Cynanchum acutum* L. podrían considerarse como un potencial agente quimioterapéutico en el tratamiento del cáncer en el futuro.

INTRODUCTION

Cynanchum acutum L. is a poisonous plant and belonging to family Asclepidaceae [1]. It is growing in several areas worldwide such as Nile Delta, Egypt and in the Blue Nile State, Southeastern Sudan [2,3]. It has different medical applications and is used in folk medicine. In Tunis, it is used to relief some dermatological and ophthalmological disorders [4], whereas in France it was used for bowel evacuation [5]. Besides, it reduced the blood glucose level in alloxan-induced diabetic rats and improved their lipids [6,7]. Also, *Cynanchum acutum* L. possessed anti-ulcer [8], anti-bacterial [9,10], and anti-inflammatory effects [11]. It contains β-sitosterol, lupeol, lupyl acetate and α-amyrin [12]. The antioxidants of *Cynanchum acutum* L. prevented the liver damage by carbon tetrachloride-induced hepatotoxicity in rats [13]. It contains antioxidant substances such as quercetin 3-O-β-galacturonopyranoside, quercetin 7-O-β-glucopyranoside, tamarixtin 3-O-β-galacturonopyranoside and kaempferol 3-O-β-galacturonopyranoside [7,10]. Furthermore, it has four flavonoid glycosides: quercetin di-O-hexoside, quercetin 3-Orhamnosyl (1→2) glyco-

KEY WORDS: Cynanchum acutum L. seeds, cytotoxicity, human cancers, HPLC-UV analysis, MTT assay.

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side, quercetin 3-O-galactoside, and quercetin 3-O-xyloside [14]. Moreover, it contains two coumarins; scopoletin and scoparone [15]. These antioxidants may cause cytotoxic effect against cancer cells [16]. Therefore, the present study has mainly focused on identifying the active principles and evaluating the cytotoxic effects of *Cynanchum acutum* L. seeds against human cancer cell lines for the first time.

MATERIALS AND METHODS

Chemicals and reagents

All the reagents and reference standards were of HPLC grade and purchased from Sigma Aldrich originate to Peypin-France, Badalona-Spain and Darmstadt, Germany. High purity water was prepared in our own lab using Milli- Q system (Millipore, MA, USA). Phenolic standards: gallic acid, pyrogallol, 3-OH Tyrosol, protocatechuic acid, catechol, *p*-OH-benzoic acid, caffeic acid, vanillic acid, *p*-coumaric acid, ferulic acid, iso-ferulic, alpha- coumaric acid, ellagic acid, coumarin, benzoic acid, 3,4,5-methoxycinnamic, cinnamic acid, catechin, apigenin-6-arabinose-8-galactose, apigenin-6-rhamnose-8-glucose, naringin, luteolin-7-glucose, rutin, hesperidin, rosmarinic acid, apigenin-7-glucose, apigenin-7-O-neohesperside, kaempferol-3-7-dirahmnoside, quercetin, kaempferol-3-glucoside-2"-*p*-coumaroyl, naringenin, acacetin-neorutinoside, acacetin-7-O-neohesperside, hesperetin, kaempferol, apigenin, chlorogenic acid, luteolin-7-O-glucoside, keampferol-3-rutinoside, isoquercitrin, and iso-rhamnetin. The purity of the standards was 98%.

Preparation of seeds extract

Seeds of *Cynanchum acutum* L. were collected during November to December 2017 from Damiatta Branch of River Nile, Egypt. The plant was authenticated at Agriculture Museum, El-Dokki, Giza, Egypt. The seeds were washed, dried, and powdered in shade. The dried powder of *Cynanchum acutum* L. seeds were extracted as reported by Moustafa *et al.* [17] with slight modifications. The seeds under investigation (100 g) were percolated in 500 mL methanol (70%), and then fully extracted by percolation at ambient temperature on shaker for 72 h. The extract was filtered using Whatman No. 1 paper and concentrated under reduced pressure at 40 °C, dried by high vacuum and the crude extract was kept in refrigerator at 4 °C until used for the experiment.

Determination of total phenolics and flavonoids contents

Quantification of phenolic compounds was carried out using Folin–Ciocalteau's method as reported by Aleksander *et al.* [18]. One g of dried powdered seeds was mixed with 40 mL of 80 % methanol, filtered through Watmann No. 1 filter paper and transferred into a volumetric flask (100 mL) with 80% methanol. An aliquot (0.2 mL) of the methanolic extract was placed into a volumetric flask (10 mL) and 0.5 mL Folin–Ciocalteu reagent (2N) was added. After 3 min, saturated sodium carbonate (1 mL) (20% in distilled water) was added to the mixture of methanolic extract and Folin-Ciocalteu reagent and the volume was completed with distilled water. After 1 h, absorbance of blue color was measured at λ_{max} 725 nm against a blank (distilled water) using Unicam UV-visible spectrophotometer 540 (Thermo Fisher Scientific, USA). The standard concentration curve was computed using gallic acid. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight (mg GAE/g D.W.).

Total flavonoid content was determined by aluminum colorimetric method according to Liu *et al.* [19]: 0.25 mL of the C. acutum methanolic extract was diluted with 1.25 mL of distilled water and placed into a volumetric flask (2.5 mL). Then 75 µL of a 5 % $NaNO_2$ solution was added to the mixture. After 6 min, 150 µL of a 10 % $AlCl3.6H_2O$ solution was added, and the mixture was allowed to stand for another 5 min. 0.5 mL 1 M Na OH was added and the volume was completed with distilled water. The solution was mixed, and the absorbance was measured against the blank at 510 nm using Unicam UV-visible spectrophotometer 540 (Thermo Fisher Scientific, USA). (+)-catechin was used as standard for a calibration curve. The results are expressed as milligrams of catechin equivalents (CE) per gram dry weight (mg CE/g D.W.).

Chromatography

Reverse-phase chromatographic separation of phenolic compounds was performed using HPLC, Agilent Series 1200 system (Agilent, USA), comprising a quaternary HP pump (series 1200) separation module for solvent delivery, autosampling injector, solvent degasser and zobrax ODS C18 column (particle size 5 µm, 250 × 4.6 mm Ø). Detection was performed using an ultraviolet (UV) detector (Agilent, 1100). The previously prepared methanol extract of the

seeds (100 mg) was dissolved in HPLC grade acetonitrile (2 mL). Separation of phenolic acids was carried out with mobile phase consisted of three solvents, A (CH_3COOH 2.5%) in water, B (CH_3COOH 8%) in water and C (acetonitrile). Gradient profile was shown in (Table 1) [20]. Chromatography was performed at 35 °C with a flow-rate of 1 mL/min. The injection volumes were 10 µl. Ultraviolet (UV) detector was set at 280 nm [20].

Time (min)	% Mobil Phase A (CH_3COOH 2.5%)	% Mobil Phase B (CH_3COOH 8%)	% Mobil Phase C (Acetonitrile)
0	95	5	0
20	90	10	0
50	70	30	0
55	50	50	0
60	0	100	0
100	0	50	50
110	0	0	100
120	0	0	100

Table 1. HPLC mobile phase gradient method for phenolic acids separation.

Flavonoid separation was done using a mobile phase consisting of 50 mM H_3PO_4, pH 2.5 (solution A) and acetonitrile (solution B) acetic acid (40:60, v/v). Gradient profile was shown in (Table 2) [21]. The temperature of the column was maintained at 35 °C. The flow rate of the mobile phase was 0.7 mL/min. The injection volumes were 5 µl. Ultraviolet (UV) detector was set at 330 nm [21]. UV spectra were recorded between 200 and 600 nm for peak characterization. Standard flavonoids and phenolic acids were prepared as 10 mg/50 mL solutions in methanol and they were diluted to make concentrations (20-40 µg/mL) and injected into HPLC. Peak area of the external standards was used to quantify the phenolic compounds of the seeds extract and the concentrations of the identified compounds were expressed as mg/100 g.

Time (min)	% Mobil Phase A (50 mM H_3PO_4)	% Mobil Phase B Acetonitrile: acetic acid (40:60, v/v)
0-5	95	5
5-55	50	50
55-65	50	50
65-67	95	5

Table 2. HPLC mobile phase gradient methods for flavonoids separation.

Cell lines

Cell lines; colon cancer (Caco-2), breast cancer (MCF-7), hepatocellular carcinaoma (HepG-2), lung cancer (A549), prostate cancer (PC3), human epithelioma cells (Hep-2) and cervical carcinoma (Hela) were obtained from tissue culture laboratory, Vacsera, Dokkey, Giza, Egypt.

MTT assay

Laminar air flow cabinet was used to maintain the sterility of the procedure. The cell culture was maintained in a Roswell Park Memorial Institute medium (RPMI 1640). It contained a 1% antibiotic-antimycotic mixture (10,000 µg/mL streptomycin sulphate, 25 µg/mL amphotericin B and 10,000 U/mL potassium penicillin), 1% L-glutamine. Fetal bovine serum (10% heat-inactivated was used to supplement the medium [17]. Culturing and subculturing were performed according to Thabrew *et al.* [22].

The cytotoxicity was evaluated by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide). The yellow MTT undergoes a mitochondrial reduction to form a purple formazan [23]. The 96-well tissue culture microplate were inoculated at a cell concentration of 1×10^5 cells per well in 100 µL of growth medium. The microplate was incubated at 37 °C (5 % CO_2) for 24 h to develop a complete monolayer sheet. Growth medium was decanted from 96 well microplate after the formation of confluent sheet of the cells. The extract of the seeds was dissolved in dimethyl sulfoxide (DMSO). Serial dilutions of the dissolved extract were prepared by RPMI–1640 medium to give a final concentration of 75, 37.5, 18.75, 9.375, 4.687, and 2.343 mg/mL; 0.1 mL of each concentration of the extract was added to confluent cell monolayers dispensed into 96-well microplate using a multichannel pipette. The treated cells were incubated at 37 °C (5 % CO_2) for 24h. Three wells were used for each concentration of the extract. Control cells were incubated without the seeds extract. MTT powder was dissolved in PBS (Bio Basic Canada Inc.) to give a solution at concentration of 5 mg/mL. After the end of the incubation period, 20 µL of the MTT solution was

No	[a] Rt	Phenolic Compounds	Conc. (mg/100 g)
		Phenolic acids	
1	6.432	Gallic acid	0.01
2	6.60	Pyrogallol	0.33
3	7.71	3-OH-tyrosol	0.14
4	8.00	Protocatechuic acid	0.15
5	9.23	Catechol	0.25
6	9.65	*p*-OH-benzoic acid	0.28
7	10.20	Caffeic acid	0.05
8	10.42	Vanillic acid	0.78
9	11.51	*p*-coumaric acid	0.05
10	11.92	Ferulic acid	0.13
11	12.31	Iso-Ferulic acid	0.19
12	13.44	Alpha- coumaric acid	0.07
13	13.52	Ellagic acid	0.06
14	14.33	Coumarin	0.04
15	14.36	Benzoic acid	0.59
16	14.45	3,4,5- methoxy- cinnamic	0.10
17	16.33	Cinnamic acid	0.19
		Flavonoids	
1	8.17	Catechin	0.32
2	11.55	Apigenin-6-arabinose-8-galactose	0.08
3	11.91	Apigenin-6-rhmnose-8-glucose	0.89
4	12.11	Naringin	0.23
5	12.22	Luteolin-7-glucose	0.51
6	12.43	Rutin	0.20
7	12.45	Hesperidin	3.48
8	12.51	Rosmarinic acid	0.28
9	12.83	Apigenin-7-glucose	0.25
10	13.02	Apigenin-7-O-neohesperside	0.45
11	13.11	Kaempferol-3-7-dirahmnoside	0.10
12	13.43	Quercetin	0.53
13	15.13	Kaempferol-3- Glucoside-2"-P-coumaroyl	5.12
14	15.26	Naringenin	0.05
15	15.31	Acacetin- neorutinoside	1.04
16	15.35	Acacetin-7-O-neohesperside	0.27
17	15.72	Hesperetin	0.46
18	15.87	Kaempferol	0.45
19	17.14	Apigenin	0.57

Table 3. Quantifications of some phenolic compounds identified in *C. acutum* L. seeds using HPLC analysis. [a]Rt: Retention time.

added to each well. The mixing was allowed at 150 rpm for 5 min using the shaker. Then, the 96-well microplate was incubated at 37 °C (5 % CO_2) for 4 h to allow the MTT metabolism. Formazan (MTT metabolic product) was resuspended in 200 µL of DMSO and placed on a shaker at 150 rpm for 5 min for a through mixing. The optical density was recorded using a Micro plate reader (Mindray-96A, China.) at 560 nm. The results were corrected using a reference wavelength 620 nm (wells without adding MTT) [22]. All experiments were carried out in triplicate.

Microscope

Inverted microscope (Nikon, 118811) with objective lens 10× and eye lens 8× was used to see the morphological features of cell lines at different concentrations of seeds extract.

Determination of IC_{50} values

GraphPad prism version 5 software, Inc., California, U.S.A was used to calculate IC_{50} (the half maximal inhibitory concentration) values of methanol extract of *Cynanchum acutum* L. seeds and doxorubicin (as a positive control) against Caco-2, MCF-7, HepG-2, A549, PC3, Hep-2, and Hela cell lines. The percentage growth inhibition was calculated using Eq. [**1**]:

$$\%Growth\ inhibition = \left(\frac{Mean\ OD\ of\ individual\ test\ group}{Mean\ OD\ of\ control\ group}\right) \times 100 \quad [\mathbf{1}]$$

RESULTS

Total phenolic compounds and flavonoids content of seeds of *C. acutum* were 39 mg gallic acid equivalents (GAE) per gram dry weight and 25 mg catechin equivalents (CE) per gram dry weight, respectively. Quantification of some phenolic compounds using the standards was performed. Seventeen phenolic acids and nineteen flavonoids were identified and quantified (Table 3).

The major phenolic acids were vanillic acid (0.78 mg/100 g), benzoic acid (0.59 mg/100 g) and Pyrogallol (0.33 mg/100 g). The other phenolic acids showed small amounts in the seeds extract. Kaempferol-3-glucoside-2"-*p*-coumaroyl (5.12 mg/100 g), hesperidin (3.48 mg/100 g), acacetin- neorutinoside (1.04 mg/100 g), and apigenin-6-rhamnose-8-glucose (0.89 mg/100 g) were the highest flavonoids in the seeds (Table 3).

The use of the MTT assay analyse the cytotoxic effect of methanol extract of *Cynanchum acutum* L. seeds on Caco-2, MCF-7, HepG-2, A549, PC3, Hep-2, and Hela cell lines. IC_{50}, the half maximal inhibitory concentration was used to estimate the seeds extract cytotoxicities against malignant cell lines (Table 4).

The IC_{50} values of the methanol extract were 3.2, 2.7, 2.3, 2.5, 2.7, 2.6, and 6.6 mg/mL against Caco-2, MCF-7, HepG-2, A549, PC3, Hep-2 and Hela cell lines, respectively. The seeds extract was effective against all cancer cell lines. However, the cytotoxic effect of the extract on cervical cancer (Hela) cell line was lesser than other cancer cell lines (Table 4). The methanol extract of the seeds killed the cancer cell lines as it was observed by the microscope (Fig. 1).

Cell Lines	[a] IC_{50} (mg/mL) *Cynanchum acutum* L. seeds	Doxorubicin (positive control)
[b] Caco-2	3.2 ± 0.8	0.06 ± 0.002
[c] MCF-7	2.7 ± 0.2	0.23 ± 0.025
[d] HepG-2	2.3 ± 0.3	0.11 ± 0.024
[e] A549	2.5 ± 0.1	0.09 ± 0.002
[f] PC3	2.7 ±0.4	0.02 ± 0.003
[g] Hep-2	2.6 ± 0.3	0.01 ± 0.003
[h] Hela	6.6 ±0.5	0.02 ± 0.002

Table 4. Cytotoxic effects of methanol extract of *Cynanchum acutum* L. seeds on cancer cell lines [a] IC_{50}: The half maximal inhibitory concentration; [b] human colon cancer (Caco-2), [c] human breast cancer (MCF-7), [d] human hepatocellular carcinaoma (HepG-2) and [e] human lung cancer (A549), [f] prostate cancer (PC3), [g] Human epithelioma cells (Hep-2) and [h] cervical carcinoma (Hela). Each value represents mean ± SD from three independent experiments.

DISCUSSION

Studies reported that phenolic acids and flavonoids have cytotoxic activity [24]. In the present study, total phenolic acids and flavonoids contents of *Cynanchum acutum* L. seeds were 39 mg gallic acid equivalent (GAE)/g D.W. and 25 mg catechin equivalent (CE)/g D.W., respectively. Phenolic acids were reported to possess cytotoxic activity [25]. In the present study, phenolic acids are OH-benzoic acids (gallic acid, p-OH-benzoic acid, protocatechuic acid and vanillic acid), hydroxycinnamic acids (ferulic acid, iso-ferulic acid, caffeic acid, coumaric acid, alpha-coumaric acid). Vanillic acid was reported to have cytotoxicity against cancer cells [26]. In this study, vanillic acid (0.8 mg/100 g) is the highest phenolic acid in the seeds of *Cynanchum acutum* L. (Table 3). Moreover, flavonoids were reported to have cytotoxic effect against cancer cells [24,27]. In this study, flavonoids are flavones (including both O- and C- glycosides with apigenin or luteolin as aglycone), flavonols (derived from the aglycones, quercetn, kaempferol and isorhamnetin), flavonones (naringenin, hespirdin and their glycosides), and only one flavan-3-ol (catechin). Kaempferol-3-Glucoside-2"-*p*-coumaroyl (5.12 mg/100 g), hesperidin (3.48 mg/100 g), acacetin- neorutinoside (1.04 mg/ 100 g) ,and apigenin-8-6-rhamnose glucose (0.88 mg/100 g) were the major flavonoids of the seeds of *Cynanchum acutum* L. (Table 3). Hesperidin was

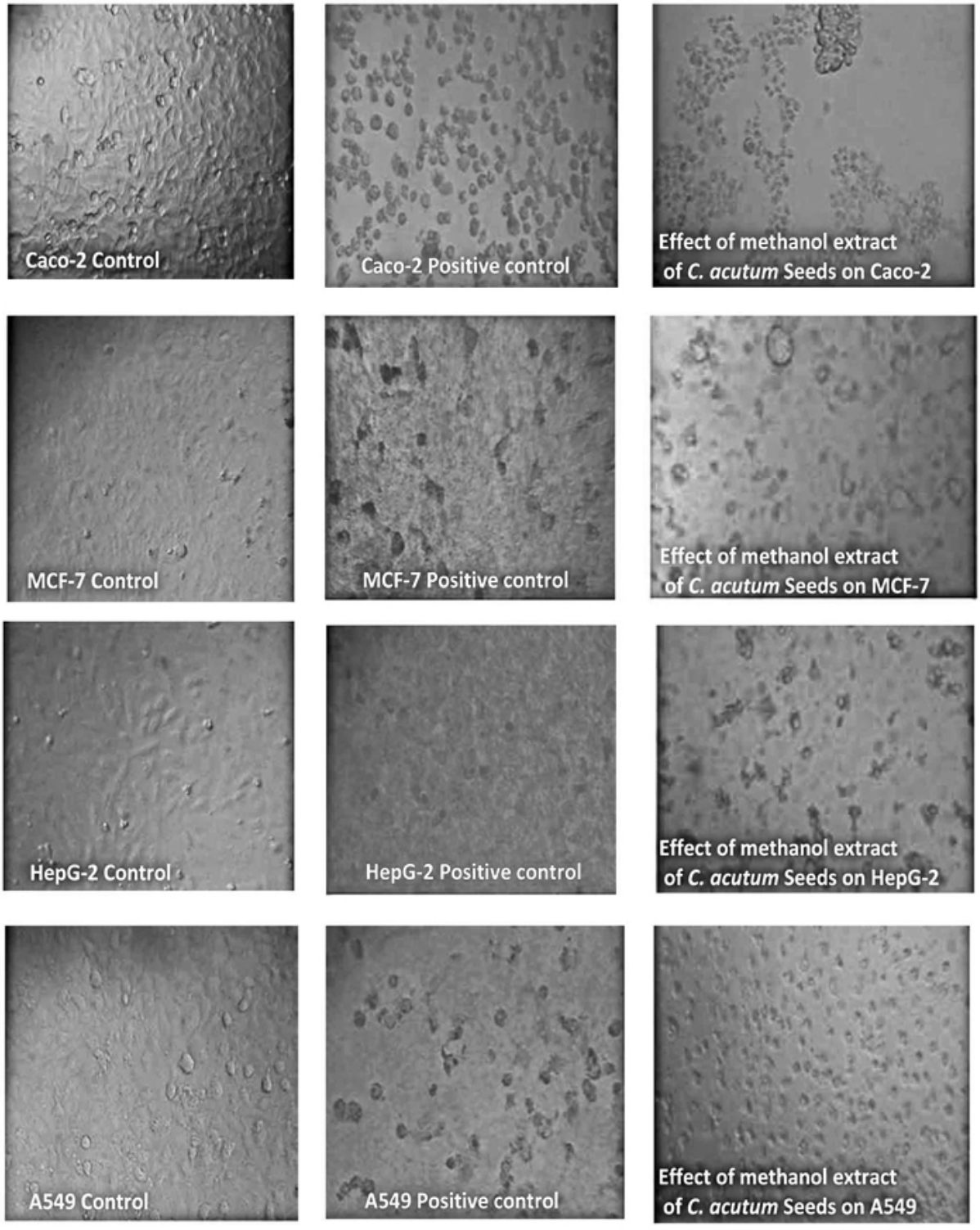

Figure 1: Effects of the seeds of extract of *C. acutum* L. on some cell lines by MTT assay.

reported to have cytotoxicity against human colon cancer cells [28].Three flavonoids: kaempferol-3-glucoside-2"-p-coumaroyl, apigenin-7-O-neohesperside. and acacetin-7-O-neohesperside were identified for the first time in *Cynanchum* genus. They were reported to have antioxidant properties [29,30]. So, the presence of C2=C3 double bond and 3-OH group in the vast majority of phenolic compounds were reported to possess antioxidant activity [31].

CONCLUSION

In this work we have evaluated the cytotoxic activity of the methanol extract of *Cynanchum acutum* L. seeds. Phenolic acids and flavonoids extracted with 70% methanol have been identified and quantified. The extract showed potential cytotoxic effect against different cancer cell lines. The current results indicate that the flavonoid and phenolic acid of extract of *Cynanchum acutum* L. seeds is a promising natural pharmaceutical for combating cancer.

Authors contribution. Ahmed Mohamed Mohamed Youssef suggested the point, designed the article, wrote this manuscript, performed HPLC analysis, and analyzed the data. Zeinab Elswaify performed seeds methanol extraction. Yousef Mohamed Al-Saraireh and Saed Mohamed AL-Dalain performed MTT assay.

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