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Colorectal cytotoxic activities of phenolic constituents isolated from *Euphorbia pseudocactus* cultivated in Egypt

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ABSTRACT

Phytochemical investigation of the ethyl acetate fraction of the *Euphorbia pseudocactus* aerial parts, family Euphorbiaceae, resulted in the isolation of eight phenolic compounds for the first time from this species. Identification was carried out using different chromatographic and spectroscopic techniques. The isolated compounds were kaempferol **(1)**, ethyl gallate **(2)**, gallic acid **(3)**, astragalin **(4)**, astragalin-6″-gallate **(5)**, nicotiflorin **(6)**, ellagic acid **(7)**, and 1,2,3,4,6-pentagalloylglucose **(8)**. The colorectal cytotoxic activity of the isolated phenolic compounds against the LS-174T and LS-513 (cecum) cell lines was tested using the sulforhodamine B assay. The results revealed that gallic acid possessed colorectal cytotoxic activity with IC_{50} values of 18.27 and 50.11 µg/ml against the LS-174T and LS-513 cell lines, respectively, while ethyl gallate possessed cytotoxic activity with IC_{50} value of 25.42 µg/ml against the LS-174T cell line. The results of the other phenolic compounds showed nonsignificant anticancer activity. These findings recommend gallic acid as an anticancer lead compound.

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent malignancies in humans, which is related to cancer mortality numbers in the world (Huanga *et al*., 2019). In 2012, CRC was diagnosed worldwide in 614,000 women and 746,000 men (Kuipers *et al*., 2015). CRC is spreading rapidly because of unhealthy diets, aging, and sedentary lifestyles. Surgery and chemotherapy are considered the conventional treatments for CRC. Chemotherapeutics can cause DNA deterioration or activate numerous signaling pathways, such as stopping cell cycle, global translation inhibition, and DNA recombination, which finally lead to cancer cell death (Woods and Turchi, 2013). However, treatment with chemotherapeutic drugs in CRC patients has different outcomes depending on the subtype of cancer, as revealed in many studies, including Malignant pleural effusion (MPE) studies. So far, there have been many problems associated with chemotherapy, mainly drug resistance, the effects of cytotoxicity, and adverse reactions. Accordingly, natural products including plants, animals, marine organisms, and microorganisms still considered to be well tolerated and have less toxicity are investigated for the identification of potential chemotherapeutic agents (Mao *et al*., 2017; Singh *et al*., 2008). Previous reports revealed that drugs extracted from natural products have broad application prospects in the treatment of CRC (Aires *et al*., 2013; Johnson and Mukhtar, 2007).

Historically, the major contribution of natural metabolites and their derivatives has been achieved in pharmacotherapy, particularly in cancer and infectious diseases (Atanasov *et al*., 2021). A large number of plants, animals, marine organisms, and microorganisms are wealthy in amazing chemical diversity, which makes nature an exceptional resource of new biologically active compounds. From January 2008 to December 2013, a total of 25 natural products and natural product-derived drugs were approved in pharmaceutical markets (Butler *et al*., 2014).

Different natural metabolites, such as polyphenols, alkaloids, polysaccharides, diterpenoids, and unsaturated fatty acids, are involved directly or indirectly in about half of all currently used

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anticancer medications (Newman and Cragg, 2016). The ongoing development of such products has opened up a new avenue for cancer prevention and treatment. Natural products are now regarded as a prolific exporter of novel anticancer medications.

Euphorbia covers a great diversity of spurge-like plants (family Euphorbiaceae), with over 2,000 species (Ernst *et al*., 2015). Great phytochemical varieties including terpenoids (Cao *et al*., 1992; Liu *et al*., 2002), phenolics (Duarte *et al*., 2008; Mueller and Pohl, 1970), and tannins (Giordani *et al*., 2001; Yashida *et al*., 1994) were discovered in the Euphorbiaceae family. So far, the same family encloses a broad spectrum of therapeutic activities like antimalarial (Zhou *et al*., 2016) and anticancer (Guo *et al*., 2018; Fu *et al*., 2016) activities against human gastric cancer cell lines of both multidrug-resistant and nonresistant. On the other hand, *Euphorbia aegyptiaca* showed anti-inflammatory activity related to its saponins, coumarins, flavonoids, tannins, sterols, and triterpenes content (Abo-dola and Lotfi, 2016). So far, flavonoids isolated from *Euphorbia tirucalli* possess anti-inflammatory and analgesic properties (Prabha *et al*., 2008).

Euphorbia pseudocactus Berger has a multibranched stem (candelabra spurge) that looks like a candelabrum, which is a dwarf, and the plant is distributed along South Africa's subtropical coast. It thrives in thorny bushlands and savannah, where it frequently forms colonies. It was reported that new abietane terpenoids which possess great antimicrobial activity have been identified in the *E. pseudocactus* hexane fraction (Abdel-Monem and Abdelrahman, 2016).

The limited review on *E. pseudocactus* drives us to explore new pharmacological activities and investigate the chemistry of this species. In this study, the ethyl acetate (EtOAc) fraction of *E. pseudocactus* was investigated for isolation and identification of pure compounds, and the new biological activity of the isolated compounds was investigated using *in vitro* colorectal cytotoxic activity.

MATERIALS AND METHODS

General

The NMR spectra were recorded on a Bruker Nuclear Magnetic Resonance (NMR) spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The NMR spectra chemical shift values were recorded in δ (ppm). Solvents used include Dimethyl sulfoxide (DMSO), $CDCl_3$, and Methanol (MeOD). Silica gel 60 (Sigma-Aldrich Chemicals, Darmstadt, Germany) and, for column chromatography (CC), Sephadex LH-20 (E-Merck) were utilized. Thin-layer chromatography (TLC) plates (Fluka precoated silica gel, F_{254}) were used for monitoring the fractions from CC. The cell lines used in the cytotoxicity assay (LS-174T and LS-513) were acquired from Nawah Scientific Inc. (Cairo, Egypt).

Cell culture

CRC cell lines LS-174T and LS-513 (cecum) were kept in Roswell Park Memorial Institute (RPMI) media complemented by 100 mg/ml of streptomycin (100 units/ml).

Cytotoxic assay

The cell viability was determined using the sulforhodamine B (SRB) assay. In 96-well plates, aliquots of 100 µl of cell suspension (5×10^3 cells) were incubated in full medium for 24 hours. Other aliquots of 100 µl media containing drugs and doxorubicin (positive control) at different concentrations (0.01, 0.1, 1, 10, and 100 µg/ml) were added to the cells. After 72 hours of drug treatment, the cells were fixed by replacing the medium with 150 µl of 10% Trichloroacetic acid (TCA) and incubating for 1 hour at 4°C. The TCA solution was taken away, and the cells were washed with distilled water five times, and then after adding aliquots of 70 µl SRB solution (0.4 % w/v), plates were incubated in a dark area for 10 minutes at room temperature. The plates were washed with 1% acetic acid three times and air-dried overnight. After that, a protein-bound SRB stain was dissolved in 150 µl of TRIS (10 mM). The absorbance at 450 nm was measured using a BMG LABTECH®-FLUOstar Omega microplate reader (Ortenberg, Germany) (Allam *et al*., 2018; Skehan *et al*., 1990).

Plant material

Euphorbia pseudocactus aerial parts were gathered in October 2014 from Cactus Farm in Tahanop, Shebin El-Qanater, Qalubiya, Egypt. The authentication of the collected plant was confirmed by Dr. Abdelhalim Mohamed (Phytotaxonomy Department, Agricultural Research Institute, Cairo, Egypt). The Pharmacognosy Department Herbarium, Faculty of Pharmacy, Beni-Suef University, received a voucher specimen (BUPD-62).

Extraction and isolation

Euphorbia pseudocactus aerial parts (7 kg) were extracted with methanol, filtered, and evaporated under reduced pressure to get a crude extract (80 g) .The dried extract was suspended in 500 ml of distilled water and partitioned into *n*-hexane (500 ml × 3), dichloromethane (500 ml × 3), EtOAc (500 ml × 3), and *n*-butanol (500 ml × 3). Evaporation of the collected extractives under reduced pressure using a rotary evaporator yielded (9 g) *n*-hexane, (2.1 g) dichloromethane, (2.3 g) EtOAc, and (6 g) *n*-butanol (Altemimi *et al*., 2017).

The EtOAc fraction (2.3 g) was chromatographed on a silica gel column ((70 g, 130 × 2.5 cm), using solvent system CH_2Cl_2 and MeOH of 5% increasing polarity, six main subfractions were collected and evaporated under reduced pressure. Fraction A (0.2 g) was chromatographed over a silica gel column (10 g, 20 × 1.5 cm) and eluted with 90% CH_2Cl_2/MeOH and then purified over Sephadex LH-20 using 100% MeOH to get compound **1** (50 mg). Fraction B (0.2 g) was chromatographed over a silica gel column (10 g, 20 × 1.5 cm), using 85% CH_2Cl_2/MeOH, followed by purification over 'Sephadex LH-20 eluted with 100% MeOH to get compound **2** (30 mg). Fraction C (0.8 g) was chromatographed on a silica gel column (40 g, 75 × 2.5 cm), eluted with 80% CH_2Cl_2/MeOH, followed by Sephadex LH-20 using 100% MeOH for purification of compound **3** (100 mg). Fraction D (0.3 g) was chromatographed over a silica gel column (15 g, 30 × 1.5 cm), eluted with 80% CH_2Cl_2/MeOH, with further purification using Sephadex LH-20, and eluted with 100% MeOH to get compound **4** (50 mg) and compound **5** (70 mg). Fraction E (0.2 g) was chromatographed using a silica gel column (10 g, 20 × 1.5 cm), eluted with 70% CH_2Cl_2/MeOH and then Sephadex LH-20 using 100% MeOH yielding compound **6** (50 mg) and compound **7** (30 mg). Finally, fraction F (0.2 g) was chromatographed over a silica gel column (10 g, 20 × 1.5 cm), eluted with 65% CH_2Cl_2/MeOH, followed by Sephadex LH-20 using 100% MeOH to get compound **8** (20 mg).

Spectral data of isolated compounds

Kaempferol **(1)**: yellow powder, ^{1}H NMR (400 MHz, MeOD) δ 8.10 (2H, d, J = 7.6 Hz, H-2′, 6′), 6.92 (2H, d, J = 7.6 Hz, H-3′, 5′), 6.41 (H, s, H-8), 6.20 (H, s, H-6).^{13}C NMR (100 MHz, MeOD) δ 146.65 (C-2), 135.74 (C-3), 175.97 (C-4), 159.14 (C-5),

97.86 (C-6), 164.17 (C-7), 93.06 (C-8), 161.10 (C-9), 103.14 (C-10), 122.32 (C-1′), 129.28 (C-2′, 6′), 114.90 (C-3′, 5′), 156.85 (C-4′).

Ethyl gallate **(2)**: off-white powder, ^{1}H NMR (400 MHz, MeOD) δ 7.08 (2H, d, J = 2.5 Hz, H-2, 6), 4.26 (2H, q, J = 7.1 Hz, H-8), 1.32 (3H, t, J = 7.1 Hz, H-9).

Gallic acid **(3)**: yellowish white needles, ^{1}H NMR (400 MHz, MeOD) δ 7.08 (1H, s, OH-4), 6.64 (2H, s, H-2, 6).^{13}C NMR (100 MHz, MeOD) δ 170.49 (C-7), 146.24 (C-3, 5), 139.18 (C-4), 121.64 (C-1), 110.33 (C-2, 6).

Kaempferol-3-O-β-D-glucoside "astragalin" **(4)**: yellow powder, ^{1}H NMR (400 MHz, MeOD) δ 8.07 (2H, d, J = 8 Hz, H-2′, 6′), 6.91 (2H, d, J = 6.8 Hz, H-3′, 5′), 6.42 (1H, s, H-8), 6.22 (1H, s, H-6), 5.27 (1H, d, J = 6 Hz, H-1″), 3.23–3.73 (m, 6H of glucose). ^{13}C NMR (100 MHz, MeOD) δ 157.67 (C-2), 134.05 (C-3), 178.10 (C-4), 161.67 (C-5), 98.53 (C-6), 164.69 (C-7), 93.37 (C-8), 157.10 (C-9), 104.31 (C-10), 121.38 (C-1′), 130.88 (C-2′, 6′), 114.67 (C-3′, 5′), 160.17 (C-4′), 102.70 (C-1″), 74.33 (C-2″), 77.01 (C-3″), 69.94 (C-4″), 76.63 (C-5″), 61.21 (C-6″).

Astragalin-6″-gallate **(5)**: yellow powder, ^{1}H NMR (400 MHz, DMSO) δ 7.94 (2H, d, J = 8.8 Hz, H-2′, 6′), 6.77 (2H, d, J = 8.8 Hz, H-3′, 5′), 6.40 (1H, s, J = 2 Hz, H-8), 6.20 (1H, s, J = 2 Hz, H-6), 5.46 (1H, d, J = 7.2 Hz, H-1″), 3.17–3.43 (m, 4H of H-2″, H-3″, H-4″ & H-5″), 4.26 (2H, d, H-6″), 6.92 (2H, s, H-2‴, 6‴) ^{13}C NMR (100 MHz, DMSO) δ 156.87 (C-2), 133.60 (C-3), 177.76 (C-4), 161.61 (C-5), 99.24 (C-6), 164.73 (C-7), 94.21 (C-8), 157.23 (C-9), 104.38 (C-10), 121.09 (C-1′), 131.23 (C-2′, 6′), 115.54 (C-3′, 5′), 160.39 (C-4′), 101.89 (C-1″), 74.54 (C-2″), 74.54 (C-3″), 69.75 (C-4″), 76.60 (C-5″), 63.21 (C-6″), 119.76 (C-1‴), 109.00 (C-2‴, 6‴), 145.93 (C-3‴, 5‴), 138.87 (C-4‴), 166.11 (C-7‴).

Kaempferol-3-O-rutinoside "nicotiflorin" **(6)**: yellow powder, ^{1}H NMR (400 MHz, MeOD) δ 8.07 (2H, d, J = 8.4 Hz, H-2′, 6′), 6.90 (2H, d, J = 8.4 Hz, H-3′, 5′), 6.39 (1H, s, H-8), 6.21 (1H, *s*, H-6), 5.12 (1H, d, J = 6.4 Hz, H-1″), 4.54 (1H, s, H-1‴), 3.27–3.82 (m, 6H of glucose and 4H of rhamnose), 1.14 (3H, d, J = 6 Hz, H-6‴). ^{13}C NMR (100 MHz, MeOD) δ 158.0 (C-2), 134.11 (C-3), 177.95 (C-4), 161.56 (C-5), 98.71 (C-6), 164.96 (C-7), 93.62 (C-8), 157.15 (C-9), 104.17 (C-10), 121.35 (C-1′), 130.98 (C-2′, 6′), 114.75 (C-3′, 5′), 160.09 (C-4′), 103.25 (C-1″), 75.78 (C-2″), 74.35 (C-3″), 70.04 (C-4″), 76.73 (C-5″), 67.18 (C-6″), 101.02 (C-1‴), 70.67 (C-2‴), 70.89 (C-3‴), 72.49 (C-4‴), 68.33 (C-5‴), 16.52 (C-6‴).

Ellagic acid **(7)**: yellow powder, ^{1}H NMR (400 MHz, DMSO) δ 7.46 (2H, s, H-5, 5′).^{13}C NMR (100 MHz, DMSO) δ 159.63 (C-7), 148.61 (C-4), 140.23 (C-3), 136.84 (C-2), 112.81 (C-1), 110.64 (C-5), 107.96 (C-6).

1,2,3,4,6-pentagalloylglucose **(8)**: yellow powder, ^{1}H NMR (400 MHz, MeOD) δ 4.29–5.45 (6H, glc. H-2,3,4,5,6), 6.39 (1H, d, glc. H-1), 6.68, 6.71, 7.03, 7.07, 7.12 (each 2H, s, galloyl H). ^{13}C NMR (MeOD, 100 MHz): δ 63.63 (glc. C-6), 70.23 (glc. C-4), 72.68 (glc. C-2), 73.94 (glc. C-3), 74.79 (glc. C-5), 94.0 (glc. C-1), 108.81, 108.92, 109.01, 109.05, 109.57, 110.31 (galloyl C-2, C-6), 119.20, 119.64, 119.78, 119.90 (galloyl C-1), 138.50, 138.53, 138.62, 138.69, 138.73 (galloyl C-4), 145.03, 145.05, 145.06, 145.11, 145.14 (galloyl C-3, C-5), 166.09, 166.63, 166.85, 167.16, 168.76 (–COO–).

RESULTS AND DISCUSSION

Identification of isolated compounds

Eight phenolic compounds were isolated and identified for the first time from the EtOAc fraction of the *E. pseudocactus* aerial parts. Structural elucidation of the isolated compounds was carried out by comparing NMR spectral data with previous reports. Consequently, the isolated and identified compounds (Fig. 1) were kaempferol **(1)** (Wu *et al*., 2012), ethyl gallate **(2)** (Ooshiro *et al*., 2009), gallic acid **(3)** (López-Martínez *et al*., 2015), kaempferol-3-O-β-D-glucoside "astragalin" **(4)** (Abdelkader *et al*., 2021; Ghareeb *et al*., 2018), astragalin-6″-gallate **(5)** (Nugroho *et al*., 2014), kaempferol-3-O-rutinoside "nicotiflorin" **(6)** (Orhan *et al*., 2007), ellagic acid **(7)** (Ghareeb *et al*., 2018), and 1,2,3,4,6-pentagalloylglucose **(8)** (Deiab *et al*., 2015). The isolated compounds' NMR spectra are provided in the supplementary file section.

Cytotoxic activity of the isolated compounds

Colorectal cytotoxic efficacy of the isolated phenolic compounds was tested against two human carcinoma cell lines, LS-174T and LS-513 (cecum), using the SRB assay and doxorubicin as the positive control with IC_{50} values of 4.50 and 5.23 µg/ml against the LS-174T and LS-513 cell lines, respectively. Results revealed that gallic acid possessed colorectal cytotoxic activity with IC_{50} values of 18.27 and 50.11 µg/ml against the LS-174T and LS-513 cell lines, respectively; also, ethyl gallate showed a moderate cytotoxic activity with IC_{50} value of 25.42 µg/ml against the LS-174T cell line, while the other phenolic compounds showed nonsignificant results. IC_{50} values and percent of carcinoma cells viability in relation to varying concentrations of gallic acid and ethyl gallate are summarized and shown in Figure 2.

CRC is third among the most frequent cancers and is the fourth leading cause of cancer-related deaths. The majority of CRC cases are found in Western countries, and its prevalence is increasing year by year (Mármol *et al*., 2017).

The great importance of the genus *Euphorbia* is because of its diverse phytochemicals, which include phenolics (Duarte *et al*., 2008) such as tannins (Giordani *et al*., 2001) and terpenoids (Liu *et al*., 2002). Previous reports highlight the chemical profile and biological importance of the hexane and dichloromethane fractions of *E. pseudocactus*; however, nothing could be traced concerning the chemistry and biology of the EtOAc fraction of the same species, which is considered our main issue in this study.

Phenolic compounds are one of the secondary metabolites which are not essential for plant growth, development, or reproduction, but they are efficient antioxidants and play a role in plant–environment interactions. Due to their effect as antioxidants (Martin and Appel, 2009) and anticancer activities (Harris *et al*., 2007), phenolics are also important components of the human healthy diet. Many classes of phenolics can be distinguished depending on the number of phenol rings and the structural features that join these rings (Stalikas, 2007).

In this study, a chemical investigation of the EtOAc fraction of the titled species was carried out, resulting in the isolation of eight phenolic and flavonoid compounds that were tested for colorectal cytotoxic activity; among the isolated phenolic constituent, gallic acid possessed colorectal cytotoxic activity. However, gallic acid (3,4,5-trihydroxybenzoic acid; GA), a natural phenolic molecule obtained from plants, has been documented to prevent the formation and progression of different cancer types (Zhang *et al*., 2019). This is the first report on the cytotoxic activity of gallic acid against these two cell lines.

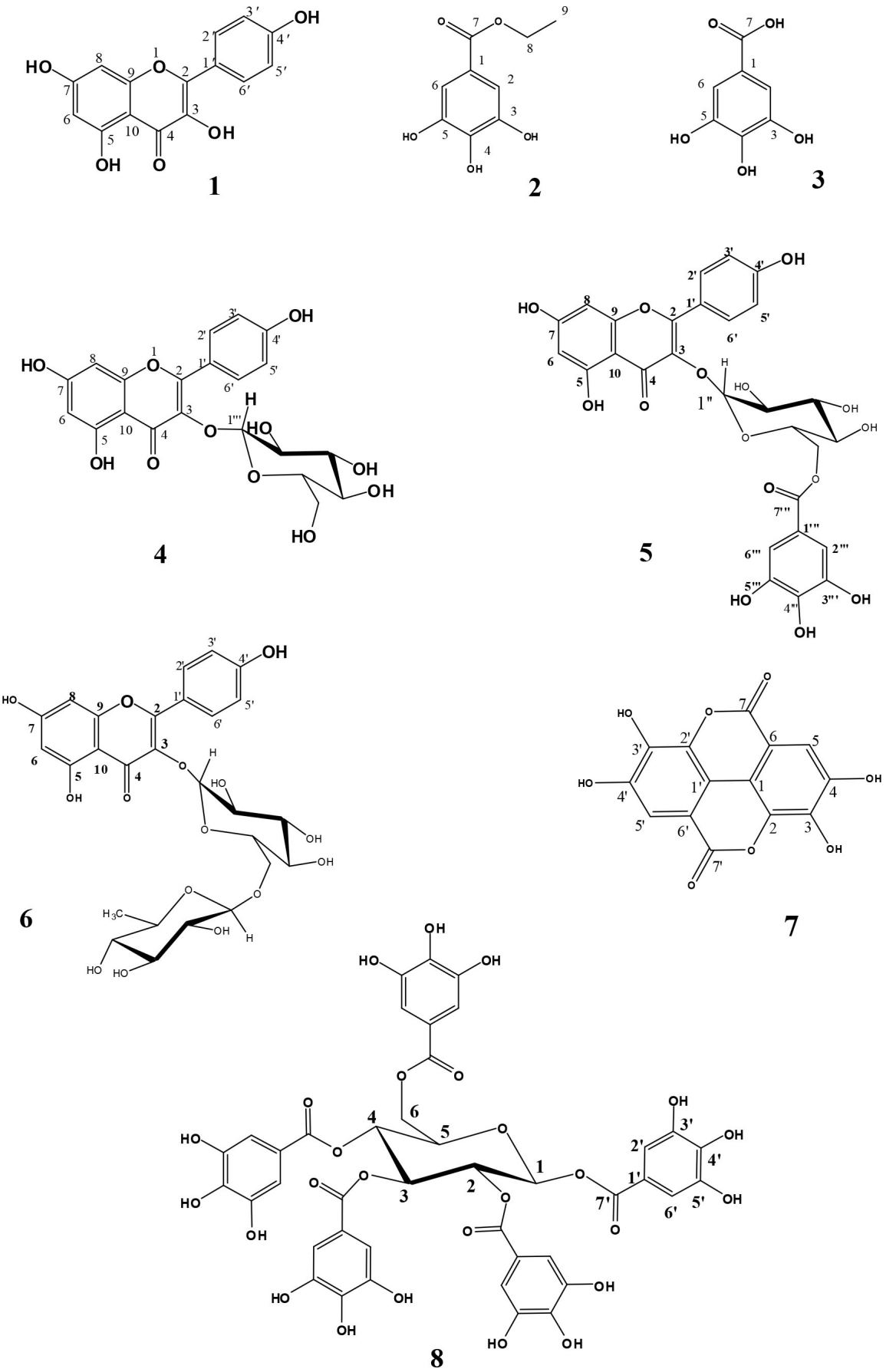

Figure 1. Structures of phenolic compounds isolated from *E. pseudocactus*.

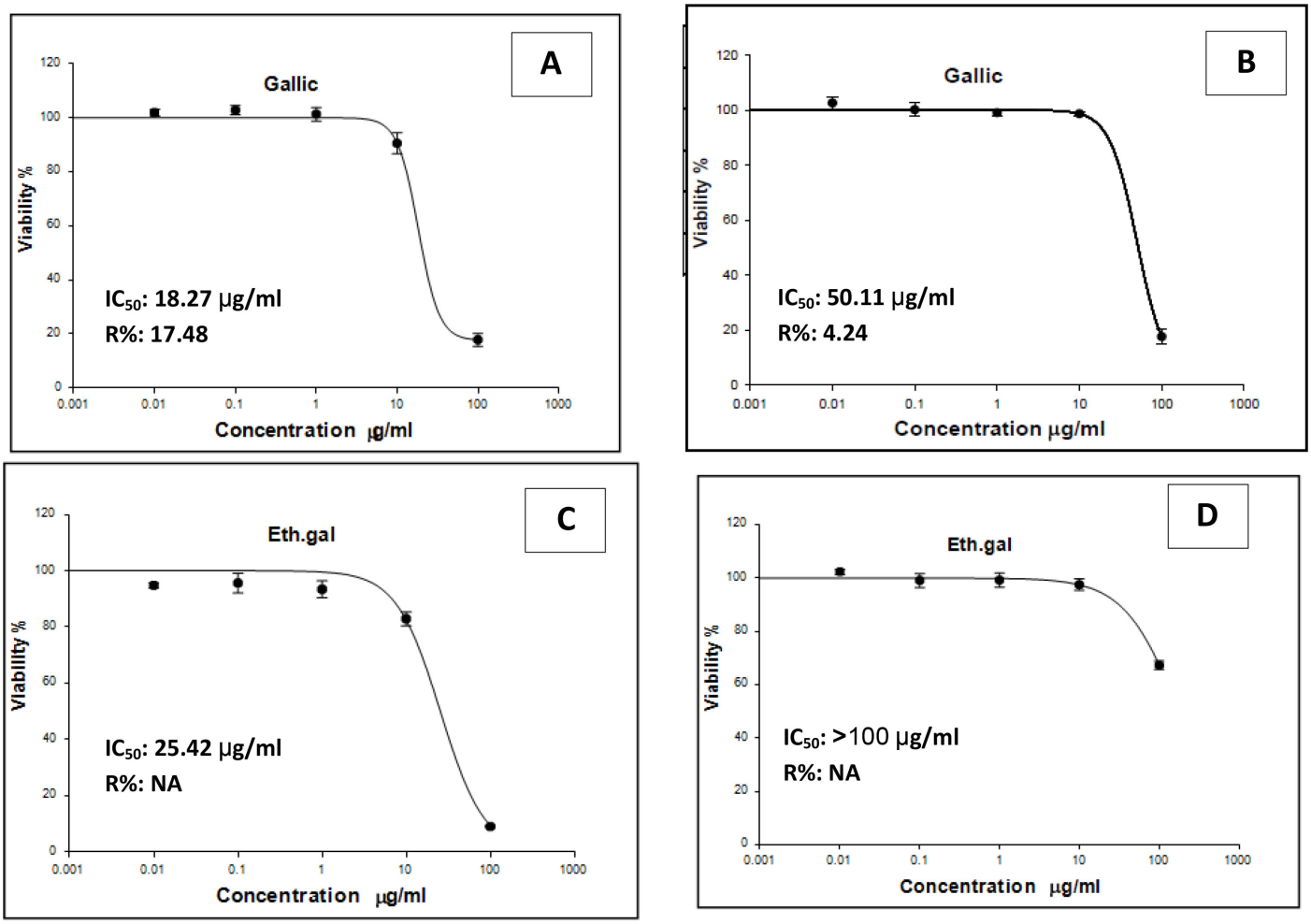

Figure 2. IC_{50} values and percent of carcinoma cells viability in relation to varying concentrations of gallic acid against LS-174T (A) and LS-513 (B) and of ethyl gallate against LS-174T (C) and LS-513 (D).

CONCLUSION

In this study, the chemical investigation of the unexplored EtOAc fraction of *E. pseudocactus* resulted in the isolation and identification of eight phenolic compounds for the first time from *E. pseudocactus*. Some of the phenolic constituents showed colorectal cytotoxic activity; interestingly, gallic acid showed inhibitory activity against CRC cell lines, and we recommend submitting gallic acid for cytotoxic activity against CRC. Last but not least, *E. pseudocactus* could be considered a large reservoir for potential activities that could be an interesting aspect in the future.

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CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

AUTHORS' CONTRIBUTION

Concept and design, data acquisition, data analysis, interpretation, funding, and final approval were created by all of the authors. The original article draft and statistical analysis were cowritten by Marwa Hassan and Mona Ismail. Rabab Mohammed, Abeer Moawad, and Mohamed Zaki contributed to supervision, technical material support, and critical revision of the manuscript.

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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