

Phytochemical analysis and antioxidant and anticancer activities of mastic gum resin from *Pistacia atlantica* subspecies kurdica

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Background: The mastic gum resin has been used in traditional Kurdish medicine for treating various disorders such as topical wound and gastric ulcer. The study designed to evaluate the total polyphenol and flavonoid content, free radical scavenging activity, and anticancer effects of mastic gum resin derived from *Pistacia atlantica* subspecies kurdica.

Materials and methods: Folin-Ciocalteu and the aluminum chloride colorimetric assays were used to determine the total phenol and flavonoid contents in the mastic gum resin respectively. Whereas, DPPH and ABTS+ assays were used to determine the antioxidant activities of mastic gum resin. Regarding anticancer activities, the MTT assay was used to study the effect of mastic gum resin on the proliferation of various cancer cells and the morphological changes were identified after Acridine Orange/Propidium Iodide staining. Flow cytometry was applied to determine the influence of mastic gum resin on the apoptosis rate by Annexin V double staining and to investigate the influence on cell cycle progression. Caspase colorimetric assay was used to estimate the hallmark enzyme of apoptosis, and finally RNA were obtained from COLO205 cells and analyzed by qRT-PCR analyses.

Results: The MTT results showed that the mastic gum resin at concentrations from 0.01 to 100 μ M induced death of cancer cells in a dose and time-dependent manner. The mastic gum resin suppressed proliferation of human cancer cells with 72 h IC₅₀ value of 15.34 \pm 0.21, 11.52 \pm 0.18, 8.11 \pm 0.23 and 5.2 \pm 0.8 μ g/mL for bile duct cancer (cholangiocarcinoma) (KMBC), pancreatic carcinoma (PANC-1), gastric adenocarcinoma (CRL-1739), and colonic adenocarcinoma (COLO205) cells, respectively. Normal human colon fibroblast (CCD-18Co) cells were not adversely affected by resin treatment. Flow cytometry showed that the mastic gum resin significantly ($P < 0.05$) arrested COLO205 cell proliferation at the G2/M phase of cell cycle. The resin caused apoptotic morphological changes in COLO205 cells. The apoptotic effect to mastic gum resin was via the mitochondrial as shown by the up-regulation of Bax, down-regulation of Bcl-2 genes, and activation of caspase-9 and -3 activities.

Conclusion: It was confirmed that the antiproliferative efficacy of the resin is positively correlated with its polyphenolic contents, suggesting a causal link related to exudate content of phenolic acid and flavonoids. The results revealed that the mastic gum resin has potential to be developed as an anticancer and antioxidant product due to its high content of polyphenol compounds.

Keywords: natural plant exudate, polyphenolic contents, free radical scavenging, apoptosis

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Introduction

Most recent data have shown that the number of cancer patients and mortality due to cancers are on the rise. Despite great advances in the development of new and innovative therapeutic strategies, cancer remains one of the leading causes of death.¹ Although new cancer therapeutic and carcinostatic agents have been developed, their effects on cancer patients are generally not obvious.

In recent years, natural herbal metabolites have gained interest as compounds for alternative remedies for various diseases.² According to the World Health Organization (WHO), almost 65% of the world's population has included plants and traditional medicine as the additional modality in health care.³ In fact, several chemical compounds isolated from plants and traditional medicine have been shown to kill rapidly dividing cells,⁴ thus revealing their great potential to be developed as anticancer agents. However, the use of these compounds is limited by their narrow beneficial index, considerable toxicity, and delivery issues during treatment.⁵

The genus *Pistacia* belongs to a cosmopolitan family Anacardiaceae that comprises approximately 70 genera and more than 600 species.⁶ The species of the genus *Pistacia* are evergreen, aromatic, nutraceutical, and deciduous resin-bearing shrubs and fast-growing xerophytic trees that can reach heights of 8–10 m.⁷ *Pistacia* plant parts including leaf, fruit, stem, exudate, and essential volatile oil have been chemically characterized and used to treat various human ailments^{8,9} because of their antiatherogenic,¹⁰ hypoglycemic,¹¹ hepatoprotective,¹² cytoprotective,¹³ antigenotoxic,¹⁴ anti-inflammatory,¹⁵ antiulcerogenic,¹⁶ antipyretic, antifungal,¹⁷ antibacterial,¹⁸ antiviral,¹⁹ antiparasitic,²⁰ antimutagenic,⁹

antioxidant,²¹ and anticancer activities,^{22–24} as well as stimulant and diuretic properties.²⁵

The *Pistacia atlantica* subspecies *kurdica*, commonly known as Daraban or Qazwan tree in Kurdish²⁶ and Baneh tree in Persian, is a medicinal and food plant that is native and endemic wild growing in Iran and in the Auramanat area of the Kurdistan province of Western Iran.^{27,28} The plant is also found in several temperate Asian countries including Armenia, Azerbaijan, Syria, Iraq, and Turkey.^{29–31} This *Pistacia* subspecies contains gums, particularly the well-known mastic gum, an oleo-resin obtained as exudate from the trunk, stem, and branches of the tree (Figure 1A).³²

Mastic gum resin (MGR) has a long history as a therapeutic agent with many reported medicinal, pharmaceutical, and biological properties.^{5,33} Ancient Greeks used MGR for the treatment of various gastrointestinal ailments such as abdominal discomfort, stomach aches, gastralgia, dyspepsia, and peptic ulcers.³⁴ MGR contains volatile oil with α -pinenes, sabinene, and limonene as the main components,^{35,36} and was reported to possess significant in vitro antibacterial and antifungal properties.²⁹ The resin is particularly effective against bacteria, such as *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus pyogenes*, that are resistant to common antimicrobial agents.^{17,18,37–39} MGR was shown to decrease the

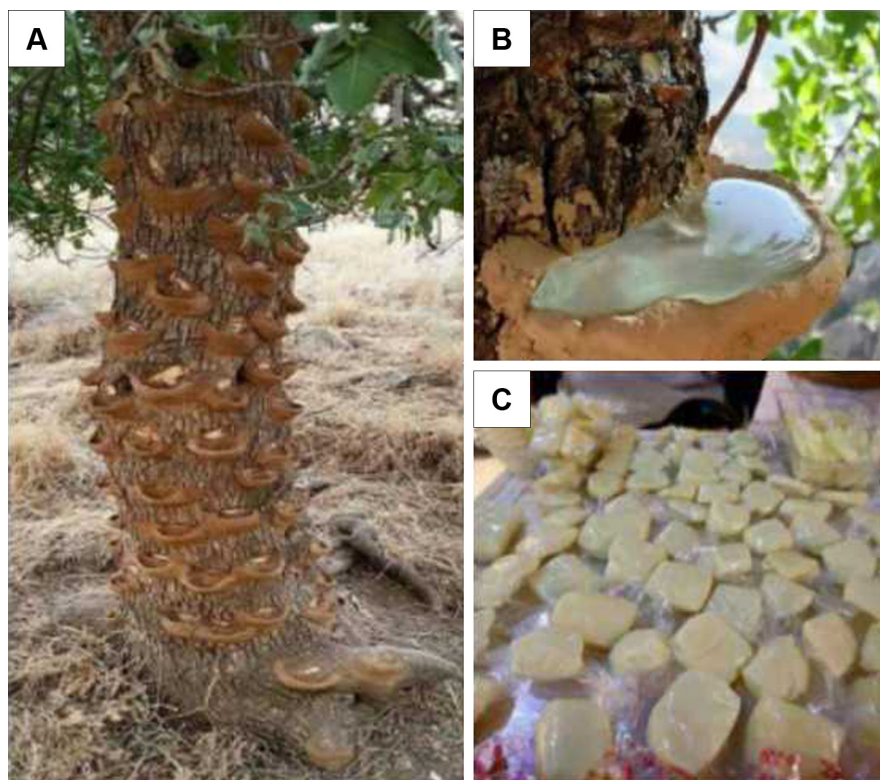


Figure 1 (A) Baneh or Daraban tree with clay cup for collecting resin. (B) The handmade muddy cup that was used for collecting exudate (resin). (C) Chewing gum produced from the natural MGR.

Abbreviation: MGR, mastic gum resin.

plasma levels of interleukin 6 and C-reactive protein,³⁶ suggesting that it may also act as an effective anti-inflammatory agent. In Kurdish culture, MGR is locally known as “bneshtal” (Figure 1B), which is used for the treatment of many health ailments including hypertension, stomach pain, gastric ulcer, and wounds.³¹ In addition, MGR is used for making a natural Kurdish chewing gum (Figure 1C) without adding any additive, preservative, or colorant.

Although the MGR of *Pistacia lentiscus* var. chia seems to be potent at inhibiting the growth of several human cancers including prostate,^{40,41} colon,²⁴ and colorectal cancers, leukemia,⁴² and Lewis lung carcinoma,⁴³ the cytotoxicity of the MGR from *P. atlantica* subspecies kurdica on both cancerous and noncancerous cells has not been fully investigated. Thus, this study is the first to report the anticancer properties of the MGR from *P. atlantica* subspecies kurdica in several digestive system-related human cancer cell lines.

Materials and methods

Plant metabolite

P. atlantica subspecies kurdica tree was identified based on the flora of the Iraq,³⁷ and MGR was collected from the trees of Penjwen area, Kurdistan region, Northern Iraq, between June and August 2016, which corresponds to the period of peak oleoresin production by the plant (Figure 1B). The gum was obtained as exudate from the trunk and branches of the plant. About 10 mg of the gum was suspended, just before use, in 1.0 mL of 0.2% (v/v) Tween 80 in distilled water (vehicle) to obtain the gum solution.¹⁹

Methods

Phytochemical analysis

The total phenol and flavonoid contents in the MGR were determined by Folin–Ciocalteu⁴⁴ and aluminum chloride (AlCl_3) colorimetric⁴⁵ assays, respectively. For the phenolic content assessment, 1.0 mL of the exudate was mixed with 1.0 mL of 10-fold diluted Folin–Ciocalteu reagent, vortexed well, and set aside for 5 minutes. Then, 10 mL of sodium carbonate solution (Na_2CO_3 ; 7.5%) was added, and the volume was made up to 25 mL with distilled water. After leaving the mixture for 60 minutes at room temperature, the absorbance was measured at 765 nm using a spectrophotometer (Hitachi, Chiyoda, Tokyo, Japan). Results were expressed in milligrams of gallic acid equivalent (GAE; 5–100 $\mu\text{g}/\text{mL}$) dissolved in distilled water per gram of exudate. On the other hand, for the flavonoid content evaluation, 1.0 mL of 2% AlCl_3 in ethanol was mixed with the same volume of resin, and a drop of acetic acid was added. Then, the volume was made up to 25 mL using distilled water. After leaving the

mixture for 45 minutes at room temperature, the absorbance was measured at 415 nm spectrophotometrically, and the results were expressed in milligrams of rutin equivalent (RE; 5–25 $\mu\text{g}/\text{mL}$) dissolved in ethanol per gram of exudate.

Determination of antioxidant activity using free radical scavenging assay

DPPH assay

Free radical scavenging activity of the MGR was determined using the DPPH reagent.²¹ Briefly, 0.5 mL of resin at various concentrations (1, 3, 10, 30, and 100 $\mu\text{g}/\text{mL}$) was added to 3.0 mL of DPPH solution (0.1 mM) and incubated in the dark for 30 minutes. Later, the optical density (OD) was measured at 517 nm using the spectrophotometer. Results were expressed as inhibition % of the DPPH. This assay was carried out in triplicate, and vitamin E (IndiaMart, Noida, India) was used as an internal control.

ABTS+ assay

ABTS reagent was used to measure the generated ABTS radicals. In brief, ABTS (7 mM) reagent and potassium persulfate (2.45 mM), at a ratio of 2:1 (v/v), were mixed thoroughly and incubated in the dark for 16–18 hours at room temperature. Later, an equal volume of resin at various concentrations (150, 300, 600, 1,200, and 2,400 $\mu\text{g}/\text{mL}$) was mixed with a previously prepared solution containing ABTS radicals, incubated at 37°C for 1 hour, and read at 732 nm using the spectrophotometer in which glutathione (Sigma-Aldrich, St Louis, MO, USA) was used as a standard.⁴⁶ The reading was used to determine the antioxidant activity of the resin.

Determination of anticancer activity

Cell culture

The most common human cancer cell lines related to the digestive system, including bile duct cancer (cholangiocarcinoma) (KMBC), pancreatic carcinoma (PANC-1), gastric adenocarcinoma (CRL-1739), and colonic adenocarcinoma (COLO205), and normal human colon fibroblasts (CCD-18Co) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were propagated in various specific growth media – RPMI-1640 (ATCC) medium for COLO205 cells, DMEM (Thermo Fisher Scientific, Waltham, MA, USA) for PANC-1 cells, F-12K (Gibco) for CRL-1739 and KMBC cells, and EMEM (Gibco) for CCD-18Co cells – to which 10% heat-inactivated FCS (Gibco), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Sigma-Aldrich) were added. As per the ATCC protocol, the cells were cultured and grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . The cultures were frequently

examined for confluency and viability. Finally, cells were cultivated as monolayer cultures and examined for viability using Trypan Blue exclusion test.

Cytotoxicity assay

The antiproliferative effect of MGR on cancer and normal cells was quantified by MTT assay.⁴⁷ Briefly, upon reaching 90% confluency, the cell concentration was determined using a hemocytometer counting chamber (Marienfeld, Lauda-Königshofen, Germany). Then, the cells, at 1×10^5 cells/mL, were cultured in 96-well microculture plates (TPP, Trasadingen, Switzerland) and treated with various concentrations of MGR in triplicates. MTT solution (Sigma-Aldrich) was added to the cells after incubation for 72 hours at 37°C. After exactly 4 hours of incubation in the dark, MTT solubilization solution (Sigma-Aldrich) was added and the plate was allowed to stand for 5 minutes at room temperature. The OD was then measured at 570 nm using an ELISA plate reader (Biotech, Inc, Alpharetta, GA, USA). The IC_{50} value was determined from absorbance versus concentration curve and compared to that of doxorubicin, the commonly used antineoplastic agent (Sigma-Aldrich), and the negative control, 0.1% DMSO (Sigma-Aldrich).

Apoptosis detection assay

The COLO205 cell death induced by MGR was investigated using the AO/PI double-staining method based on the standard protocol and was viewed using confocal microscopy (Leica, Wetzlar, Germany). The COLO205 cells, at 1×10^6 cells/mL, were treated with MGR and incubated at 37°C under 5% CO₂ for 24, 48, and 72 hours. Then, the cells were trypsinized, collected, and centrifuged at $200 \times g$ (32R; Hettich, Tuttlingen, Germany) for 8 minutes, and the supernatant was removed. PBS was used to wash the cells twice after centrifuging at $200 \times g$ (32R; Hettich) for 5 minutes, and excess medium was removed. About 15 μ L of the cell pellets was stained with 15 μ L mixture, containing equal amounts (100 μ g/mL) of AO and PI mixture, for 3 minutes, and then, 15 μ L of stained cell suspension was placed on a glass slide, covered with a glass slip, and examined within 30 minutes before the fluorescence faded.⁴⁸

Annexin V-FITC assay

COLO205 cell apoptosis caused by MGR treatment was determined with the annexin V-FITC kit (Sigma-Aldrich) without modifications. COLO205 cells, at 1×10^6 cells/mL, were treated with MGR at a concentration of 5.2 ± 0.8 μ g/mL, while control cells were not treated. After 24, 48, and 72 hours of incubation, the treated cells were harvested by trypsinization and centrifuged at $200 \times g$ (32R; Hettich) for 8 minutes.

Approximately 2 mL ice-cold PBS was used to wash the cell pellet twice before recentrifugation at $200 \times g$ for 8 minutes. The cells were resuspended in 0.5 mL ice-cold 1X binding buffer to which a mixture of 5 μ L of annexin V-FITC conjugate and 10 μ L of PI was added. The suspension was gently mixed by vortexing, allowed to stand in the dark at 25°C for 15 minutes, and then analyzed using a flow cytometer (BD FACSCalibur) equipped with an argon laser (BD Biosciences, San Jose, CA, USA) under laser-emitting excitation light at 488 nm.

Cell cycle assay

The cytotoxic effect of MGR on COLO205 cells was further confirmed using cell cycle analysis by means of flow cytometry. In brief, approximately 2.5×10^6 COLO205 cells/mL treated with MGR at a concentration of 5.2 ± 0.8 μ g/mL were incubated for 24, 48, and 72 hours. The cells were harvested by trypsinization and centrifugation at $200 \times g$ (32R; Hettich) for 5 minutes and washed with 1.0 mL ice-cold PBS (pH 7.4). To prevent cell clumping and aggregation, 500 μ L of 80% ice-cold ethanol was added to the cell pellet, gently mixed by vortexing, and then stored at -20°C. After 5–7 days, the cells were washed twice with 1.0 mL PBS each time and centrifuged at $200 \times g$ (32R; Hettich) for 5 minutes, and the ethanol was discarded. Finally, the cell pellets were stained with staining solution containing 0.1% Triton X-100, 10 mM EDTA, 50 μ g/mL RNAase A, and 3 μ g/mL PI and incubated on ice in the dark for 30 minutes. The BD FACSCalibur flow cytometer equipped with argon laser (BD Biosciences) was used to analyze the cell suspension under laser-emitting excitation light at 488 nm.⁴⁹

Caspase assays

Caspase-3 is the hallmark enzyme of apoptosis that is required for DNA fragmentation, while caspase-9 is an enzyme involved in the mitochondrial intrinsic pathway and precedes caspase-3. Thus, a colorimetric assay kit (GenScript, Piscataway, NJ, USA) was used to estimate the activities of caspase-3 and -9 in the COLO205 cells. COLO205 cells, at approximately 2×10^6 cells/mL, treated with MGR were incubated for 24, 48, and 72 hours; untreated cells which were similarly incubated served as controls. The cells were trypsinized and centrifuged for 5 minutes at 2,000 rpm (32R; Hettich). The medium was discarded, and the pellet was washed twice with ice-cold PBS and recentrifuged at 2,000 for 5 minutes. Approximately 50 μ L cold lysis buffer containing 0.5 μ L DTT and 0.25 μ L PMSF was used to lyse cells, and the cell suspension was allowed to stand on ice for exactly 60 minutes with vortexing at 10-minute intervals. The cell lysates were centrifuged for 1 minute at 10,000 rpm (32R; Hettich) at 4°C, and the supernatant was collected for

Table 1 Primer sequences in one-step SYBR Green quantitative real-time PCR

Primer	Forward sequence	Reverse sequence
Bcl-2	5cl CCAGACTCATTCAACCAGACA-3A	53A GATGACTGAGTACCTGAACCG-3T
Bax	5ax TTTGCTACAGGGTTTCAT-3T	53T CTCCATATTGCTGTCCAG-3C
Cyt-c	5yt GTCTTATGCTTGCCTCCCTT-3C	53C CGTCTGTCTTCGAGTCCGA-3T
β3T CGT	53T CGAGATGTGATGAAGGAGATG3-3A	53A CCTGTTGACTGGTCATTACAA-3T
GAPDH	5AP CGGGACCTAATGAAACTCCA-3G	53G AATCTCCACTTTGCCACTGC-3T

Note: Highly purified salt-free primers were designed by Next Gene Scientific Sdn. Bhd. and synthesized by AITBiotech.

determination of protein concentrations using the Bradford assay. Then, 200 µg protein in a 50 µL solution was mixed with 50 µL 2X reaction buffer containing 0.5 µL DTT and 0.25 µL PMSF. Approximately 5 µL caspase substrate was added, and the suspension was transferred to a 96-well plate (TPP), wrapped with aluminum foil, and incubated in the dark at 37°C for 4 hours. Finally, the caspase activities were determined spectrophotometrically in a microplate reader (Universal Microplate Reader; Biotech, Inc) at 405 nm.

qRT-PCR assay

RNeasy® lipid tissue mini kit (Qiagen NV, Venlo, the Netherlands) was used to extract total RNA from COLO205 cells prior to conducting qRT-PCR analysis. The nanophotometer (Implen GmbH, München, Germany) was used to quantify the RNA before aliquoting and storing at -80°C. The expression of Bax, Bcl-2, and Cyt-c genes was determined by qRT-PCR assay using GAPDH and β-actin genes as references.

Highly purified salt-free primers were designed by Next Gene Scientific Sdn. Bhd. (Puchong, Malaysia) and synthesized by AITbiotech (Singapore, Singapore) (Table 1). QIAGEN® One-Step RT-PCR SYBR Green kit (Qiagen NV) was used to prepare the reaction mix according to instructions of the manufacturer. Thermal cycler (BioRad®, CFX96; BioRad Laboratories, Inc., Hercules, CA, USA) amplification program conditions for temperature gradient PCR were as follows: 50°C for 10 minutes (reverse transcription), 1 cycle of 95°C for 5 minutes (initial activation), and 39 cycles of 95°C for 10 seconds (denaturation) and 50°C–60°C for 30 seconds (combined annealing and extension). The melting curve analysis was performed between 70 and 95°C, with 0.5°C/5 seconds increments. CFX Manager™ software, version 1.6 (BioRad Laboratories, Inc.) was used for quantitative gene transcription analyses in triplicate.⁵⁰

Statistical analysis

The data were expressed as mean ± SD. Using SPSS software, version 21.0 (SPSS Inc., Chicago, IL, USA), the data were analyzed for significant differences at $P < 0.05$. All experiments were done in triplicate.

Results

MGR is rich in polyphenolic compounds

The phytochemical investigation of the exudate revealed the presence of flavonoid and phenolic compounds. The total phenolic content was 147.11 ± 0.25 mg GAE/g extract, whereas the flavonoid content was 45.55 ± 3.2 mg RE/g extract, with $P = 0.0004$ in both cases (Figure 2).

MGR scavenged free radicals

The antioxidant activity of the resin was evaluated by its ability to scavenge DPPH and ABTS+ free radicals. The resin showed DPPH scavenging activity with a percentage increase from 26.44, 60.37, 75.44, 83.66, and 95.56, respectively, at 1, 3, 10, 30, and 100 µg/mL (Figure 3A), with an IC₅₀ value of 2.5 µg/mL. In contrast, the resin exhibited ABTS+ radical scavenging activity with a percentage increase from 25.11, 30.08, 55.3, 68.77, and 89.9 at concentrations of 150, 300, 600, 1,200, and 2,400 µg/mL, respectively, with an IC₅₀ value of 500 µg/mL (Figure 3B).

MGR showed cytotoxicity toward cancer cells

The colorimetric MTT assay was used to investigate the cytotoxic effects of the MGR on human bile duct cancer (cholangiocarcinoma) (KMBC), pancreatic carcinoma (PANC-1), gastric adenocarcinoma (CRL-1739), colonic

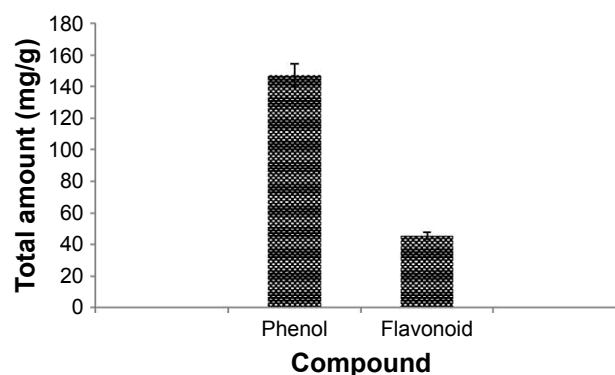


Figure 2 Total phenolic and flavonoid contents of MGR from *Pistacia atlantica* subspecies *kurdica*. Each value is the average of relative SD of 3 analyses.

Abbreviation: MGR, mastic gum resin.

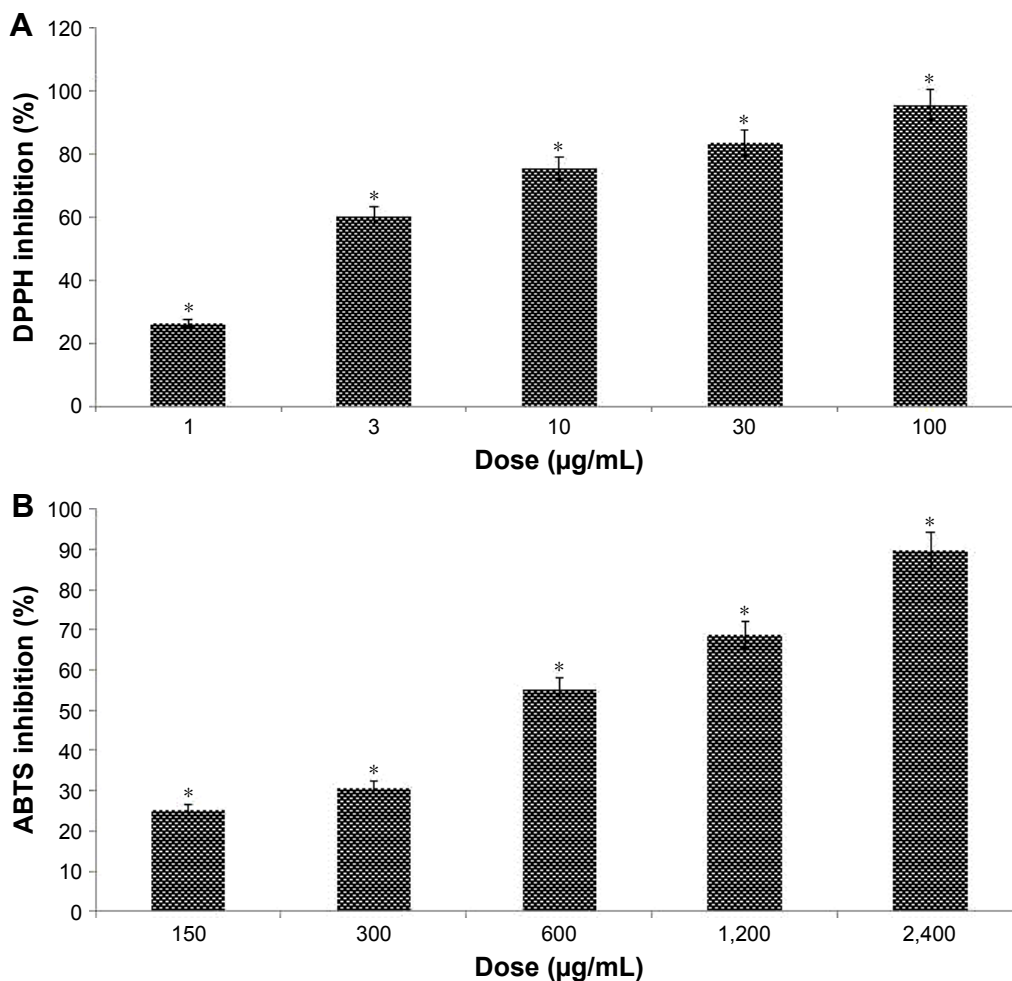


Figure 3 Increase in radical scavenging activity of MGR: (A) DPPH and (B) ABTS+. *Significant difference from control ($P < 0.05$).
Abbreviation: MGR, mastic gum resin.

adenocarcinoma (COLO205), and normal human colon fibroblast (CCD-18Co) cells. The IC_{50} values were 15.34 ± 0.21 , 11.52 ± 0.18 , 8.11 ± 0.23 , and $5.2 \pm 0.8 \mu\text{g/mL}$ at 72 hours of treatment for KMBC, PANC-1, CRL-1739, and COLO205

cells, respectively (Figure 4A). On the other hand, treatment with various doses of MGR did not adversely affect the normal CCD-18Co cells (Figure 4B). Thus, the results showed that MGR significantly inhibited proliferation of

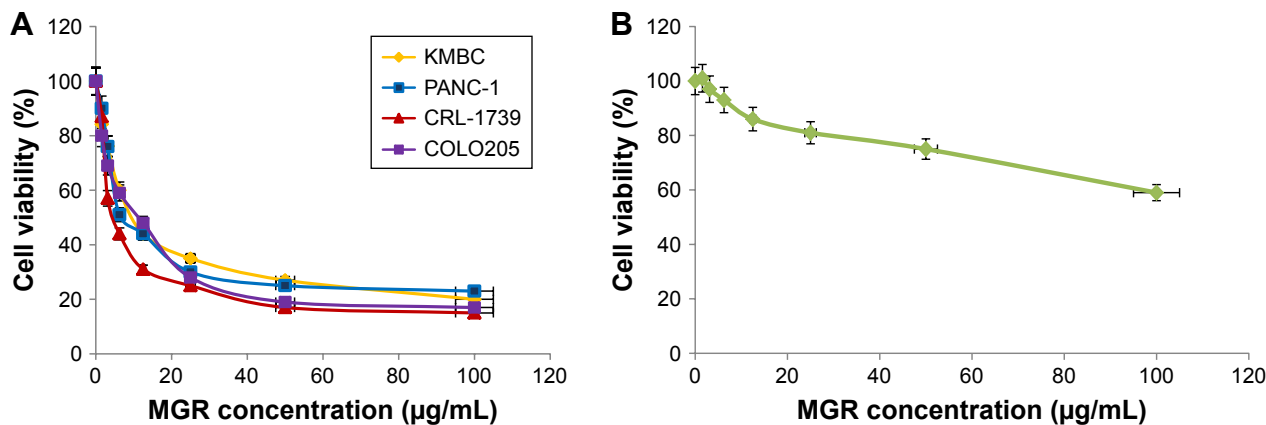


Figure 4 (A) IC_{50} value of 15.34 ± 0.21 , 11.52 ± 0.18 , 8.11 ± 0.23 , and $5.2 \pm 0.8 \mu\text{g/mL}$ at 72 hours of treatment with MGR for bile duct cancer (cholangiocarcinoma) (KMBC), pancreatic carcinoma (PANC-1), gastric adenocarcinoma (CRL-1739), and colonic adenocarcinoma (COLO205) cells. (B) The normal human colon fibroblasts (CCD-18Co) did not show any adverse effect after treatment with various doses of MGR.
Abbreviation: MGR, mastic gum resin.

human cancerous cells while being innocuous to the normal fibroblasts. Based on MTT results, we used an IC_{50} of $5.2 \pm 0.8 \mu\text{g/mL}$ to further determine the antiproliferative activities of MGR on COLO205 cells.

MGR induced apoptosis effects on colon cancer cells

The effect of MGR on COLO205 cells was determined after treatment for 24, 48, and 72 hours, using AO/PI assay and confocal laser scanning microscopy. After 24 hours, the cells became apoptotic, which was evident by the development of membrane blebs. After 48 and 72 hours of treatment, the cells entered early and late apoptotic phases showing nuclear margination, chromatin condensation, and nuclear fragmentation or apoptotic body formation (Figure 5). These features indicated that MGR time-dependently reduced viability and caused the death of COLO205 cells. After treatment with MGR, normal human colon fibroblast CCD-18Co cells

remained viable, normal in size and morphology, and with the regular cell membrane.

MGR induced externalization of phosphatidylserine in colon cancer cells

The effect of MGR on COLO205 cells was further determined by flow cytometry using the annexin V-FITC/PI apoptosis detection kit. The treatment caused a significant ($P < 0.05$) increase in the number of apoptotic cells with a consequential decrease in viable cells. Both early and late apoptotic cells increased with duration of treatment (Figure 6 and Table 2).

MGR promoted cell cycle arrest at the G2/M phase in colon cancer cells

MGR induced changes in COLO205 cell cycle phases (Figure 7). The cells mostly accumulated at the G2/M phase, which was particularly evident after 48 hours of treatment. After 72 hours of treatment, the cells significantly ($P < 0.05$) accumulated at the

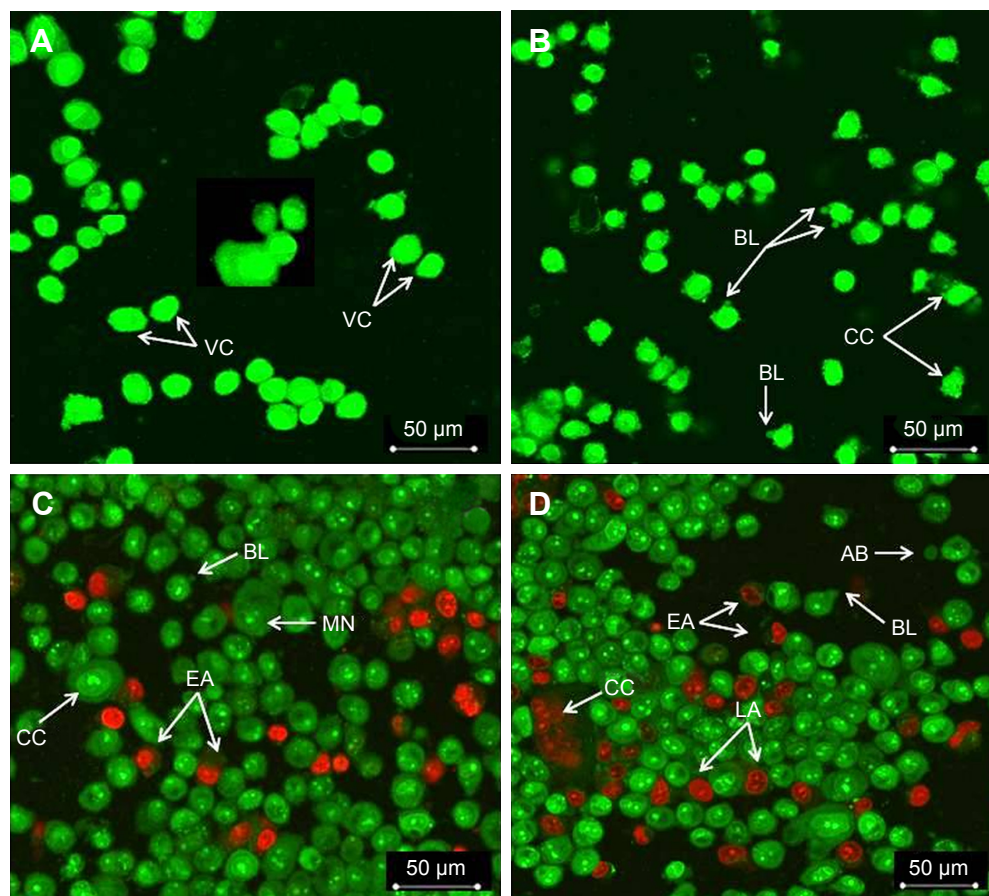


Figure 5 Confocal micrographs of AO and PI double-stained COLO205 cells. Cells were treated with IC_{50} of MGR in a time-dependent manner. (A) Untreated cells showed normal structure without prominent apoptosis and necrosis. (B) Early apoptosis features were seen after 24 hours representing intercalated AO (bright green) among the fragmented DNA. (C) BL and nuclear margination were noticeable after 48 hours of treatment (arrows). (D) Late apoptosis was seen after 72 hours of treatment (a positive staining of orange color represents the hallmark of late apoptosis).

Abbreviations: VC, viable cells; BL, blebbing; CC, chromatin condensation; MN, margination of the nucleus; EA, early apoptosis; AB, apoptotic body; LA, late apoptosis; MGR, mastic gum resin; PI, propidium iodide; AO, acridine orange.

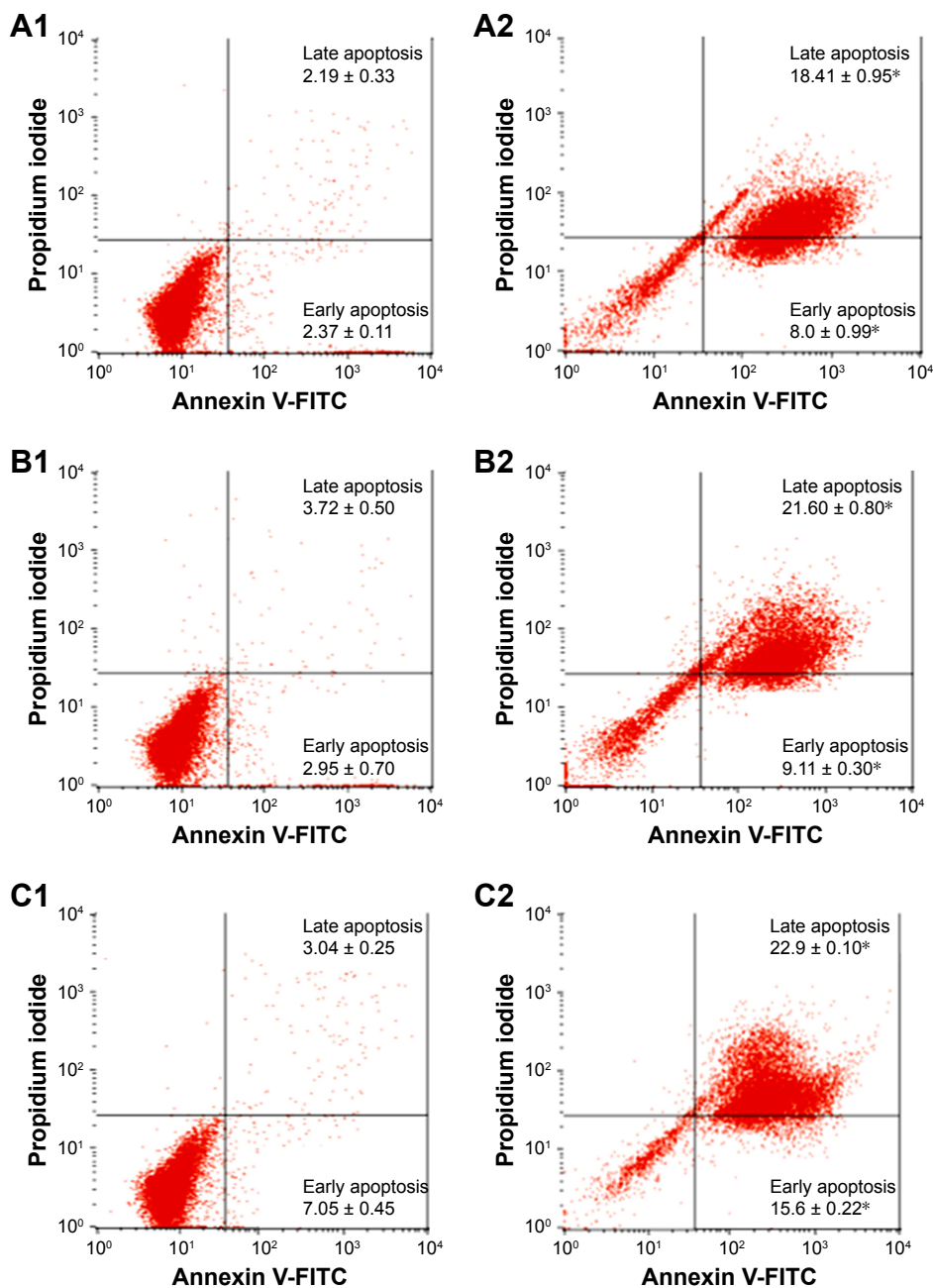


Figure 6 Induction of apoptosis in colonic adenocarcinoma (COLO205) cells by MGR which was observed by staining with FITC-conjugated annexin V-FITC. **(A1–C1)** Untreated (control) COLO205 cells at 24, 48, and 72 hours. **(A2–C2)** COLO205 cells after treatment with MGR at 12, 24, and 48 hours. *Significant ($P < 0.05$) increase in apoptotic cells in the MGR-treated groups compared to untreated controls.

Abbreviation: MGR, mastic gum resin.

Table 2 Flow cytometry analysis of COLO205 cells after treatment with MGR, for which the cells were stained with FITC-conjugated annexin-V and PI and incubated at 37°C for 24, 48, and 72 hours

Cell condition	24 hours		48 hours		72 hours	
	Control	Treated	Control	Treated	Control	Treated
Viable cells	95.4 ± 0.74	71.59 ± 0.65	91.35 ± 0.55	69.28 ± 0.15	89.90 ± 0.25	61.5 ± 0.13
Early apoptosis	2.37 ± 0.11	8.0 ± 0.99*	2.95 ± 0.70	9.11 ± 0.30*	7.05 ± 0.45	15.6 ± 0.22*
Late apoptosis and/or necrosis	2.19 ± 0.33	18.41 ± 0.95*	3.72 ± 0.50	21.6 ± 0.80*	3.04 ± 0.25	22.9 ± 0.10*

Notes: Values are expressed as mean ± SD of three different experiments. Data were analyzed using post hoc comparison test one-way ANOVA, and mean were compared by Tukey's *b* test. *Significant ($P < 0.05$) increase in apoptotic cells in the MGR-treated groups compared to untreated controls.

Abbreviation: MGR, mastic gum resin.

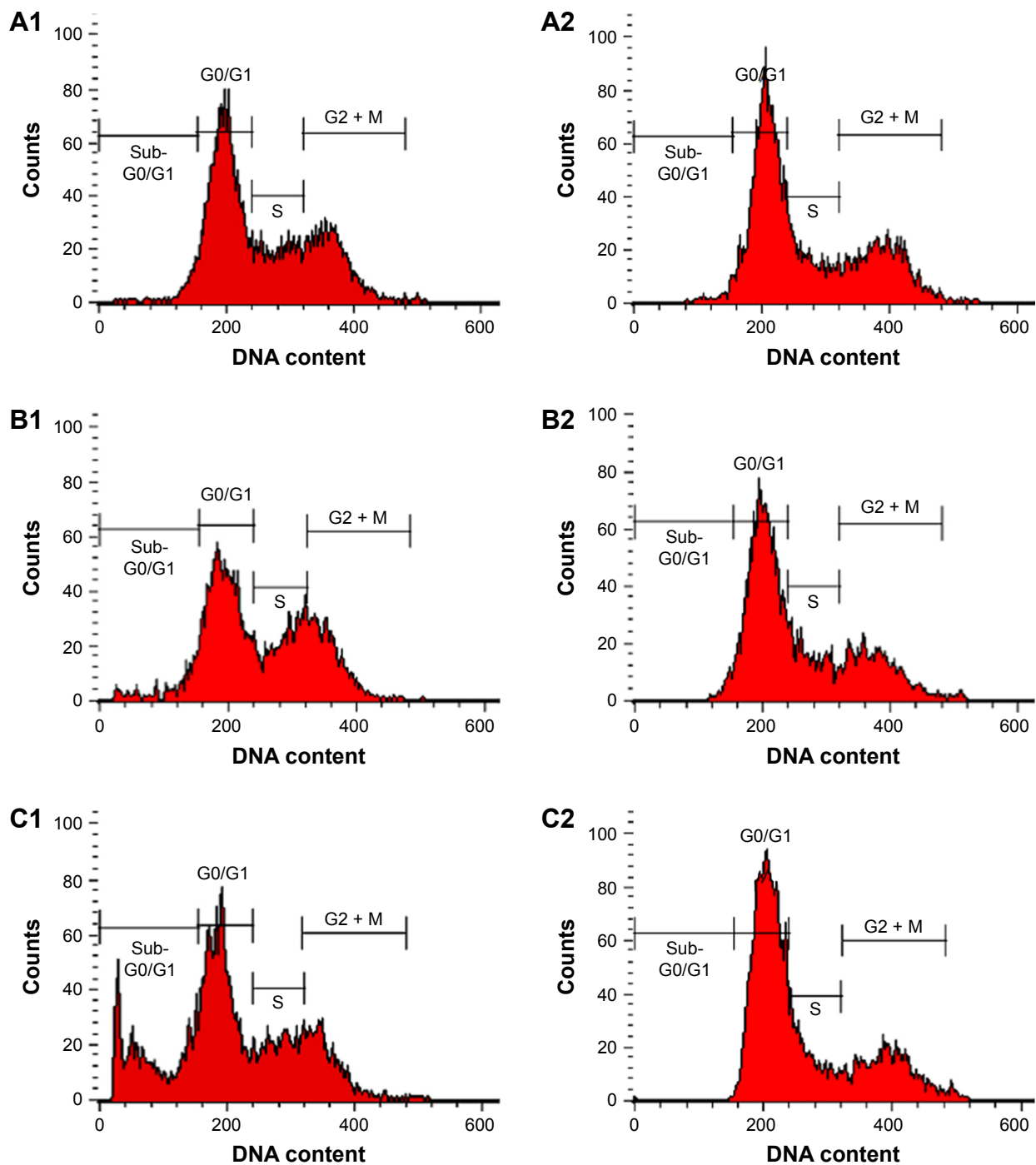


Figure 7 The cell cycle of colonic adenocarcinoma (COLO205) cells treated with MGR after staining with PI. (A1–C1) Untreated (control) COLO205 cells at 24, 48, and 72 hours. (A2–C2) COLO205 cells treated with resin at 12, 24, and 48 hours. G0/G1, G2/M, and S are cell cycle phases, and sub-G0/G1 is the apoptotic cell population. **Abbreviation:** MGR, mastic gum resin.

G0/G1 phase while the sub-G0/G1 cells decreased in number. However, no significant change was observed in the S-phase cell population at all treatment periods (Table 3).

MGR activated caspase-3 and -9 in colon cancer cells

The activities of caspase-3 and -9 in COLO205 cells treated with MGR are shown in Table 3. The treated cells significantly

($P < 0.05$) expressed the caspases, and the expression increased with treatment period (Figure 8 and Table 4).

MGR elicited gene expressions in colon cancer cells

The qRT-PCR analysis was performed using the comparative threshold cycle method to determine gene expression normalized to GAPDH and β -actin as the reference

Table 3 Flow cytometry analysis of COLO205 cells after treatment with MGR, for which the cells were stained with PI and incubated at 37°C for 24, 48, and 72 hours

Cell cycle phases	24 hours		48 hours		72 hours	
	Control	Treated	Control	Treated	Control	Treated
G0/G1	54.05 ± 0.06	43.70 ± 0.45	52.10 ± 0.29	54.61 ± 0.52	30.04 ± 0.32	45.70 ± 0.68*
G2/M	10.65 ± 0.76	18.00 ± 0.41*	13.96 ± 0.26	22.70 ± 0.35*	14.30 ± 0.22	16.05 ± 0.93*
Synthesis	34.01 ± 0.06	37.30 ± 0.33	29.24 ± 0.06	20.93 ± 0.12	37.50 ± 0.61	35.37 ± 0.18
Sub-G0/G1	1.2 ± 0.23	1.00 ± 0.20	4.60 ± 0.34	1.80 ± 0.20*	18.20 ± 0.46	2.85 ± 0.56*

Notes: Values are expressed as mean ± SD of three different experiments. Data were analyzed using post hoc comparison test one-way ANOVA, and mean were compared by Tukey's *b* test. *Significant ($P < 0.05$) increase in cells in sub-G0/G1 phase in the MGR-treated groups compared to untreated controls.

Abbreviation: MGR, mastic gum resin.

housekeeping genes. As shown in Figure 9, the proapoptotic Bax gene in the COLO205 cells was upregulated 1.66-fold by MGR treatment whereas the antiapoptotic Bcl-2 and Cyt-c genes were downregulated 1.21-fold and 1.33-fold, respectively.

Discussion

Plants constitute an important source of active natural compounds such as phenols and flavonoids, which differ widely in terms of structure and biological properties. They play a remarkable role in the traditional medicine in different countries, especially in the prevention of cancer, hypertension, diabetes, and cardiovascular diseases.^{4,6}

The protective effects of plants can be due to the presence of flavonoids and phenolic compounds. The main phytochemicals in the *Pistacia* spp. are gallic acid, quercetin, 1,2,3,4,6-pentagalloyl glucose, and α -tocopherol. In this current study, it is confirmed that the Baneh extract contains more polyphenols and flavonoids than the extracts of many

plants belonging to the same genus including *Pistacia terebinthus*, *P. lentiscus* fruits,²¹ and *P. atlantica* Desf. leaf extracts.⁸ Since phenolic compounds have good antioxidant and radical scavenging properties,⁵¹ the Baneh extract rich in these compounds has the potential to be developed into a natural antioxidant.

Antioxidants have attracted much interest with respect to their protective effect against free-radical damage that may be the cause of many diseases including cancer, and these compounds have been shown to inhibit cancer cell growth through cell cycle arrest and activation of apoptosis.⁵² In this study, the antioxidant activities of MGR were evaluated by measuring the generated free radicals using DPPH and ABTS assays, and the results were in agreement with previously conducted researches on the various species of the genus *Pistacia*.^{8,9,13,21}

Over the past 2 decades, cancer treatment has evolved from using relatively nonspecific cytotoxic agents to compounds of greater selectivity and specificity. Many of the cancer therapeutics are obtained from plants. Plants belonging to the genus *Pistacia* are considered as the most popular medicinal plants worldwide, including the Iraqi Kurdistan region.¹⁸ The antioxidant and anti-inflammatory properties of various parts of *Pistacia* tree including fruit, leaves, volatile oil, resin, and gum have been determined.²¹ Recent studies have shown that these portions of the tree also have potential anticancer effects, inducing cytotoxicity in several tumor cell lines, such as Lewis lung carcinoma (LLC) cells, mouse skin melanoma (B16F10) cells, human lung cancer (H292) cells,¹³ human breast cancer (MCF-7) cells,⁵⁴ human melanoma (Skmel-28 and Mewo) cells, human oral cancer (YD-10B) cells,²³ human leukemia (K562) cells,⁵⁵ human prostate cancer (PC-3) cells,⁴⁰ and human colon carcinoma (HCT116 and HT29) cells.^{52,56}

The antiproliferative effect of MGR seems to be specific to cancer cells. The resin did not affect the viability of normal

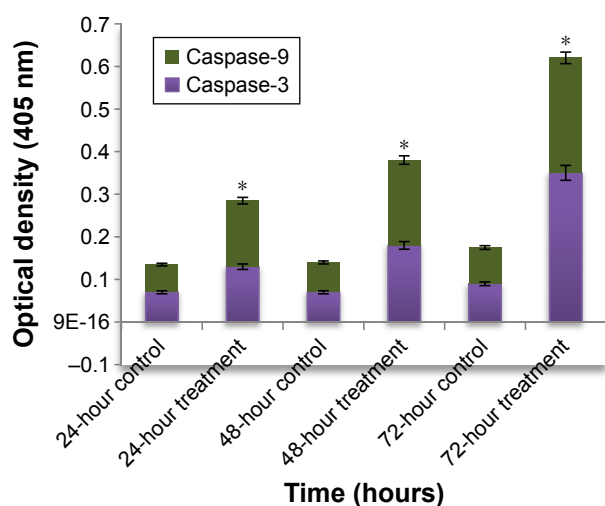


Figure 8 Activities of caspase-3 and -9. The total cell lysates from COLO205 cells treated with MGR for 24, 48, and 72 hours were analyzed using caspase colorimetric protease assay kits. *Significant difference from control ($P < 0.05$).

Abbreviation: MGR, mastic gum resin.

Table 4 Spectrophotometric analysis of caspases in COLO205 cells after treatment with MGR for 24, 48, and 72 hours

Caspase	24 hours		48 hours		72 hours	
	Control	Treated	Control	Treated	Control	Treated
Caspase-3	0.058 ± 0.020	0.12 ± 0.02*	0.073 ± 0.041	0.18 ± 0.07*	0.099 ± 0.032	0.21 ± 0.06*
Caspase-9	0.07 ± 0.061	0.16 ± 0.03*	0.074 ± 0.01	0.19 ± 0.05*	0.09 ± 0.02	0.25 ± 0.035*

Notes: Values are expressed as mean ± SD of three different experiments. Data were analyzed using post hoc comparison test one-way ANOVA, and mean were compared by Tukey's *b* test. *Significant ($P < 0.05$) increase in apoptotic cells in the MGR-treated groups compared to untreated controls.

Abbreviation: MGR, mastic gum resin.

fibroblasts. At 72-hour IC_{50} of $5.2 \pm 0.8 \mu\text{g/mL}$, the antiproliferative effect of MGR on human colonic adenocarcinoma COLO205 cells was very potent. The resin causes cancer cell apoptosis in a time-dependent manner, increasing the effect with the time of exposure. On the other hand, recently Rahbar Saadat et al⁵⁷ reported that after 24 hours of treatment with *P. atlantica* MGR, cytotoxicity was induced in a dose-dependent manner with an IC_{50} of about 182, 215, and 90 $\mu\text{g/mL}$ for NIH-3T3 cells, KB cells, and HUVEC cells, respectively.

Cell cycle analysis showed MGR affects G1 checkpoint and arrests cells at the G2/M phase, particularly after 48 hours of treatment. There was also a significant decrease in COLO205 cells at the sub-G0/G1 phases with resin treatment, possibly as a result of elimination through apoptosis.

Although the anticancer effect of MGR was obvious, the molecular mechanism of the effect is still not clear. A previous study showed that the antiproliferative effect of MGR

is exhibited through targeting of NF- κ B signaling pathway.⁴⁰ MGR also showed antiproliferative effect on LNCaP and PC-3 cells by suppressing the expression and function of *PSA* and *hK2* promoter genes in the androgen receptor.^{41,58}

It is suggested that the cancer cell antiproliferative effect is mediated via simultaneous upregulation of proapoptotic genes and downregulation of antiapoptotic genes. The proapoptotic Bax protein in the endoplasmic reticulum membrane upon stimulation undergoes conformational changes, causing the release of calcium into cytoplasm and activation of mCalpain.⁵⁹ Additionally, the upregulation of Bax triggered a release of cytochrome c from mitochondria and induced caspase activation into the cytosols. Several studies had suggested that Bax could reduce the mitochondrial membrane potential by creating pores in the membrane for cytochrome c to escape and eventually cause cell death. Moreover, the release of cytochrome c was upstream of the initiation of caspase cascade including caspase-3 and -9 in

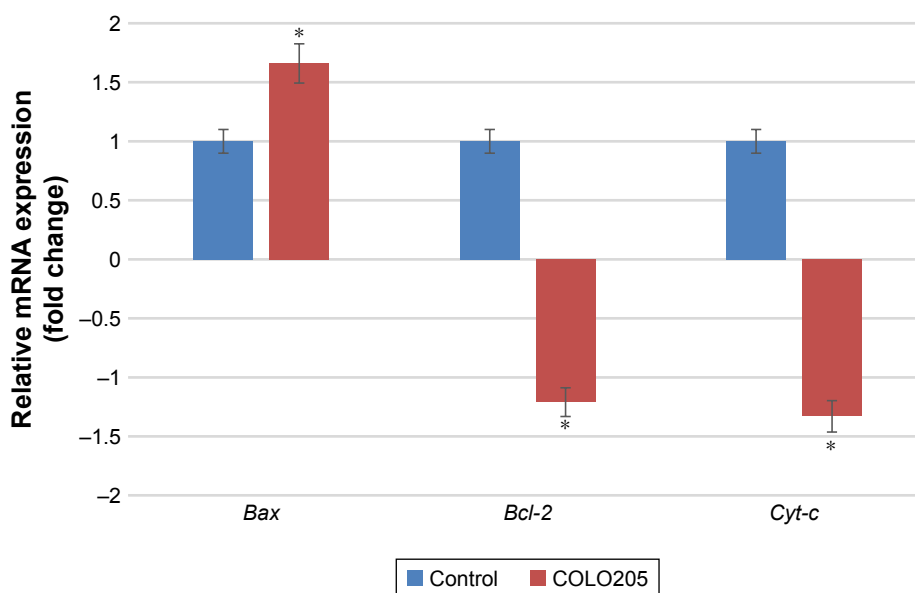


Figure 9 mRNA expression levels of *Bcl-2*, *Bax*, and *Cyt-c* normalized to the transcription levels of β -actin and *GAPDH*. The qRT-PCR analysis was performed on COLO205 cells treated with $5.2 \pm 0.8 \mu\text{g/mL}$ MGR. The experiment was done in triplicates, and data are expressed as mean ± SD. *Significant difference from control ($P < 0.05$).
Abbreviations: MGR, mastic gum resin; qRT-PCR, quantitative real-time polymerase chain reaction.

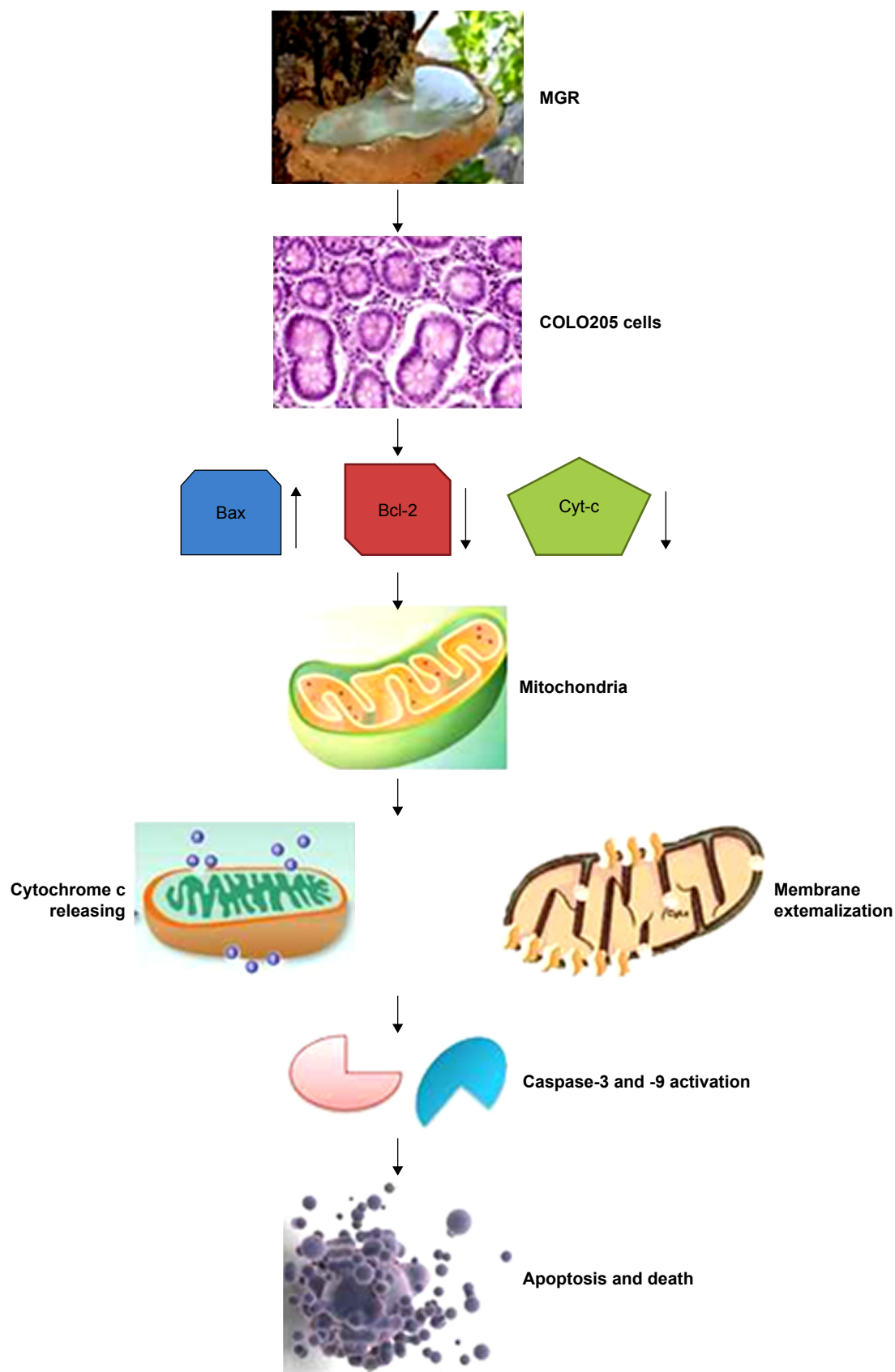


Figure 10 Proposed model of MGR mechanism of action behind its antiproliferative and apoptosis effects against colon adenocarcinoma in vitro.
Abbreviation: MGR, mastic gum resin.

enhanced apoptosis. In this study, cytochrome c is released into the cytoplasm to stimulate the apoptosome consisting of Apaf1, ATP, and procaspase 9. Activation of procaspase 9 leads to activation of caspase-3, followed by DNA fragmentation and cell death.^{60,61} Interestingly, this study showed that MGR upregulated the proapoptotic Bax while downregulated the antiapoptotic Bcl-2 gene. This shows that antihuman colonic adenocarcinoma effect of MGR is through activation of the mitochondrial pathway of apoptosis (intrinsically triggered cell death) (Figure 10).

Conclusion

The present study demonstrated that MGR induced human colon cancer toxicity and apoptosis in vitro in a time-dependent manner through the mitochondrial apoptosis pathway. Thus, MGR can potentially be developed into a safe product for the treatment of colon cancers, although some people in the middle east use it daily as a chewing gum to protect themselves from digestive diseases, especially colon cancer. Nonetheless, a further in-depth analysis will be done in our future study through in vivo experiments to test this hypothesis and also to investigate further roles of MGR as a potential anticancer agent.

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Disclosure

The author reports no conflicts of interest in this work.

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