Mechanism of Anticancer Effect of Gambogic Acid on Gastric Signet Ring Cell Carcinoma

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Abstract

Background

Gambogic acid has demonstrated inhibitory effects on the growth of various cancer cell types, such as breast cancer, pancreatic cancer, prostate cancer, lung cancer, and osteosarcoma. This study aims to investigate the antiproliferative activity of Gambogic acid on SNU-16 cells derived from gastric signet ring cell carcinoma and elucidate the underlying mechanisms.

Material and Methods

The cytotoxic effect of gambogic acid was evaluated in SNU-16 cells by treating them with different concentrations of the compound, and the XTT cell viability assay was employed to assess cell viability. ELISA was used to measure bax, BCL-2, caspase 3, PARP, and 8-oxo-dG levels. Additionally, immunofluorescence staining was applied to assess 8-oxo-dG and LC3β levels in SNU-16 cells.

Results

It was observed that gambogic acid exerted a dose-dependent and statistically significant antiproliferative effect on SNU-16 cells. The IC_{50} value of gambogic acid in SNU-16 cells was found to be 655.1 nM for 24 hours. Subsequent investigations conducted using the IC_{50} dose revealed a significant upregulation of apoptotic proteins including cleaved caspase 3, Bax, and cleaved PARP ($p < 0.001$), along with a downregulation of BCL-2 ($p < 0.001$), an anti-apoptotic protein. Moreover, the administration of this drug led to an upregulation of 8-oxo-dG ($p < 0.001$), a widely acknowledged biomarker indicating oxidative damage in DNA, as well as an increase in LC3β levels ($p < 0.05$), a marker associated with autophagy.

Conclusion

The antiproliferative effect of gambogic acid against gastric signet ring cell carcinoma is attributed to its ability to induce apoptosis and autophagy. This discovery highlights the promising potential of gambogic acid as a treatment option for gastric signet ring cell carcinoma.

Introduction

Gastric cancer (GC) is a highly prevalent global malignancy and is a major contributor to cancer-related fatalities. Based on estimates provided by the GLOBOCAN database, the incidence of GC surpassed 1 million cases in 2020, leading to over 768,793 recorded deaths [1]. While the overall occurrence of gastric cancer has declined in recent years, there has been a consistent rise in the incidence of signet-ring cell carcinoma (SRCC) in Asia, the United States, and Europe [2]. Chemotherapy is a widely employed

cancer treatment that utilizes pharmaceutical drugs to eliminate cancer cells. Herbal medicines, derived from natural sources, have long been recognized for their diverse therapeutic properties and have shown significant potential in combating cancer, particularly in terms of their anti-cancer activity. Numerous plant-derived active ingredients hold significant promise for the development of therapeutic drugs in cancer treatment. These compounds have the potential to extend survival, minimize side effects, and enhance the quality of life for individuals with cancer. Xanthone-containing drugs have garnered considerable attention due to their ability to inhibit tumor growth. Xanthones, a type of natural product initially extracted from plants and microorganisms, exhibit a range of biological activities including anti-tumor, anti-hypertensive, and anti-thrombotic properties [3]. Gambogic acid is a naturally occurring compound belonging to the xanthone family, known for its significant pharmaceutical implications [4]. Derived from the Garcinia hanburyi tree as a dry resin, gambogic acid exhibits diverse anticancer activities, including apoptosis, autophagy, cell cycle arrest, and inhibition of invasion, metastasis, and angiogenesis. Gambogic acid has demonstrated inhibitory effects on the growth of various cancer cell types, such as breast cancer, pancreatic cancer, prostate cancer, lung cancer, and osteosarcoma. These effects are thought to be mediated through modulation of signaling pathways involving c-Jun N-terminal kinase-1 (JNK-1), protein kinase B (AKT)/mammalian target of rapamycin (mTOR), AKT/forkhead box protein O1 (FOXO1)/BIM, nuclear factor kappa-B (NF-κB), and others [5]. In vitro and in vivo investigations have revealed that general gambogic acids possess inhibitory effects on Heps, S180, and EC in Kunming strain mice. Furthermore, gambogic acids has demonstrated inhibitory activity on human hepatocellular carcinoma cells SMMC-7721 and BEL-7402, as well as human pulmonary carcinoma cell SPC-A1[6]. Various research studies have consistently demonstrated the distinct impact of gambogic acid on different phases of the cell cycle in diverse types of cancer cell lines. Gambogic acid exhibits evident abilities to induce cell cycle arrest at either the G2/M or G0/G1 phase in a range of cancer cell lines, including MCF-7 cells, K562 cells, U2OS cells, and others [7]. However, It was demonstrated that gambogic acid induced apoptosis and produced anticancer effect in human gastric cancer line BGC-823 derived from a poorly differentiated adenocarcinoma tumor of the stomach [8]. To date, the antiproliferative effect of gambogic acid on SNU-16 cells, which are derived from a signet ring cell carcinoma, has not been investigated or studied extensively. The objective of this study is to investigate the antiproliferative impact of gambogic acid on SNU-16 cells and assess the involvement of its apoptotic and autophagic effects in achieving this outcome.

Materıal and Methods

Cell line and cell culture

The SNU-16 gastric signet ring cell carcinoma cell line (CRL-5974) was obtained from the American Type Culture Collection (ATCC) located in the USA. To culture these cells, they were routinely grown in Roswell Park Memorial Institute Medium (RPMI) at a temperature of 37°C, in a humidified atmosphere containing 5% CO2. The culture medium utilized in this study consisted of 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. Gambogic acid, a compound of interest, was liquefied in

DMSO and then diluted in the culture medium. Care was taken to adjust the final concentration of DMSO in the culture medium to be less than 0.1% before treating the cells. All the materials used in the experiment, excluding the cell line, were acquired from Sigma-Aldrich.

Cell viability assay

The impact of gambogic acid on the viability of the SNU-16 cell line was evaluated by conducting the XTT assay. The cells were initially seeded at a density of $1x10^4$ cells per well and allowed to incubate for 24 hours. Following that, the SNU-16 cells were subjected to treatment using different concentrations of gambogic acid, (200, 400, 800, and 1600 nM). Untreated cells served as the control group. After the incubation period, each well received 50 µL of an XTT mixture. Following a 4-hour incubation, the cells were agitated, and the absorbance at 450 nm was quantified using a microplate reader provided by Thermo Fisher Scientific in Altrincham, United Kingdom. The experiment was conducted in triplicate, and the cell viability was determined by calculating the percentage of live cells relative to the untreated control cells [9].

Determination of Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-Hydroxy-deoxyguanosine levels

In order to assess the levels of Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-hydroxy-deoxyguanosine (8-oxo-dG) in both gambogic acid-treated and untreated SNU-16 cells, specific ELISA kits were employed. The human ELISA kits of Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-Hydroxy-Desoxyguanosine (BT Lab Shanghai, China) were utilized for their respective measurements. To perform the experiments, SNU-16 cells were seeded in a 6-well plate and exposed to gambogic acid at dose of 655.1 nM (IC_{50}) for 24 hours. Following the treatment, both the gambogic acid-treated and untreated SNU-16 cells were collected, diluted with PBS, and subjected to multiple freeze-thaw cycles to induce cellular damage. The levels these markers in the resulting cell lysates were quantified according to the instructions provided by the manufacturer. Furthermore, the total protein concentrations of both the gambogic acid-treated and untreated SNU-16 cells were assessed using the BCA assay from Pierce Biotechnology in the United States [10,11].

Immunofluorescence staining

The cells prepared as a suspension culture were transferred onto a slide using a pipette. After the transfer process, they were dried for 8 hours. After this step, the cells were fixed with methanol at -20ºC for 5 minutes and then washed with PBS. Subsequently, they were incubated in PBS containing 0.1% Triton X-100 at room temperature for 15 minutes. After the washing process, the cells were incubated at room temperature for 60 minutes in PBS containing 2% BSA. Following another washing step, the cells were incubated overnight at + 4ºC with primary antibodies, including monoclonal anti-MAP LC3β (Santa Cruz, Catalog no. sc-271625) and monoclonal anti-8-OHdG (Santa Cruz, Catalog no. sc-66036), at a dilution ratio of 1/200. Cells washed with PBS were incubated with goat anti-mouse FITC secondary antibody at a dilution ratio of 1/50 for 1 hour at room temperature in the dark, compatible with the primary antibodies

used. Finally, the cells were examined under a fluorescence microscope after applying 4',6-diamidino-2-phenylindole (DAPI) on them. During the evaluation, the positivity of the cells in the entire field was assessed semiquantitatively as negative (-), mild (+), moderate (++), and intense (+++).

Statistical analysis

The laboratory findings were presented as mean ± standard error. XTT assay findings analyzed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey to assess multiple comparison tests. ELISA and immunofluorescence staining findings analyzed using Student T-test. A p-value of less than 0.05 was chosen for accepting statistically significant differences. The statistical evaluations were performed using the SPSS Statistics Program v.22.

Results

The Antiproliferative Effect of gambogic acid in SNU-16 Cells

The cells were exposed to gambogic acid in a concentration range of 200 to 1600 nM for 24 hours, and the viable cell count was determined using the XTT cell viability assay. gambogic acid did not show any effect at a dose of 200 and 400 nM, but exhibited a dose-dependent antiproliferative effect on SNU-16 cells at doses of 800 and 1600 nM ($p < 0.001$; Fig. 1). The IC_{50} value of gambogic acid was calculated as 655.1 nM using GraphPad Prism.

The impact of gambogic acid on BCL-2, cleaved caspase 3, Bax and cleaved PARP levels in SNU-16 cells

To assess the levels of apoptosis-related proteins in SNU-16 cells, ELISA was employed. The expression of various proteins, including Bcl-2, cleaved caspase 3, Bax, and cleaved PARP, was analyzed using this method. The exposure to gambogic acid at dose of 655.1 nM for 24 hours resulted in a significant increase in the levels of cleaved caspase 3 from 393,28 ± 2,18 to 757,17 ± 9,62 pg/mg protein ($p < 0.001$, Fig. 2), Bax from 9,84 ± 0,30285 to 18,14 ± 0,25 ng/mg protein ($p < 0.001$, Fig. 2) and cleaved PARP from 487,39 ± 8,996 to 562,67 ± 6,35 pg/mg protein ($p < 0.001$, Fig. 2). On the other hand, gambogic acid significantly decreased BCL-2 level from 35,96 ± 1,05 to 46,298 ± 0,61 ng/mg protein ($p < 0.001$, Fig. 2).

The impact of gambogic acid on 8-oxo-dG level in SNU-16 cells

In order to evaluate the DNA-damaging effects of gambogic acid, ELISA was employed to measure the expression of 8-oxo-dG in SNU-16 cells. The exposure to gambogic acid at dose of 655.1 nM for 24 hours resulted in a significant increase in the level of 8-oxo-dG from 38,48 ± 0,82 to 65,0159 ± 0,56 ng/mg protein ($p < 0.001$, Fig. 3).

The impact of gambogic acid on 8-oxo-dG and LC3β levels in SNU-16 cells using immunofluorescence staining

In both the gambogic acid and control groups, a total of 6 samples were stained. The analysis revealed statistically significant differences in the quantities of 8-oxo-dG and LC3β between the two groups ($p < 0.05$), as illustrated in Fig. 4. In the group where SNU-16 cell line was used as the control, the expression of LC3β was found to be mild, while in the gambogic acid group, the expression of LC3β was severe (Fig. 5). The expression of 8-OHdG was observed to be mild in the control group, whereas it was moderate in the gambogic acid treatment group (Fig. 6). It was determined that the detected fluorescence positivity was located intracytoplasmically.

Discussion

In recent times, there has been a surge of interest in gambogic acid as a potential candidate for cancer treatment. Its anti-cancer effects are progressively being verified, while the precise mechanisms underlying its action are still being investigated. In this investigation, we examined the impact of gambogic acid on the growth, programmed cell death (apoptosis), cellular self-digestion (autophagy), and DNA damage in an in vitro model using the SNU-16 cell line, which is derived from a type of gastric cancer known as signet ring cell carcinoma. To assess the dose-dependent cytotoxic impact of gambogic acid on SNU-16 cells, we performed XTT experiments as a preliminary step. The obtained experimental results demonstrated a substantial concentration-dependent inhibition of SNU-16 cell proliferation by gambogic acid. Notably, after 24 hours of exposure, the IC50 value for gambogic acid was determined to be 655.1 nM. Consistent with our findings, Liu et al. demonstrated a notable dose-dependent inhibition of the low differential human gastric cancer cell line BGC-823 following incubation with gambogic acid [8]. Furthermore, Zhao et al. conducted a study revealing the ability of gambogic acid to dose-dependently impede the proliferation of MGC-803 cells, which are derived from moderately differentiated gastric adenocarcinoma [12]. Hatami et al. conducted a study revealing that gambogic acid had a potential to enhance the anticancer activity of gemcitabine in non-small cell lung cancer [13]. Seo et al. demonstrated that gambogic acid induced cell death in cancer cells through vacuolization, a process associated with the formation of vacuoles, by interfering with thiol proteostasis [14]. According to Suksen's report, gambogic acid effectively blocked the wnt/β-catenin signaling pathway and triggers apoptosis in human cholangiocarcinoma by inducing endoplasmic reticulum (ER) stress [15]. In contrast, Xia and Tang's findings indicated that gambogic acid did not display any harmful effects on a normal human bronchial epithelial cell line called 16HBE [16]. The importance of apoptosis in the molecular development of cancer and its impact on the effectiveness of chemotherapy and radiation therapy is widely acknowledged [17]. ELISA studies were conducted to assess the impact of gambogic acid on apoptosis in SNU-16 cells by measuring the levels of Bax, cleaved caspase 3, BCL-2, and cleaved PARP. Promoting apoptotic cell death, which serves as a crucial defense mechanism against cancer development and progression, is a primary objective of cancer therapy [18]. The pro-apoptotic protein Bax plays a role in disturbing the integrity of the mitochondrial membrane, leading to the liberation of cytochrome c. The released cytochrome c combines with caspase-9 and Apaf-1 to form a complex known as the

apoptosome, which triggers the activation of effector caspases and initiates the process of apoptosis. Conversely, the anti-apoptotic protein BCL-2 safeguards the stability of the membrane, preventing the release of cytochrome c and impeding apoptosis [19]. Caspases, a group of enzymes crucial in executing apoptosis, exhibit characteristic activation during the apoptotic process. These enzymes are initially synthesized as inactive forms called zymogens, which can be cleaved to generate active enzymes when apoptosis is induced [20]. Caspase 3, the most prominent member among caspases, plays a pivotal role in apoptosis. It triggers the activation of endonuclease CAD (Caspase-activated DNAse), leading to the degradation of chromosomal DNA and the condensation of chromatin [21]. Moreover, PARP (Poly ADP-ribose polymerase) is a significant protein involved in DNA repair pathways, specifically in the mending of base excisions. However, when PARP is cleaved or inhibited, it exploits a flaw in DNA repair and triggers cell death [22]. This particular protein has also been extensively researched as one of the most thoroughly studied targets of activated caspases [18]. During the course of this investigation, it was observed that the administration of gambogic acid at a concentration of 655.1ng/mL had a pronounced effect on the upregulation of pro-apoptotic Bax, as well as the activation of cleaved caspase 3 and cleaved PARP. Simultaneously, there was a notable reduction in the levels of the anti-apoptotic protein BCL-2. These alterations exerted a significant influence in favor of promoting cell apoptosis. In agreement with our findings, gambogic acid was identified as a counteractive agent against BCL-2 family proteins in Hela cells [23]. In MCF-7 human breast cancer cells, gambogic acid was observed to suppress the expression of BCL-2 through enhanced p53 activity, leading to cellular apoptosis [24]. Gambogic acid has the potential to initiate an intrinsic pathway of apoptosis by directly impacting the mitochondria, resulting in a swift depolarization and fragmentation of the mitochondrial membrane. This process leads to the release of cytochrome c, activation of caspase-3 and caspase-9, cleavage of PARP, and an elevated Bax/BCL-2 ratio [25–29]. To investigate the potential association between the cytotoxic effect of gambogic acid and DNA damage, we performed an analysis of DNA fragmentation in SNU-16 cells following a 24-hour treatment with gambogic acid. We utilized the 8-oxo-dG ELISA and Immunohistochemical staining methods, which are commonly used to measure levels of 8-oxo-dG, a well-known biomarker of oxidative damage in DNA [30,31]. Our findings revealed that treatment with gambogic acid significantly increased the levels of 8-oxo-dG in SNU-16 cells. This observation provides additional support for the cytotoxic and apoptotic effects of gambogic acid. Elevated levels of 8-oxo-dG indicate the occurrence of oxidative damage to DNA, which can potentially lead to DNA fragmentation and cell death. Consistent with our observations, a study conducted by Suksen et al. also reported the ability of gambogic acid to induce DNA damage activation [15]. Furthermore, the study conducted by Rong et al. revealed that the administration of gambogic acid initiates DNA damage signaling, resulting in the activation of the p53/p21Waf1/CIP1 pathway through the ATR-Chk1 pathway [32]. Studies conducted in preclinical models indicate that metabolic stress within human tumors can trigger autophagy as a response to meet the increased energy demands during the progression of cancer. In the event that detrimental metabolic circumstances persist beyond a critical threshold necessary for cell survival, it is possible for autophagic cell death to occur, which can be a targeted goal in the context of anticancer therapy. Multiple anticancer agents have been found to induce autophagy as part of their mechanism of action [33]. Under normal conditions, the autophagic protein LC3β is evenly distributed

throughout the cytoplasm as observed in immunofluorescence. However, during the process of autophagic induction, LC3β undergoes a change in its appearance, adopting a granular or punctate pattern. This alteration occurs as LC3β integrates into the autophagosome membrane during its formation. Subsequently, the autophagosome merges with the lysosome, giving rise to an autophagolysosome, which facilitates the degradation of the autophagosome and its contents. Microscopic visualization of LC3β proteins can be employed as a method to investigate autophagic induction [34]. Our findings revealed that treatment with gambogic acid significantly increased the levels of LC3β in SNU-16 cells. Increased levels of LC3β serve as an indication of autophagy occurrence, which could potentially contribute to the antiproliferative effects of gambogic acid. Our findings are consistent with previous observations, as gambogic acid was found to induce autophagy in NCI-H441 cells through the upregulation of Beclin 1 (a key factor in autophagosome formation) and the conversion of LC3 I to LC3 II (an autophagosome marker) [35]. Likewise, in glioblastoma multiforme (GBM) cells, gambogic acid induced a noticeable association with autophagy, as evidenced by the increased expressions of Atg5, Beclin 1, and LC3-II [36]. Furthermore, in A549 cells, treatment with gambogic acid stimulated autophagy by inhibiting the Akt/mTOR signaling pathway, leading to a significant decrease in the phosphorylation levels of Akt, mTOR, and S6 [37].

In summary, the treatment of SNU-16 cells with gambogic acid exhibited a concentration-dependent inhibition of cell growth. This effect was accompanied by significant increases in pro-apoptotic markers such as cleaved caspase 3, Bax, and cleaved PARP, while anti-apoptotic protein BCL-2 levels decreased. Moreover, gambogic acid treatment led to a significant elevation in 8-oxo-dG levels, indicating the induction of oxidative DNA damage. Additionally, gambogic acid treatment resulted in increased levels of LC3β, an autophagy marker. These findings suggest that gambogic acid holds promise as a potential therapeutic agent for signet ring cell carcinoma. Nevertheless, further in vivo and clinical studies are required to validate these findings.

Declarations

Conflict of Interest

The authors disclose that they do not possess any conflicts of interest.

Acknowledgment

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Figures

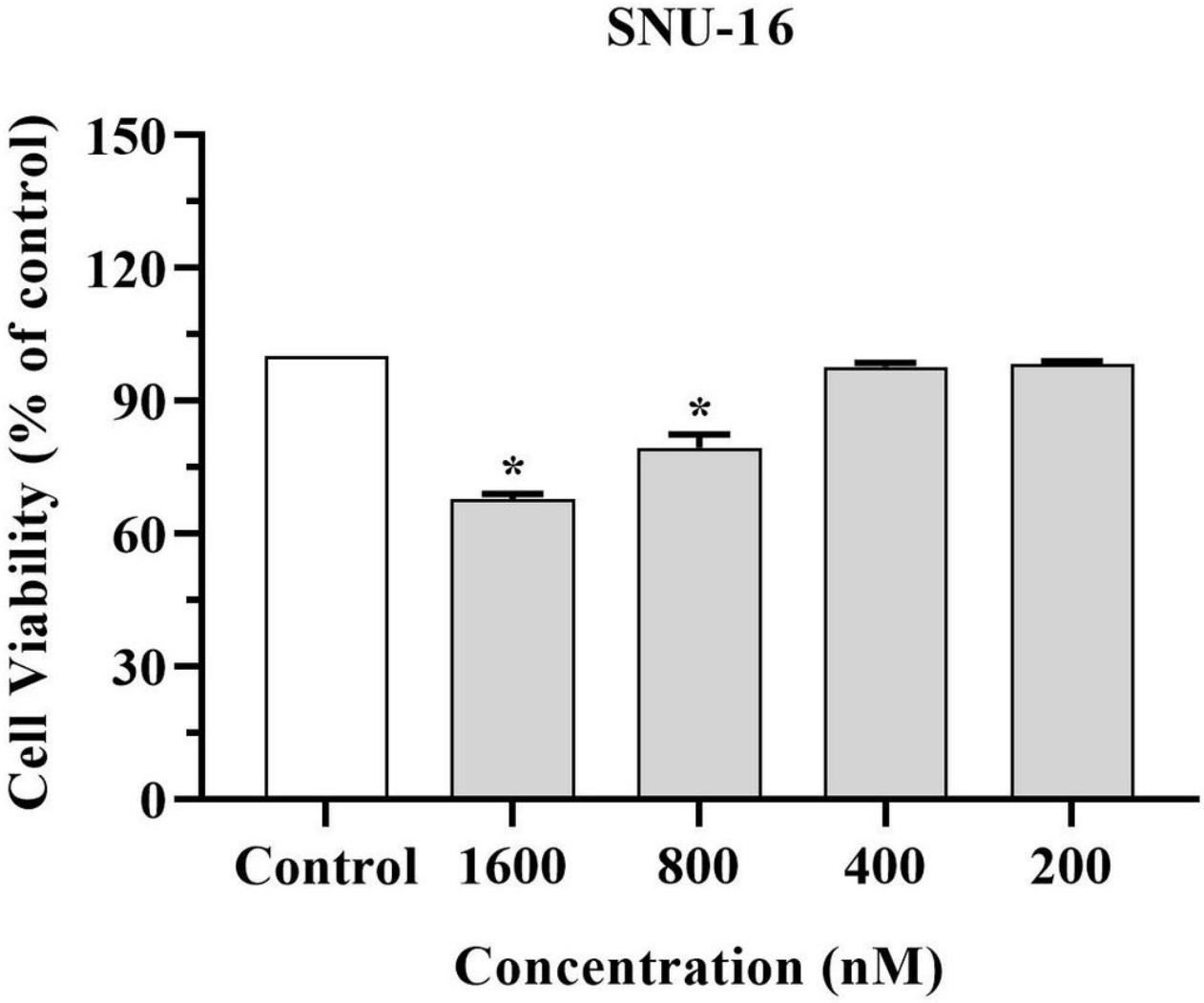

Figure 1

The impact of gambogic acid on the proliferation of SNU-16 cells was evaluated by determining the percentage of viable cells compared to the control group. The results are expressed as the mean ± SEM of six samples. *$p<0.001$ as compared to the control group.

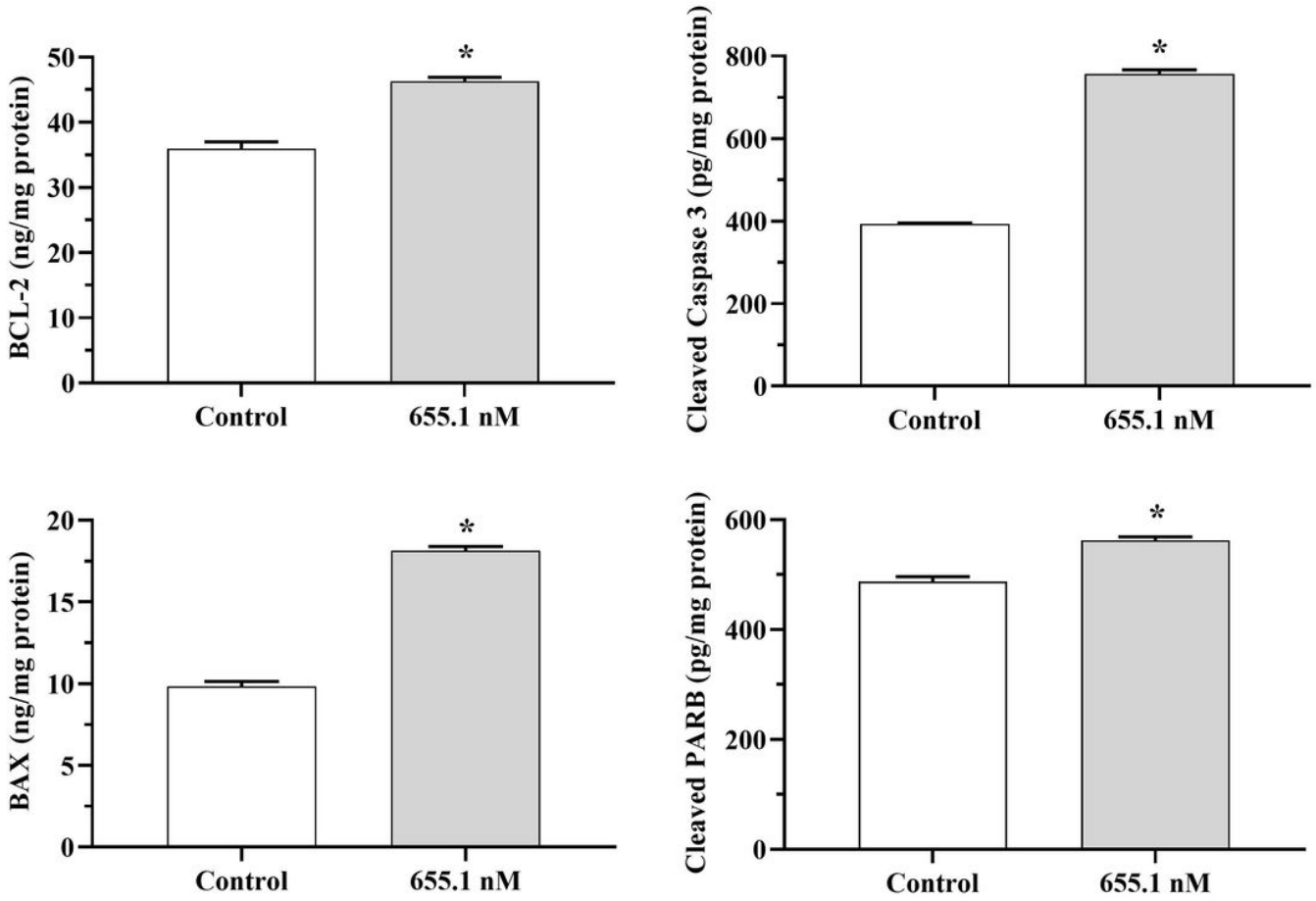

Figure 2

The treatment of SNU-16 cells with gambogic acid at a concentration of 655.1 nM resulted in an increased apoptosis rate. The levels of Bcl-2, cleaved caspase 3, Bax, and cleaved PARP were quantified using ELISA kits. The results are expressed as mean ± SEM of six samples. *p< 0.001 as compared to the control group.

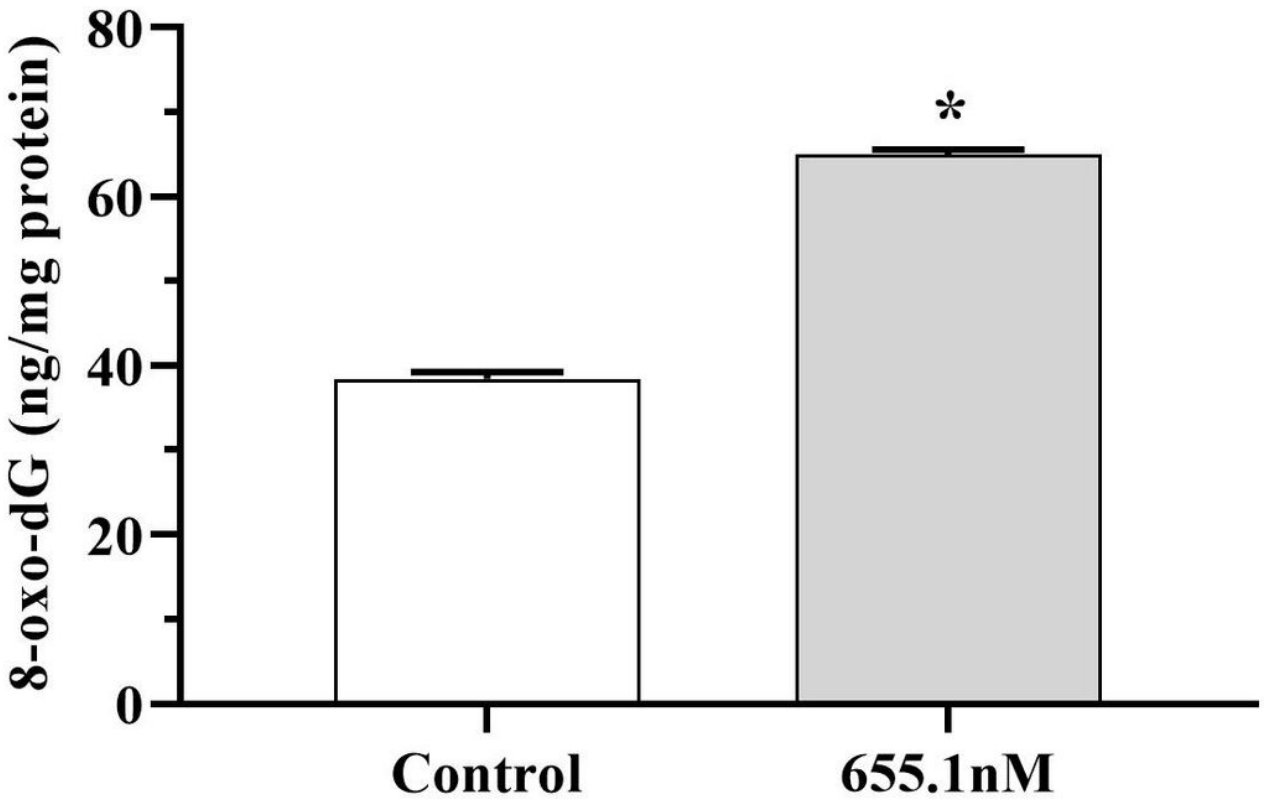

Figure 3

The treatment of SNU-16 cells to gambogic acid at a concentration of 655.1 nM led to an augmentation of DNA damage. The level of 8-oxo-dG was measured using an ELISA kit. Results are expressed as mean ± SEM of six samples. *p<0.001 as compared to the control group.

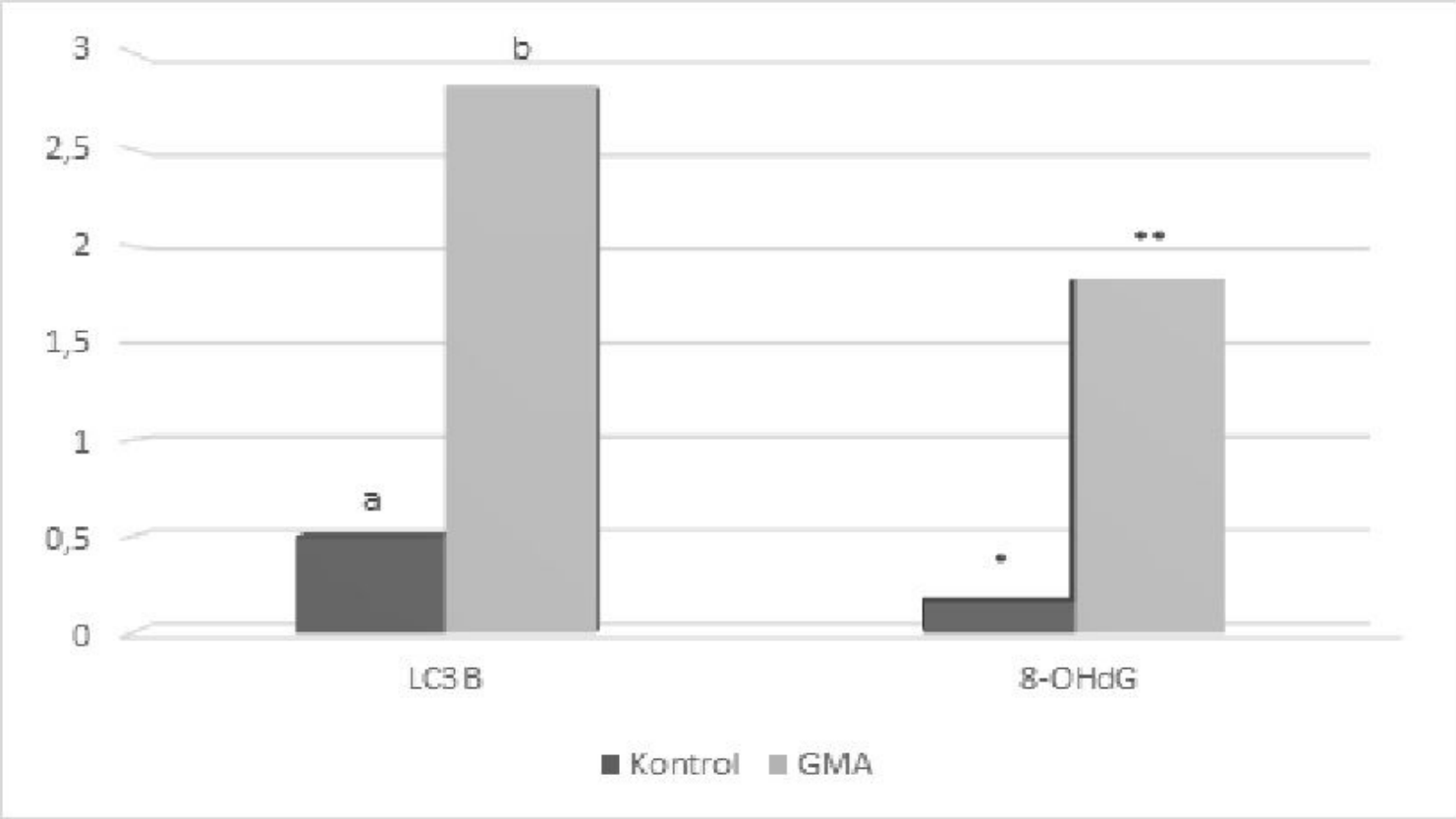

Figure 4

Statistically, the expression levels of LC3β and 8-OHdG showed significant differences among the groups (a, b, *, ** indicate differences between groups, $p < 0.05$). The results were obtained through semi-quantitative analysis of the immunofluorescence staining.

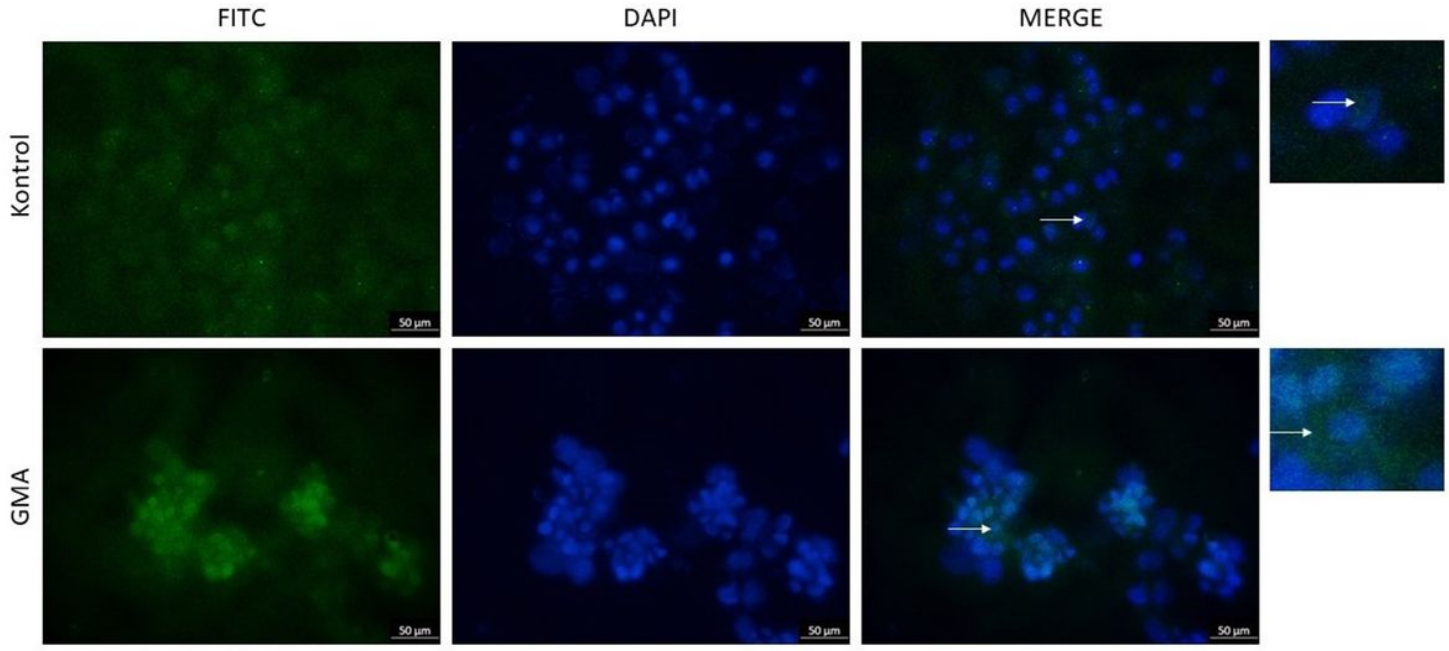

Figure 5

The impact of gambogic acid (GMA) on LC3β levels in SNU-16 cells using immunofluorescence staining in the control group, there was a mild level of LC3β immunofluorescence positivity, while in the GMA group, there was a severe level of LC3β immunofluorescence positivity (arrows).

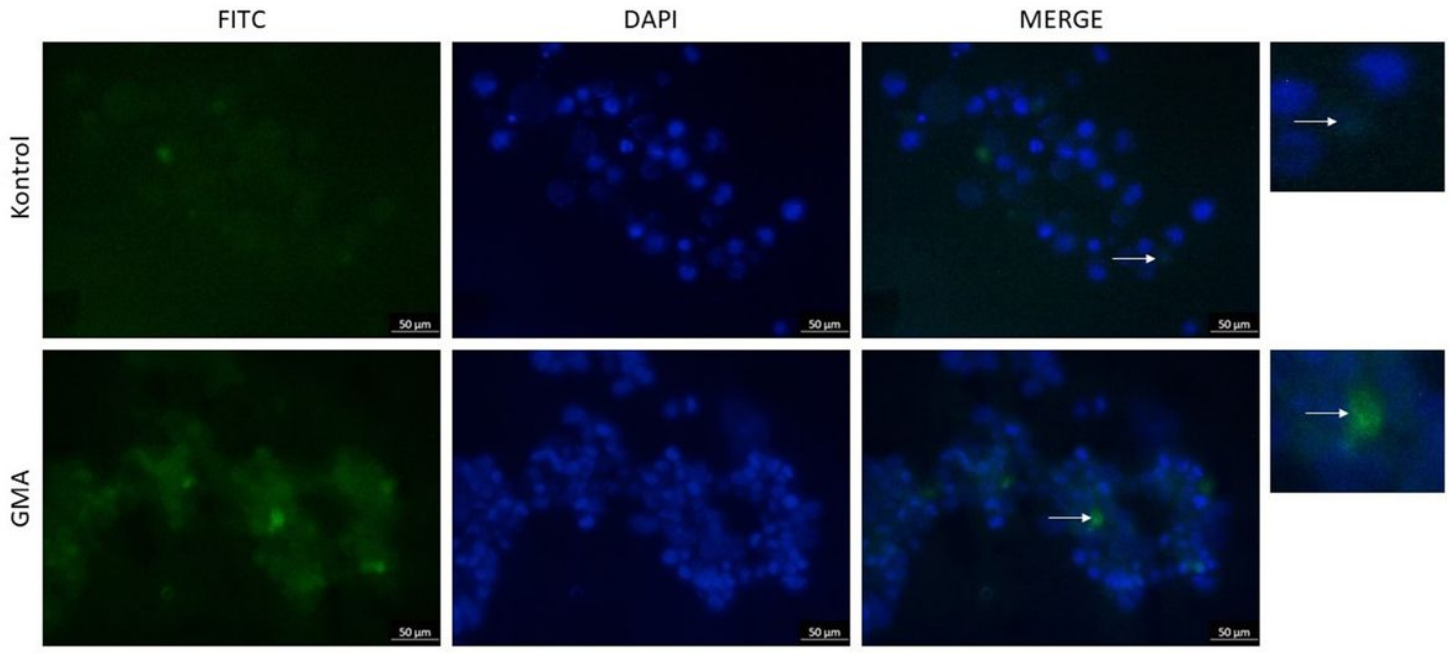

Figure 6

The impact of gambogic acid (GMA) on 8-oxo-dG levels in SNU-16 cells using immunofluorescence staining in the control group, there was a mild level of 8-oxo-dG immunofluorescence positivity, while in the GMA group, there was a moderatelevel of 8-oxo-dG immunofluorescence positivity (arrows).