

Genipa americana lectin (GaBL) induces inhibition of growth, migration, invasion, and regulation of the expression of caspase-mediated apoptosis and proteins related to the development of cancer in human head and neck cells

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Research Article

Keywords: caspase-3, E-cadherin, lectin

Posted Date: March 12th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4030027/v1>

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Additional Declarations: No competing interests reported.

Abstract

The antitumor activity of *Genipa americana* bark lectin (GaBL) was evaluated for the first time against cell lines of human skin cancer (A431), melanoma (B16), and squamous cell carcinoma of the tongue (SCC9). Cancer cell lines were treated with 10 µg/ml of GaBL to assess cell viability, cell migration and invasion, as well as the identification of cell membrane alterations associated with apoptosis. Real-time polymerase chain reaction for caspase-3 was performed to verify if apoptosis is activated by lectin treatment. The mRNA expression of proteins (E-cadherin, type I collagen) related to the epithelial-mesenchymal transition was also analyzed. GaBL decreased (27.5–50%) cell proliferation and reduced cell migration in all strains evaluated. Additionally, the lectin decreased the invasion of SCC9 cells. Apoptosis was higher against B16 and SCC9 cells treated with the lectin. GaBL induced the upregulation of caspase-3, E-cadherin and suppression of type I collagen in all strains tested, indicating lower cancer development. GaBL induces inhibition of growth, migration, invasion, and regulation of the expression of caspase-mediated apoptosis and proteins related to the development of cancer in human head and neck cells.

1. Introduction

Data published in 2020 by the International Agency for Research on Cancer highlighted a wide rise in the numbers of new cancer cases and deaths worldwide. According to the publication, in 2020 the worldwide incidence of cancer was over 19.2 million new cases and 9.9 million deaths, and these numbers tend to increase even more, reaching more than 50 million new cases and 13 million deaths in the next five years (Globocan, 2020).

Cancer metastasis is the primary cause of cancer mortality, accounting for about 90% of cancer deaths. Metastasis is dependent on several steps, including vessel formation, cell adhesion, migration, invasion, and cell proliferation (Majidpoor and Mortezaee, 2021). It occurs when genetically unstable neoplastic cells adapt to a tissue microenvironment far from the primary neoplastic mass. This process involves the selection of pathways advantageous for neoplastic cells and the ability to recruit stromal pathways that accommodate these cells (Follain et al., 2020).

Due to this heterogeneity, cancer treatment is still fraught with limitations. Cytotoxic chemotherapy, one of the main forms of treatment, is limited by cell resistance to drugs, in addition to causing many adverse side effects (Patriota et al., 2021, Lin et al., 2020, Petrović et al., 2020). Thus, tumors are highly adaptable, as they can activate resistance signaling routes and inactivate the death signaling cascade, leading to resistance to treatment. There is a need to develop new therapeutic strategies that are more selective in targeting specific pathways, sparing healthy cells from exposure to these drugs.

Plant species are widely used in studies on the prevention, development, and treatment of cancer, as most natural compounds have low toxicity compared to conventional therapies used for treatment (Wang et al., 2020). Among the studied compounds in a plant extract, there are lectins, proteins that have high

specificity in carbohydrate recognition and can bind in a reversible way to monosaccharides or oligosaccharides through the non-catalytic domain (Bhutia et al., 2019, Hasan et al., 2021). Due to the wide structural diversity, these biomolecules have several biological activities, such as anti-inflammatory, antibacterial, antiviral, antifungal, immunomodulatory, and antitumor properties (da Silva et al., 2018, Silva et al., 2019, Bhutia et al., 2019, Wang et al., 2020, Santos et al., 2020, Sohrab et al., 2020, Mesquita et al., 2021).

Some lectins have greater affinity for carbohydrates found in tumor cells than for those present in normal cells. Therefore, lectins can be used as effective anticancer agents and/or as biomarkers. Several studies use plant lectins to identify malignant cancer cells through the analysis of glycosylated patterns or by using microarrays of these proteins (Bhutia et al., 2019, Petrović et al., 2020, Cavada et al., 2021). These molecules are also capable of reducing proliferation and inducing tumor cell apoptosis, migration, and invasion and can be used in the treatment of malignant neoplasms (Shi et al., 2017, Hammouda et al., 2018, Bellé et al., 2018, Islam et al., 2018, Ramos et al., 2019, Petrović et al., 2020, Wang et al., 2020).

Barks of genipap (*Genipa americana* L.) have a lectin (GaBL) with a molecular mass greater than 200 kDa. GaBL has broad stability at pH and temperature, remaining stable in temperature and pH values found in the human body. It was also detected that GaBL showed high specificity to lactose and fetuin glycoprotein (Costa et al., 2018). Increased level of fetuin is described as a potential marker for metastatic disease diagnosis (Mintz et al., 2015, Silva et al., 2016, Neri-Numa et al., 2020). Considering the characteristics presented by this isolated biomolecule, along with the descriptions of the use of this plant as a phytotherapeutic product, the objective of the present work was to evaluate the anti-tumor activity of the lectin from the bark of *G. americana* on the human skin cancer cell line (A431), melanoma (B16), and tongue squamous cell carcinoma (SCC9) phenotypes.

2. Materials and Methods

2.1 Collection of plant material and protein purification

The bark of genipap (*G. americana*) was collected in Coruripe, located on the Southern coast of Alagoas, Brazil, with the authorization of the National System of Genetic Heritage and Management of Associated Traditional Knowledge (SISGEN) (process number A87C1CC) and one sample was sent to the Environmental Institute of Alagoas (IMA) for identification, obtaining registration number 64576. The plant material was stored, in plastic packaging, and taken to the Laboratory of Metabolism and Proteomics - LAMP, at the Federal University of Alagoas.

GaBL was isolated as described by Costa et al [21]. The bark, after being dried at room temperature, was weighed and ground. Then, 1:2 extraction buffer (50 mM Tris-HCl, pH 8.0) was added and the solution was kept under stirring for 16 hours. After this time, the solution was filtered and centrifuged at 15,000g, for 15 minutes, at 4°C, using a refrigerated centrifuge (HERMLE-Z236K). The supernatant was collected and stored at – 20°C.

For the purification process, the supernatant obtained during the extraction procedure was treated with ammonium sulfate at 20% saturation, for 4 hours, followed by centrifugation at 15,000 g, at 4°C, for 15 min. The precipitate was resuspended in Tris-HCl buffer pH 8.0, 50 mM and dialyzed against distilled water (4 h) and 50 mM Tris-HCl pH 4.0 (2 h).

The precipitated fraction was subjected to liquid chromatography with a molecular exclusion column, composed of a stationary phase formed by Sephacryl S-100 HR resin (16 mm x 60 cm), equilibrated with 50 mM Tris-HCl buffer pH 8.0, with 0.5 M NaCl as a mobile phase. The flow was adjusted to 0.1 mL/min, with a total column volume of 50 mL, connected to a fast performance liquid chromatography system (AKTA purifier 25 M1, General Electric). A volume of 300 µL of the precipitate resuspended in Tris-HCl buffer pH 8.0, 50 mM was applied and 2 mL per fraction were collected, which were evaluated for protein concentration and hemagglutinating activity (HA) and the active fractions were called GaBL.

2.2 Protein concentration and hemagglutinating activity assay

Protein concentration was estimated according to Bradford (1976), using bovine serum albumin (0.009–250 µg/mL) as a standard. The HA assay was performed on 96-well microtiter plates using a suspension of rabbit erythrocytes (2.5% v/v) previously treated with glutaraldehyde, as previously described by Bing et al. (1967) and Silva et al (2016). For this purpose, a serial dilution of 50 µL of the sample was performed (in duplicate) in 0.15 M NaCl in a row of the plates and, immediately afterwards, 50 µL of the erythrocyte suspension was added. The number of units of HA corresponded to the reciprocal of the highest dilution of the sample that promoted agglutination of erythrocytes. Specific HA was defined as the ratio between HA and protein concentration (mg/mL). The erythrocyte collection was approved by the Animal Experimentation Ethics Committee of the Federal University of Pernambuco (process 23076.033782 / 2015-70).

2.3 Cell culture and treatment

The immortalized cell line of tongue squamous cell carcinoma (SCC9) (ATCC, Manassas, VA, USA), the human epidermoid carcinoma cell line A431 (ATCC® 1555™, USA) and murine metastatic melanoma cells of the B16 line, provided by the Federal University of Minas Gerais, were used. B16, A431 and SCC-9 cells were cultured at 37°C in 5% CO₂ in a Ham's F-12 nutrient mixture (Invitrogen, Carlsbad, CA, USA) supplemented with 400 ng/ml hydrocortisone (Sigma, St. Louis, MO) and 10% fetal bovine serum. Mouse embryonic fibroblasts (3T3 cell line) were kept in medium Dulbecco's modified Eagle medium, containing 10% fetal bovine serum, L-glutamine (2 mM) and gentamicin (40 µg/mL) in incubator at 37°C and 5% CO₂.

2.4 Cell viability assay

To explore the cytotoxic activity of GaBL to noncarcinogenic cells, the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was pursued with fibroblastic (3T3 cell line), with an incubation period of 24 and 48h at 37°C in a CO₂ humidity incubator. A 96-well plate at a

density of 1×10^4 cells/well were treated with the serial concentrations of the compound GaBL (0.5, 1, 5, 10, and 50 $\mu\text{g/ml}$). At the end of the treatment, 5 mg/mL of MTT (Sigma-Aldrich – EUA) in phosphate-buffered saline was added, and the cells were incubated for another 4 h. Dimethyl sulfoxide (200 μl) was added to each well after removal of the supernatant. After shaking the plate for 10 min, cell viability was assessed by measuring the absorbance at 490 nm using an Enzyme-labeling instrument (EX-800 type). All measurements were resulted of an experiment performed in triplicate. Cell viability was expressed as a percentage of untreated cells (control, 100%). The cytotoxic effect was considered when the highest concentration gave a response that was < 70% of the control.

The cytotoxic effect against tumor cells (SCC9, A431 and B16 cell lines) was evaluated through MTT assay. A 96-well plate at a density of 1×10^4 cells/well, cultured for 12 h under 37°C in 5% CO₂, was treated with 10 $\mu\text{g/ml}$ of GaBL for 24 h. Cell growth curve was completed using time as the abscissa and A value (mean $\pm$ standard errors) as the ordinate.

2.5 Cell migration assays

Cell migration was monitored in a wound scratch assay as described previously (Guimaraes et al., 2016). Briefly, a scratch was made in the center of the well with a sterile pipette tip in a confluent cell layer and washed twice in phosphate-buffered saline to remove debris and smooth the edge of the scratch. Then, fresh medium was added to the cells with or without GaBL (10 $\mu\text{g/ml}$). The plates were then incubated at 37°C in 5% CO₂ for up to 24 h. Wells were photographed at the beginning of the experiment and after 24 hours. Pictures were obtained with a camera SC30 (Olympus, Center Valley, PA, USA) in an IX81 inverted microscope (Olympus, Center Valley, PA, USA). ImageJ software was used for analysis (Schneider et al., 2012). To calculate the wound healing ratio, the initial area (in pixels) was divided by the final cell-free area (in pixels). Each experiment was carried out in triplicate.

2.6 Invasion assay

For the transwell invasion assay, a polycarbonate membrane with pores of 8 μm in diameter was used for the upper chamber covered with 50 μl of Matrigel (BD Biosciences, USA). Then, in the top chamber, 2×10^5 SCC9 cells were plated onto each well and treated with 10 $\mu\text{g/ml}$ of lectin. In lower compartment, 10% fetal bovine serum was added as a chemoattractant factor for cells. The multiwell plate was covered and incubated for 24 h at 37°C in CO₂ incubator. Then the upper chamber was inverted to remove both cells that had not invaded and culture medium from the upper chamber. Only the cells that had migrated were fixed in 4% formaldehyde in PBS and stained with 2% Crystal Violet. Images were acquired at a scale of 200 μm with the camera SC30 (Olympus) in an IX81 inverted microscope (Olympus). The number of cells that invaded was determined through counting. The experiment was performed in triplicate.

2.7 Cell apoptosis assay

Dual acridine orange/ethidium bromide (AO/EB) fluorescent staining, visualized under a fluorescent microscope, can be used to identify apoptosis-associated changes of cell membranes during the process of apoptosis. This method can also accurately distinguish cells in different stages of apoptosis. Dual

AO/EB staining was used to examine apoptosis in a human oral cancer cell line treated with GaBL (10 µg/ml). The samples in a 96-well plate were divided into 2 groups, with 24 well samples in each group. Dual fluorescent staining solution (1 µl) containing 100 µg/ml AO and 100 µg/ml EB (AO/EB, Sigma, St. Louis, MO) was added to each well. The morphology of apoptotic cells was examined after 24h and 500 cells were counted within 20 min using a fluorescent microscope. Intense EB (Ex360-370nm, Em420-460nm, filter DM400) staining indicates cell death, while extreme AO (Ex460-495nm, Em510-550nm, filter DM505) indicates live cells. Dual acridine orange/ethidium bromide (AO/EB) staining method was repeated three times.

2.8 Analyses of gene expression

To verify the effect of lectin treatment on gene expression in A431, B16 and SCC9 cells, RNA was isolated from tumor tissue using the Trizol reagent (GIBCO Invitrogen), according to the manufacturer. Total RNA was reverse transcribed, and the subsequent cDNA was heated at 95°C to terminate the reaction. For real-time polymerase chain reaction (PCR), 2 µl of the cDNA was added to TaqMan Universal PCR Master Mix (Applied Biosystems) with the E-cadherin, Collagen type 1 alpha 1 (Col1A1), and Caspase-3 specific primer/probe set (Applied Biosystems) as described in Online Resource 1 (Table S1). Amplification was performed on an ABI 7000 Sequence Detection System. All reactions were done in triplicate, and 18S rRNA served as an internal control. The results were quantified as Ct values, where Ct was defined as the threshold cycle of PCR at which the amplified product is first detected and defined as relative gene expression (the ratio of target/control).

2.9 Statistical analysis

All studies were conducted in triplicate. Analyses were performed using GraphPad Prism software (Version 5.0, GraphPad Software Inc., San Diego, CA, USA). Data were presented as the means ± standard errors. Mean differences between the experimental and control groups were analyzed statistically using unpaired two tailed t-test when comparing two experimental groups. The differences were considered significant when the P-values were < 0.05.

3. Results

3.1 Lectin isolation

GaBL was successfully isolated following the previous established protocol. The crude extract showed specific HA of 640 and was submitted to treatment with ammonium sulphate. Chromatography of the dialyzed supernatant fraction (specific HA of 1561) on Sephacryl S-100 HR resin yielded isolated GaBL with specific HA of 1838 (purification fold: 2.9). These data are in accordance with the previous results reported by Costa et al. (2018).

3.2 Effect of lectin on cell viability

GaBL was investigated for cytotoxicity on normal fibroblast cell line (3T3) before the evaluation of antitumoral activity since compounds with unselective cytotoxicity are not viable for use in chemotherapy. Treatment for 24 h with pure lectin at concentrations of 0.5, 1, 5 and 10 µg/mL did not change fibroblast viability (Fig. 1). However, cells treated with 50 µg/mL of the lectin showed a 31% ($p < 0.05$) reduction in cell viability. A similar effect on viability was observed with the 48 h treatment with the lectin ($p < 0.05$). Therefore, the concentration of 10 µg /mL was used for the remaining tests.

To elucidate whether lectin contributes to the viability of tumor cell lines, MTT assay was performed on the A431, B16 and SCC9 cells treated with 10 µg/ml of lectin for 24h. The lectin significantly decreased the percentage of treated cells. After treatment with lectin, the viability was decreased in both A431, B16, and SCC9 (Fig. 2). These results indicate that lectin elicits a cytotoxic effect in tumor cells.

3.3 Effects of lectin on migration and invasion

Wound-scratch assay was performed on the cancer cells. Cell migration assays were performed with confluent monolayers of human A431, B16, and SCC9 cells treated with GaBL, which were mechanically scratched and subsequently allowed to re-populate the wounded cell-free areas. Application of lectin resulted in a significant decrease of scratch area closure, consistent with decreased cell migration against all tumor cells evaluated (Fig. 3). Invasiveness is an important characteristic of cancer cells and a target for the development of anticancer agents. According to matrigel invasion assay, lectin significantly reduced invasiveness in SCC9 treated cancer cells (Fig. 4).

3.4 Effect of lectin (GaBL) on apoptosis and on caspase-3, E-cadherin, and Col1A1 expression

Acridine Orange/Ethidium Bromide Cell death assay reveals that the lectin significantly increased the number of apoptotic cells when compared to control, specifically in B16 and SCC9 cancer cells (Fig. 5). To determine if apoptosis could be activated because of lectin treatment, a reverse transcription-PCR for caspase-3 was performed. The lectin increased caspase-3 transcription when compared with non-treated B16 and SCC9 cells. In A431 cells, the lectin did not alter caspase- 3 mRNA levels. Additionally, a quantitative real-time PCR was performed to evaluate the mRNA expression levels of epithelial-to-mesenchymal transition-related proteins (E-cadherin and Col1A1). GaBL induced the upregulation of E-cadherin and the suppression of Col1A1 in both A431, B16, and SCC9 cells (Fig. 6).

4. Discussion

In a previous study, it was demonstrated that *G. americana* L. has a lectin called GaBL with wide stability in pH and temperature. This lectin was not inhibited up to 120°C, losing its activity only at pH 2. GaBL is also able to bind to various carbohydrates and glycoproteins. These characteristics make GaBL a desirable candidate for potential use in the treatment of infectious diseases in humans (Costa et al., 2018).

Viable biomolecules in cancer therapy have become a promising target in the field of biotechnology. Carbohydrate-specific binding proteins play an important therapeutic and biotechnological role (Islam et al., 2023). Lectins can recognize carbohydrate or glycoconjugate fractions and bind reversibly to cancer cells with high specificity and without changes in the covalent structure (Figueiroa et al., 2017). The commercially acquired lectins DBA, LCA, PHA-L, and WGA showed high recognition for cancer cells through binding to glycans present on the cell surface (Szydlak et al., 2023). In another study, Freitas et al. (2019) described that the lectins E-PHA and L-PHA bind to cancer-related N-glycans, and the AAL lectin binds to fucose glycans. It is important to highlight that glycoconjugates present in extracellular vesicles play versatile roles in cancer (Islam et al., 2023). A study by Surman et al. (2018) showed that a group of lectins can bind specific glycan epitopes in extracellular vesicles compared to parental cell membranes. It is noteworthy to describe that, to the best of our knowledge, this is the first study showing the association between this lectin (GaBL) and cancer cells.

In this study, it was observed that GaBL did not show cytotoxicity for normal fibroblast cells. However, GaBL had an antiproliferative effect on cancer cells. This lectin could reduce both migration and invasion. These findings were supported by the mRNA expression levels of E-cadherin and Col1A. These data are consistent with the literature regarding the roles of some described plant lectins with antitumor action, such as DVL, a lectin purified from *Dioclea violacea* seeds, which showed cytotoxic activity against human cells, acting on mechanisms of cell migration and proliferation (Nascimento et al., 2019). The lectin from *Canavalia ensiformis* seeds (ConA) exhibited antitumor action, capable of inhibiting the proliferation of cancer cells, reducing tumor growth, and inducing apoptosis in tumor cells (Huldani et al., 2022).

The increase in E-cadherin expression is important in regulating cell adhesion and maintaining tissue integrity, preventing disintegration and possible metastasis in vivo. In this work, it was observed that E-cadherin expression was increased by lectin treatment, while Col1A1 was reduced. It has been demonstrated that the loss of E-cadherin expression/function is associated with the development and progression of different types of cancer. The loss of E-cadherin is often associated with epithelial-mesenchymal transition (EMT) and a more aggressive cancer phenotype (Mendonsa et al., 2018). Both the positive regulation of Col1A1 and the reduction or loss of E-cadherin expression are considered characteristics of EMT, a complex developmental program that allows carcinoma cells to suppress their epithelial characteristics and shift to mesenchymal ones (Ma et al., 2019, Sommariva and Gagliano, 2020). These effects (positive regulation of E-cadherin and negative regulation of Col1A1) shown by GaBL may lead to the inhibition of metastatic capacity.

The lectin can activate the EGFR pathway mediated by Ras-Raf-MEK-ERK, inducing apoptosis and autophagy in human MCF-7 tumor cells (Ouyang et al., 2014). Mistletoe lectins (MLs) have antiproliferative mechanisms, being potent cytotoxic anti-tumor lectins and inducing apoptosis by efficiently inhibiting protein synthesis in acute lymphoblastic leukemia and hepatocarcinoma cells (Lyu et al., 2002, Seifert et al., 2008). In this study, it was observed that the lectin (GaBL) induced the death of cancer cells. The data indicated that treatment with the lectin (GaBL) markedly triggered caspase-3

transcription. A study conducted by Batcha et al., (2023) showed that the lectin extracted from bananas (BanLec) triggered apoptosis, manifesting through distinct morphological changes. These included the formation of bubbles in the cell membrane, reduction in cell volume, chromatin condensation, cell budding, and nucleus fragmentation. Similarly to GaBL, the expression of the caspase-3 gene was notably positively regulated, clearly suggesting the induction of the apoptotic process during treatment with BanLec.

The lectin from the tuberous rhizome of *Kaempferia rotunda* also exhibited anticancer activity against SW480 and SW48 colon cancer cells, acting on the activation of caspase-3 and caspase-9, preventing cell proliferation, and inducing the apoptotic process in the mitochondrial intrinsic pathway (Islam et al., 2019). The lectin isolated from the hemolymph of *Gluttonous beatus* exerted cytotoxic effects in vitro on human breast cancer cells (MCF-7). This lectin can induce apoptosis by activating caspase-3 (Ponraj et al., 2017). Apoptosis is an important mechanism in controlling cell proliferation as part of normal development.

5. Conclusion

GaBL was able to reduce proliferation and cell migration in human skin cancer cell lines (A431), melanoma (B16), and tongue squamous cell carcinoma (SCC9). Apoptosis and cell invasion were also altered for some of the tested cell lines. Furthermore, the positive regulation of E-cadherin and negative regulation of Col1A1 show that GaBL can lead to the inhibition of metastatic capacity. GaBL is a candidate for future *in vivo* antitumor assays.

Declarations

Acknowledgement

The authors would like to thank the government development agency *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) for the postgraduate scholarships (Ricardo Bezerra Costa, Monizy da Costa Silva, and Ana Kelly da Silva Fernandes Duarte) and the government development agency *Fundação de Amparo à Pesquisa do Estado de Alagoas* for financial support (n. E:60030.0000000161/2022).

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability

All data generated or analysed during this study are included in this published article.

Conflict of interest

Authors declare no conflict of interest.

CRediT author statement

Ricardo Bezerra Costa: Data Curation; Formal analysis; Investigation; Methodology; Software; Writing - original draft; Writing - review & editing; Visualization. **Monizy da Costa Silva:** Formal analysis; Investigation; Roles/Writing - original draft; Visualization; Validation. **Emisael Stênio Batista Gomes:** Investigation; Software; Visualization; Writing - review & editing. **Rogério Gonçalves da Rocha:** Investigation; Software; Visualization; Writing - review & editing. **Ana Kelly da Silva Fernandes Duarte:** Software, Data curation, Validation; Roles/Writing - original draft. **Stella Freitas de Queiroz:** Formal analysis; Validation; Data curation; Roles/Writing - original draft. **Marta Angelo dos Santos:** Formal analysis; Validation; Data curation; Roles/Writing - original draft. **André Luiz Sena Guimarães:** Conceptualization; Resources; Supervision, Funding acquisition; Methodology; Writing - review & editing. **Hugo Juarez Vieira Pereira:** Conceptualization; Supervision; Resources; Methodology; Project administration; Writing - review & editing. **Carlos Alberto de Carvalho Fraga:** Conceptualization; Supervision, Funding acquisition; Methodology; Project administration; Roles/Writing - original draft. **Francis Soares Gomes:** Conceptualization; Resources; Funding acquisition; Supervision; Methodology; Project administration, Roles/Writing - original draft; Writing - review & editing.

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Figures

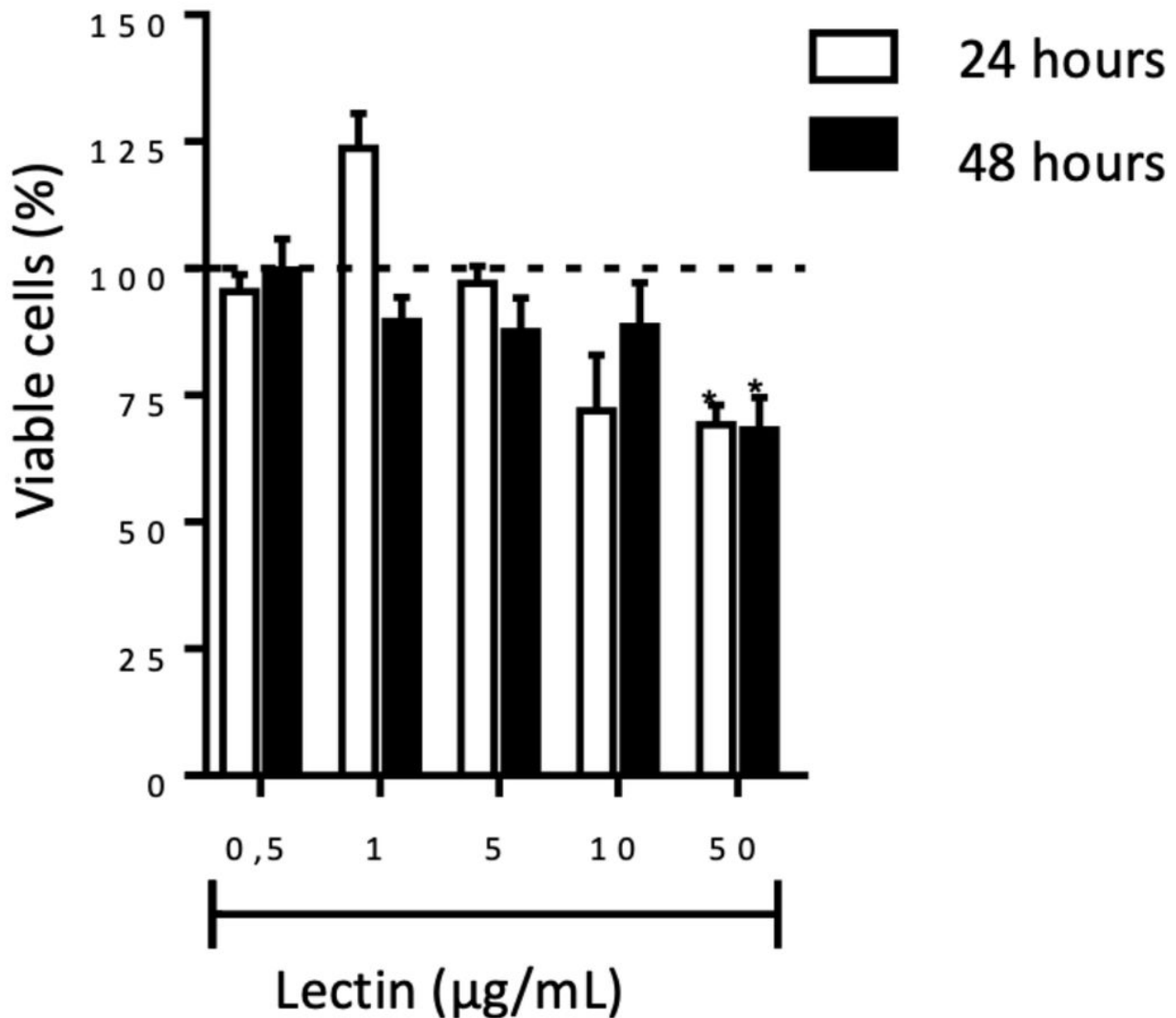


Figure 1

The effect of lectin (GaBL) on the proliferation of fibroblastic cell line 3T3 through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A 96-well plate, seeded at a density of 1×10^4 cells/well, were treated with the serial concentrations of GaBL (0.5, 1, 5, 10, and 50 µg/ml) during 24h (white bar) and 48h (black bar). Untreated cells served as controls. Cell viability was expressed as a percentage of untreated cells (control, 100%). The cytotoxic effect was considered when the highest concentration gave a response that was < 70% of the control. The results are the means $\pm$ SD of an experiment performed in triplicate. Bars = SD. Mean differences between the experimental and control groups were analyzed statistically using unpaired two tailed t-test when comparing two experimental

groups. The differences were considered significant when the P-values were <0.05.* Statistical significance

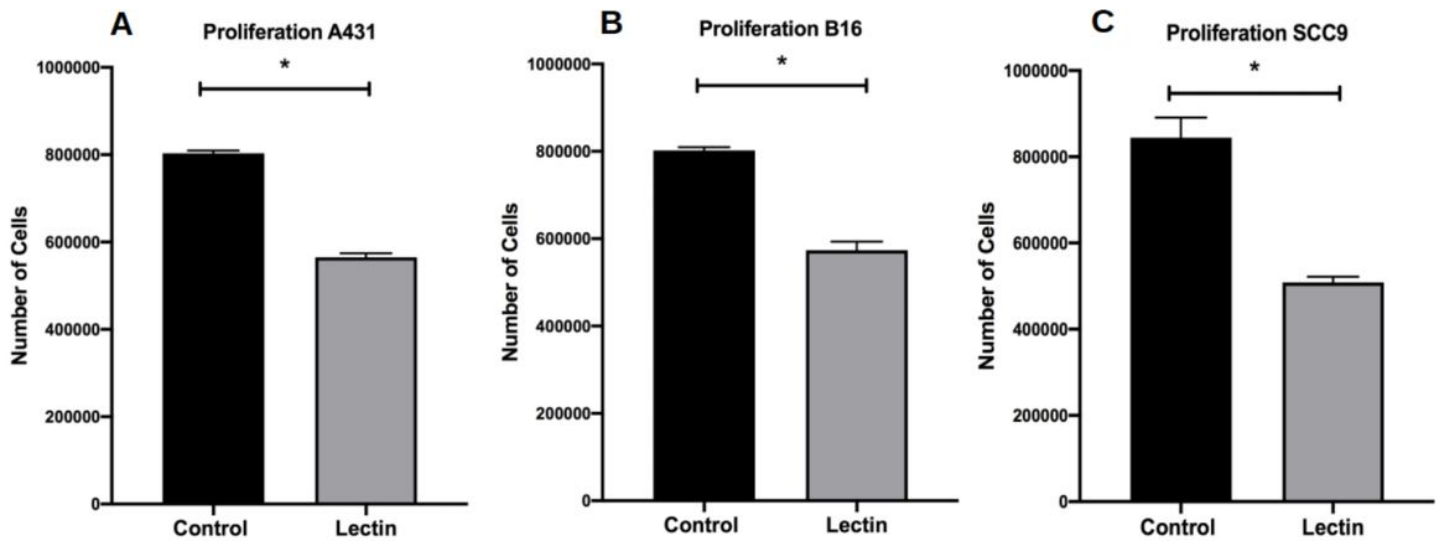


Figure 2

The effect of lectin (GaBL) on the proliferation of A431, B16 and SCC9 cancer cells through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A 96-well plate at a density of 1×10^4 cells/well were treated with 10 $\mu\text{g/ml}$ of GaBL during 24h. Untreated cells were used as controls. Cell viability was expressed as a percentage of untreated cells (control, 100%). The cytotoxic effect was considered when the highest concentration gave a response that was < 70% of the control. The results are the means $\pm$ SD of three independent experiments performed in triplicate. Bars = SD. Mean differences between the experimental and control groups were analyzed statistically using unpaired two tailed t-test when comparing two experimental groups. The differences were considered significant when the P-values were <0.05.*Statistical significance.

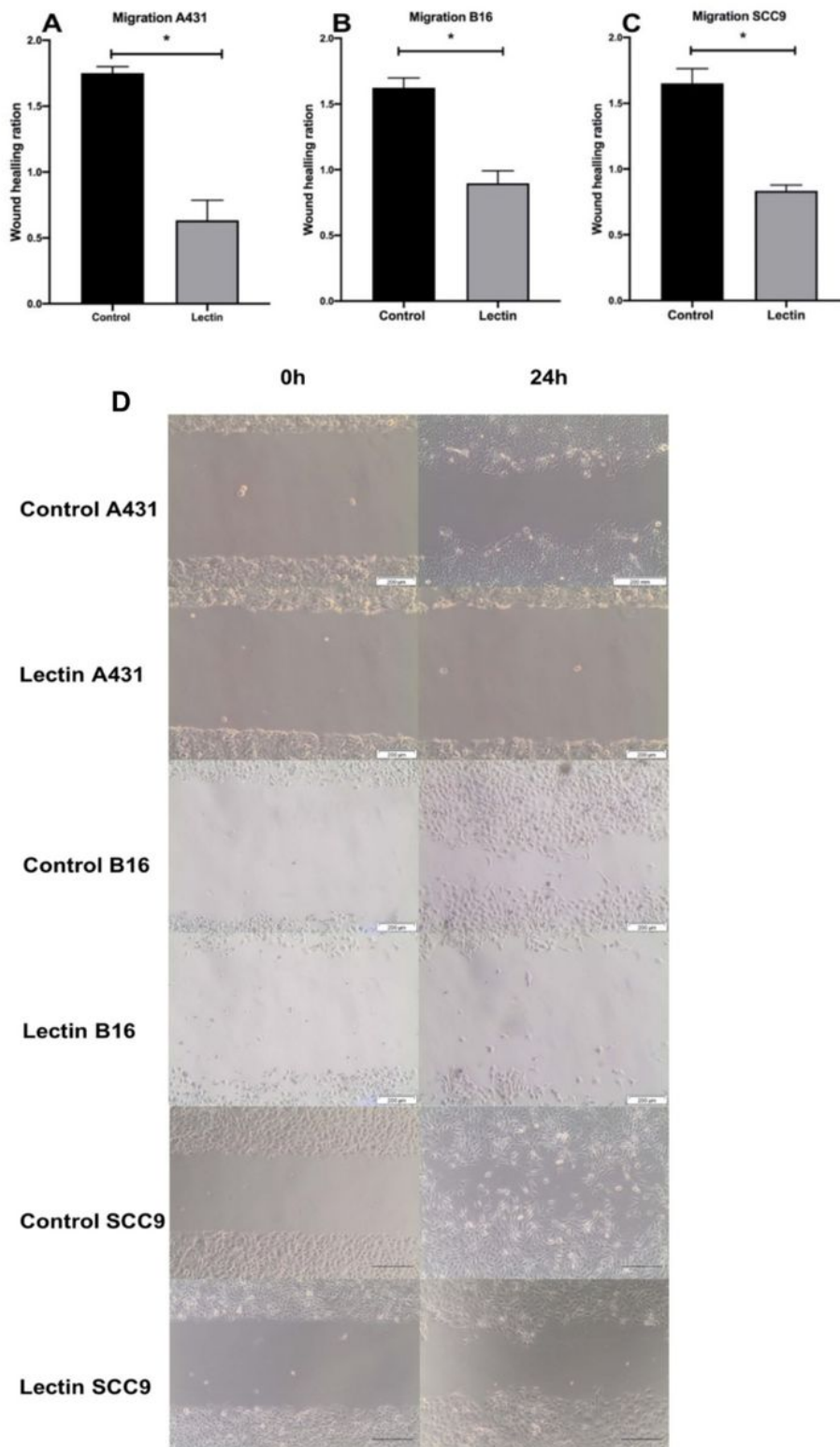
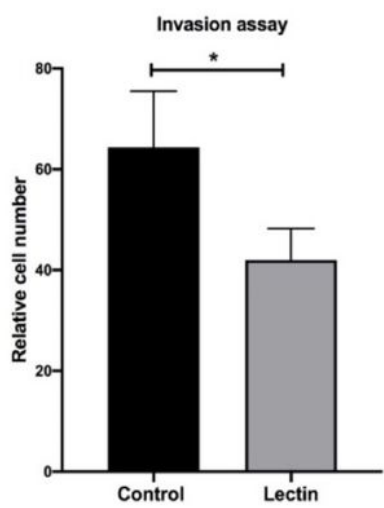


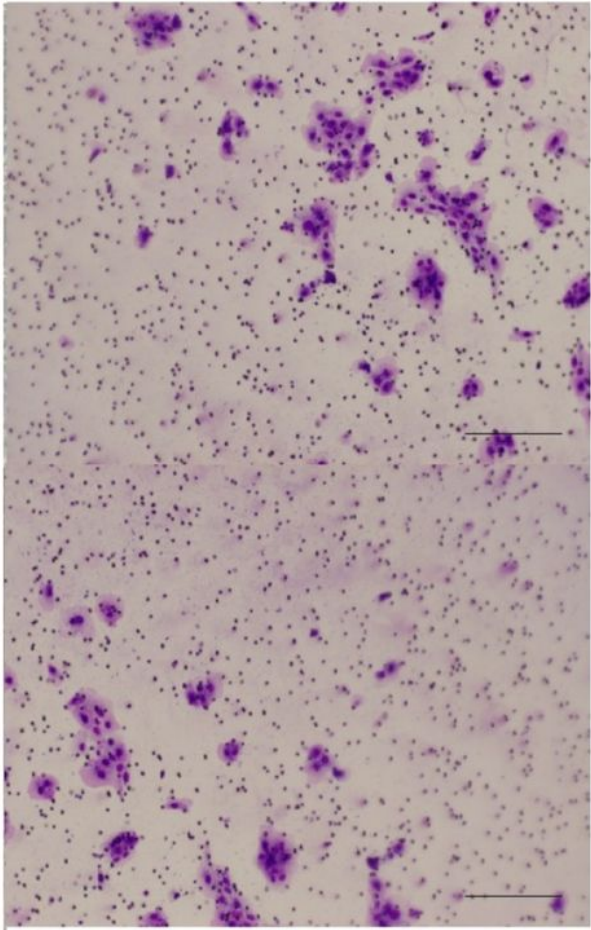
Figure 3

The effect of lectin (GaBL) at 10 $\mu\text{g/ml}$ on the migration of cancer cells A431 (A), B16 (B) and SCC9 cells (C) compared to the control assay without GaBL through wound-scratch assay. The wound healing ratio was quantified by dividing the initial area (in pixels at 0h) by the final cell-free area (in pixels at 24h). Panel D illustrates the wound-scratch assay of the A431, B16 and SCC9 cells lines with GaBL at 10 $\mu\text{g/ml}$ and without any treatment (control) at the start of the experiment and after 24h. The scale is 200 μm . The

experiment was performed in triplicate. Mean differences between the experimental and control groups were analysed statistically using unpaired two tailed t-test when comparing two experimental groups. * $P < 0.05$, significance compared with the control group.



Control SCC9



Lectin SCC9

Figure 4

The effect of lectin (GaBL) on SCC-9 cells invasiveness assessed by transwell invasion precoated with Matrigel. A density of 2×10^5 SCC9 cells was plated onto each well and treated with 10 $\mu\text{g}/\text{ml}$ of GaBL for 24 hours. Invading cells were fixed using formaldehyde and stained with 2% Crystal Violet. The relative number of invading cells was determined through counting. The scale is 200 μm . The experiment was performed in triplicate. Mean differences between the experimental and control groups were analyzed statistically using unpaired two tailed t-test when comparing two experimental groups. * $P < 0.05$, significance compared with the control group.

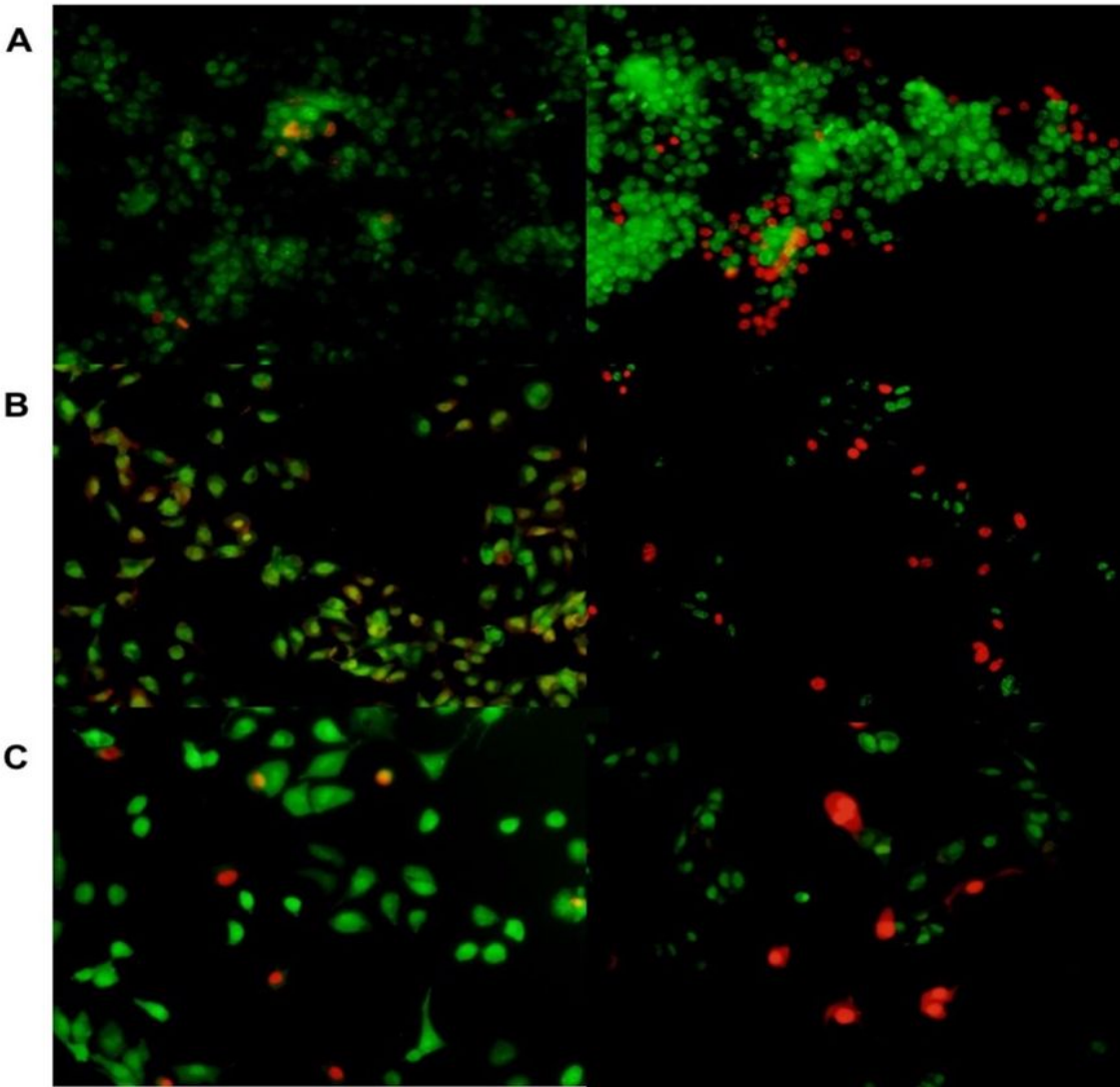
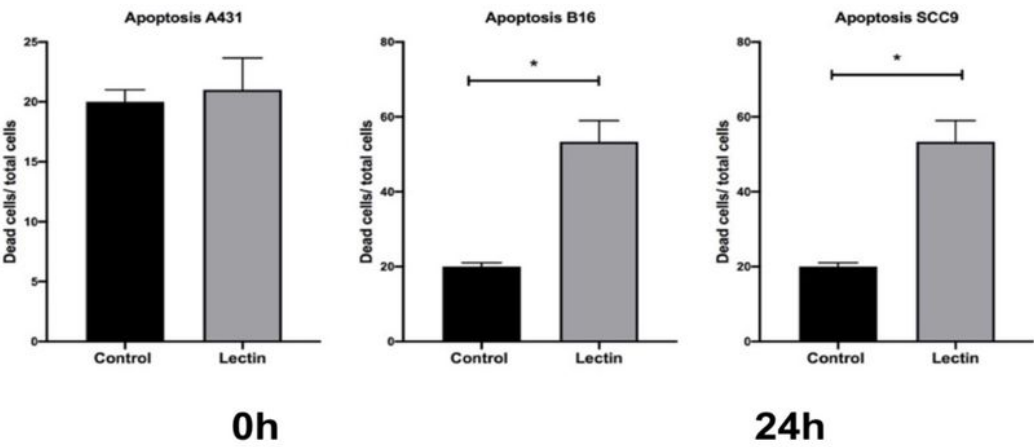


Figure 5

The effect of lectin (GaBL) on cell death. Dual acridine orange/ethidium bromide (AO/EB) fluorescent staining was used to examine apoptosis of the human oral cancer cell line A431 (A), B16 (B) and SCC9 (C) treated with GaBL (10 µg/ml) and without any treatment (control). The morphology of apoptotic cells was examined after 24h. 500 cells were counted within 20 min using a fluorescent microscope. Intense EB staining indicates cell death, while extreme AO indicates live cells. The scale of 200 µm. The experiment was performed in triplicate. Mean differences between the experimental and control groups were analyzed statistically using unpaired two tailed t-test when comparing two experimental groups. * $P < 0.05$, significance compared with the control group.

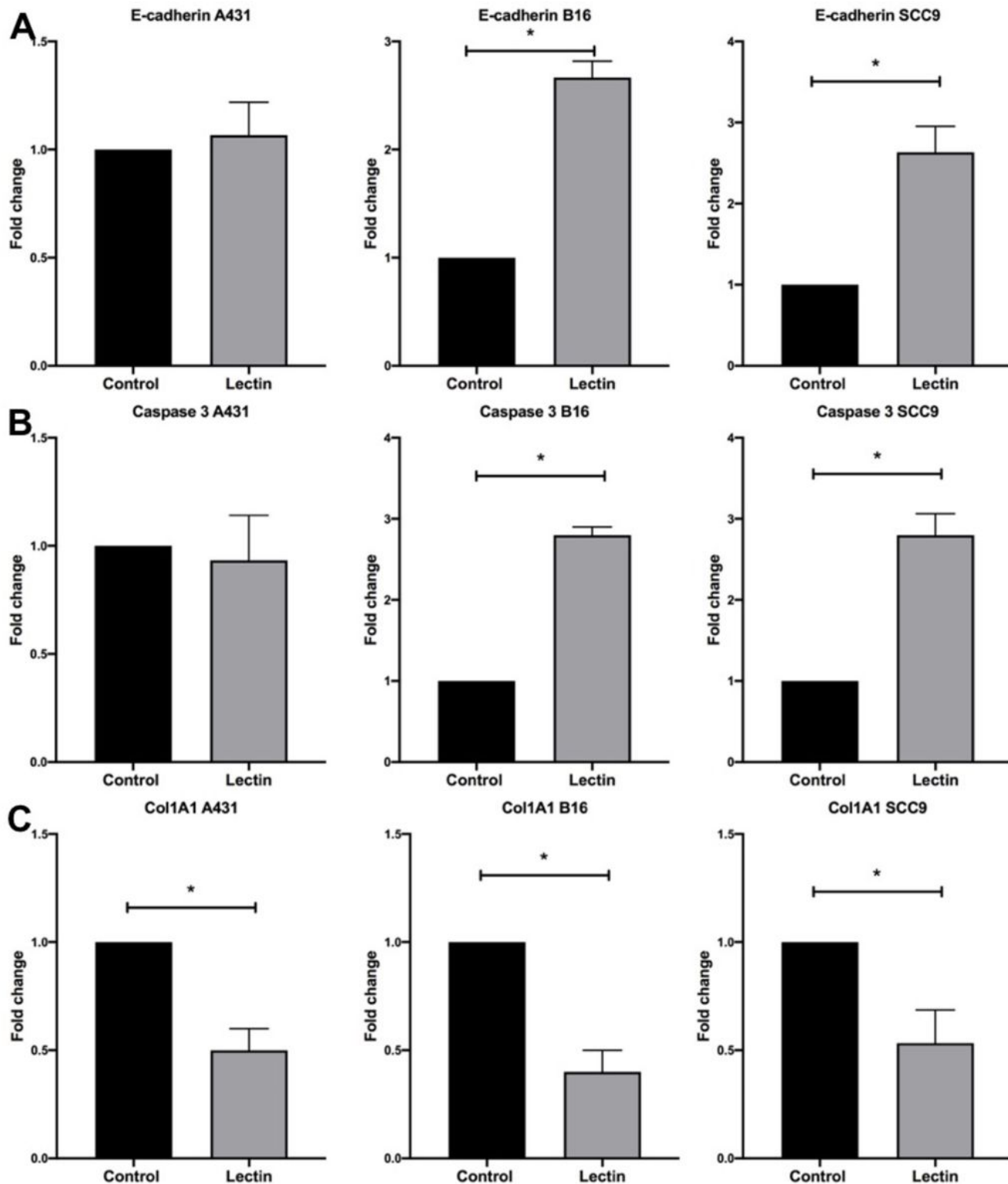


Figure 6

Quantification of the effect of lectin (GaBL) on E-cadherin (A), Caspase-3 (B) and Collagen type 1 alpha 1 (C) on expression of mRNA through real-time polymerase chain reaction in human oral cancer cell line A431, B16 and SCC9, respectively. 18S rRNA served as an internal control. The results were quantified as Ct values, defined as the threshold cycle of PCR at which the amplified product is first detected and defined as relative gene expression (the ratio of target/control). All reactions were done in triplicate. Mean

differences between the experimental and control groups were analyzed statistically using unpaired two tailed t-test when comparing two experimental groups. * $P < 0.05$, significance compared with the control group.

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