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RESEARCH ARTICLE

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Lectins CGL and MTL, representatives of mytilectin family, exhibit different antiproliferative activity in Burkitt's lymphoma cells

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Abstract

Lectins are carbohydrate-binding proteins, whose biological effects are exerted via binding to glycoconjugates expressed on the surface of cells. Exposure to lectins can lead not only to a change in the structure and properties of cells but also to their death. Here, we studied the biological activity of lectins from the mussels *Crenomytilus graynus* (CGL) and *Mytilus trossulus* (MTL) and showed that these proteins can affect the proliferation of human lymphoma cells. Both lectins suppressed the formation of colonies as well as cell cycle progression. The mechanism of action of these lectins was not mediated by reactive oxygen species but included damaging of mitochondria, inhibition of key cell cycle points, and activation of MAPK signaling pathway in tumor cells. Computer modeling suggested that various effects of CGL and MTL on lymphoma cells may be due to the difference in the energy of binding of these lectins to carbohydrate ligands on the cell surface. Thus, molecular recognition of residues of terminal carbohydrates on the surface of tumor cells is a key factor in the manifestation of the biological action of lectins.

KEYWORDS

antiproliferative activity, apoptosis, bivalve lectins, cell cycle, computer modeling

1 | INTRODUCTION

Cancer is one of the main causes of death worldwide.[1] The development of methods for accurate and early diagnosis of oncological diseases, as well as the search for bioreception molecules with diagnostic and therapeutic potential substantially ultimately define the treatment success. Carbohydrates are essential components of cell structures and intercellular structures.[2] As part of cell membranes, carbohydrates form the cellular glycocalyx, which plays an important role in maintaining the living organism. It is known that the neoplastic transformation of the cells is accompanied by a change in glycosylation and expression of carbohydrate moieties on the surface of tumor cells.[3]

The vast majority of lectins are complex multidomain proteins that can recognize and bind specific

carbohydrates. Carbohydrate-recognition domains are the specific regions of the lectin molecule which are capable of carbohydrate binding.[4,5] Animal lectins play an important role in various physiological processes such as cancer cell metastasis, apoptosis, and immunomodulation.[6,7] Many lectins isolated from animal tissues have been investigated as apoptosis-inducing agents, immunomodulatory, antiviral, and anticancer drugs.[7–9] Some lectins are capable of inhibition of proliferation and activation of cell death pathways of various types of cancer cells while having low or no cytotoxicity in non-malignant cells. This fact is associated with the expression of different glycans on the surface of tumor and normal cells, which allows lectins to specifically recognize and target malignant cells.[10,11]

Bivalve mollusks (mussels, scallops, and oysters) are of particular interest to researchers since they are mass species used in mariculture in many countries of the world. In bivalves, lectins with characteristic structures have been reported, including C-type lectins, galectins, fibrinogen-, C1q-binding lectins, and F-type lectins (ficolins).[12] These findings confirm that bivalves, and specifically mussels, are a promising source of new and known lectins.

Previously, our group has reported and isolation and characterization of several new lectins with a wide spectrum of biological activities, including antibacterial, cytokine-stimulating, and anti-HIV.[13–15] We identified sources of lectins among the most common commercial species of mussels of the Mytilidae family, which have a vast habitat and are widely used in mariculture. Gal/GalNAc-specific lectins were previously isolated by us from mussels *Crenomytilus grayanus* (CGL)[16] and *Mytilus trossulus* (MTL).[17] Further protein sequencing showed that CGL and MTL have a high degree of homology and are representatives of a new class of lectins[18,19]—mytilectins. This lectin family also includes MytiLec-1, -2, and -3 from the mussel *Mytilus galloprovincialis*[20,21] and MCL isolated from the mussel *Mytilus californianus*.[22] A common structure feature of mytilectins is three tandem repeats with similar sequences and three carbohydrate binding sites in each subdomain.[23] Very recent investigations showed that mytilectins have a very broad taxonomic distribution.[24]

Previously we found that CGL recognizes globotriose (Gb3), a trisaccharide consisting of Galα1-4Galβ1-4Glc, on the surface of Burkitt's Raji lymphoma cells which express high levels of Gb3, resulting in a dose-dependent cytotoxic effect, G2/M cell cycle arrest, and apoptosis.[25] Despite a high degree of homology, CGL and MTL have point substitutions at their carbohydrate-binding sites and therefore may have different ligand specificities. In the current study, we report different antiproliferative activities and mechanisms of action of CGL and MTL in human cancer cells.

2 | MATERIALS AND METHODS

2.1 | Materials

CGL and MTL were isolated as described previously.[16,17] Human Burkitt's lymphoma cells were from the Russian Cell Culture Collection (Saint Petersburg, Russia) (Raji RRID:CVCL_0511) and American Type Culture Collection (Rockville, MD, USA) (EB-1 RRID:CVCL_2027 and Daudi RRID:CVCL_0008). RPMI 1640 medium was purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Gemini Bio-Products (Calabasas, CA, USA); penicillin/streptomycin and gentamycin were from Bio-Whittaker (Walkersville, MD, USA); L-glutamine was from Mediatech, Inc. (Herndon, VA, USA). Cell Titer 96 Aqueous One Solution Reagent (MTS) kit for the cell viability assay was from Promega (Madison, WI, USA). FITC Annexin V Apoptosis Detection Kit was from BD Biosciences (San Diego, CA, USA); Mitochondrial Membrane Potential Assay Kit (II) and antibodies for western blotting were purchased from Cell Signaling Technology (Beverly, MA, USA); 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA) was from Sigma (Germany); bisBenzimide H 33342 trihydrochloride (Hoechst 33342) was from Sigma, St. Louis, MO, USA; and Fluorescein isothiocyanate (FITC) was bought in Thermo Scientific (Germany).

2.2 | Computer modeling

Analysis of the amino acid sequence of MTL (Uniprot ID A0A0G2UGT2) was performed using the MOE 2020.09 software (Molecular Operating Environment [MOE], 2020.09; Chemical Computing Group ULC, Montreal, QC, Canada). The crystal structure of CGL in a complex with globotriose (PDB ID 5F90_B) was used as a prototype when constructing a model of the spatial structure of MTL by comparative modeling. The structure of the complex of MTL with globotriose was obtained by the method of 3D-superposition with the complex of CGL with globotriose. The binding energy of globotriose with MTL and CGL was calculated using the GBVI/WSA ΔG function. The model structure was optimized using the Amber10:EHT force potential. The analysis of contacts in the complexes was carried out using the Ligand Interaction module of the MOE software.

2.3 | Cell culture

Burkitt's lymphoma (Raji, EB-1 and Daudi) cells were cultured at 37°C and 5% CO_2 in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

2.4 | Cell viability assay

The effect of lectins on cell viability was evaluated using MTS reduction into its formazan product.[26] The cells were cultured for 12 h in 96-well plates (6000 cells/well) in the corresponding media (100 μL/well) containing 10% FBS. The solution in culture media of CGL and MTL (100 μL) at various concentrations was added, and the cells were incubated for 48 h. Then, 20 μL of MTS reagent was added into each well and MTS reduction was measured 2–4 h later spectrophotometrically at 492 and 690 nm as background.

2.5 | Agglutination of tumor cells

Raji cells were cultured for 12 h in 96-well plates (6000 cells/well) in the corresponding media (50 μL/well) containing 10% FBS. Then 50 μL of lectin solution in media (CGL and MTL) at 100 μg/mL was added. After incubation for 1 h agglutination of Raji cells was observed using EVOS XL Core Cell Imaging System (Thermo Fisher Scientific, Bothell, WA, USA).

2.6 | Preparation of fluorescein isothioycanate (FITC)-labeled lectins

Lectins (1 mg CGL or 1 mg MTL) in 1 mL sodium bicarbonate buffer (0.1 M $NaHCO_3$, pH 9.0) were incubated with 50 μL of FITC solution (1 mg/mL in DMSO) for 8 h in darkness at 4°C. The reaction was stopped by adding NH_4Cl to a final concentration of 50 mM. Unconjugated FITC molecules were removed by gel filtration on a Sephadex G-25 column. The FITC-labeled lectins were then dialyzed for 12 h against PBS.

2.7 | Fluorescence confocal microscopy

To visualize lectin internalization, Raji cells were cultured in RPMI 1640 with various concentrations of FITC-labeled lectins for 24 h at 37°C and 5% CO_2 in a 12-well plate. After the treatments, the cells were washed twice with PBS, mounted on glass slides, and visualized by LSM 710 LIVE Axio Observer confocal microscope (Carl Zeiss GmbH, Germany). FITC was excited at 495 nm and detected at 520 nm. Cell nuclei were stained using a Hoechst 33342 fluorescent dye at a concentration of 5 M. Fluorescence was excited at 488 nm, and emission was recorded at 493–652 nm. Processing and subsequent analysis of cell images was performed using the ZEN 2011 software (Carl Zeiss GmbH, Germany).

2.8 | Cell cycle assay

The cell cycle was analyzed by flow cytometry using propidium iodide (PI) staining as described previously.[25] Briefly, cells, 1×10^6/well, in culture medium were treated with various concentrations of CGL (0–50 μg/mL) and MTL (0–100 μg/mL). After incubation, cells were washed with PBS by centrifugation at 1000 rpm for 5 min, and resuspended in 300 μL of PBS and 700 μL of cold ethanol (70%) was added dropwise while gently vortexing to fix the cells. After 1 h on ice cells were washed with PBS, resuspended in 250 μL of PBS and 5 μL of 10 mg/mL RNase A was added. Cells were incubated at 37°C for 1 h. Then, 10 μL of 1 mg/mL PI was added, and the cells were analyzed by flow cytometry using the NovoCyte flow cytometer system (Agilent, Santa Clara, CA, USA).

2.9 | Western blotting

Raji cells treated with CGL and MTL for 24 h were harvested, washed with ice-cold PBS, and treated with lysis buffer containing 50 mM Pipes/HCl (pH 6.5), 2 mM EDTA, 0.1% Chaps, 5 mM DTT 20 μg/mL leupeptin, 10 μg/mL pepstatin A, and 10 μg/mL aprotinin. The concentration of proteins in each lysate was determined using Bio-Rad Protein Assay Reagent (Bio-Rad Lab., Richmond, CA, USA) following manufacturer protocol. Then, 25 μg of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Lab., Richmond, CA, USA). The membranes were blocked with 5% skim milk in PBS (blocking buffer) for 1 h at 25°C and incubated with primary polyclonal antibodies (1:1000 dilution), directed against target proteins, in a blocking buffer overnight at 4°C. The membranes were incubated with the secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) for 1 h at 25°C. The signals were detected using Clarity Western ECL Substrate (Bio-Rad Lab., Richmond, CA, USA) following manufacturer protocol. Quantitation of western blotting was performed using Image Lab (Bio-Rad Lab.,

Richmond, CA, USA) and ImageJ software. MAPK activation was determined by analyzing the p- and t-MAPK (phosphorylated and total MAPK, respectively) signals in each sample and comparing the p-/t-MAPK ratios obtained from lectin-treated and control cells. All signals were normalized to total β-actin.

2.10 | Cell fractionation

The cells were seeded in T75 culture flasks (4×10^6 cells/bottle in 20 mL/bottle). The following day, the media was replaced with fresh media containing the lectins at the indicated concentrations. Cells were incubated for 48 h, harvested by scraping and the further separation of cytosolic, mitochondrial, and nuclear fractions was performed using the Cell Fractionation Kit (ab109719; Abcam, Cambridge, MA) as previously reported.[27] Additional homogenization of nuclear fractions was performed using the QIAshedder kit (QIAGEN, Hilden, Germany). Mitochondrial and cytosolic fractions were further concentrated using Amicon® Ultra-2 Centrifugal Filter device (Cat. No. UFC203024; Merck, Darmstadt, Germany). Further protein concentration determination as well as Western blotting analysis were performed as described above (see Section 2.9). Unfractionated treated and untreated cells were used as the total protein lysate fraction.

2.11 | Mitochondrial membrane potential assay

The loss of mitochondrial membrane potential (Δψm) was measured by flow cytometry technique using the mitochondrial membrane potential assay kit. Raji cells were seeded in 12-well plates (1×10^5 cells/well in 1 mL/well) and incubated overnight. Then, the media was changed to media (1 mL/well) containing lectins. After 24 h of incubation (37°C, 5% CO_2, in the dark) the cells were washed twice with PBS, resuspended in 100 μL of 2 μM TMRE, incubated for 1 h (37°C, 5% CO_2, in the dark) and applied for cytometry using NovoCyte flow cytometer system (Agilent, Santa Clara, CA, USA). CCCP (carbonyl cyanide 3-chlorophenylhydrazone) was used as a positive control.

2.12 | Detection of ROS generation

Raji cells (10,000 cells/well) were incubated for 1 h with lectins or hydrogen peroxide (200 μM) for 30 min. Then wells were washed twice with PBS and 5 μM 2′,7′-dichlorofluorescein diacetate in PBS was added and incubated for 30 min. The fluorescence intensity of 2′,7′-dichlorofluorescein, the oxidation product of 2′,7′-dichlorofluorescein diacetate, was detected at an excitation wavelength of 488 nm and an emission wavelength of 529 nm using a microplate absorbance reader.

2.13 | Soft agar colony formation assay

The soft agar colony formation assay was performed according to Borowicz et al.[28] with some modifications on Raji cells. Solutions of different lectin concentrations were prepared in 0.3% basal medium Eagle agar containing 10% FBS and 3 mL was added to a 6-well culture plate. After 30 min at room temperature, 1 mL of cell suspension (2×10^4 cells/mL) in 0.3% basal medium Eagle agar containing 10% FBS was placed on the top (final 8000 cells/well). The culture was maintained at 37°C and 5% CO_2 for 2 weeks and the cell colonies were scored using EVOS XL Core Cell Imaging System (Thermo Fisher Scientific, Bothell, WA, USA) and ImageJ software. Concentration leading to 50% inhibition of colony formation (INCC50) was calculated.

2.14 | Statistical analysis

Experimental results are presented as mean ± SD. Differences between means were evaluated by two-tailed Student's *t*-test, with $p < .05$ considered to be statistically significant.

3 | RESULTS

3.1 | Computer modeling

The crystal structure of CGL was established earlier and revealed a β-trefoil fold that dimerizes into a dumbbell-shaped quaternary structure.[29] Using the in silico homologous modeling approach, the structure of the carbohydrate-binding sites of lectins was predicted, and the mechanisms of binding of lectins to ligands were analyzed. The amino acid sequence of MTL (Uniprot ID A0A0G2UGT2) was analyzed using the MOE 2020.09 program (Molecular Operating Environment 2020.09), which showed that CGL lectin could be used as a prototype. The amino acid sequence identity of CGL and MTL is 83%,[19] which indicates the possibility of constructing a theoretical model of high accuracy. To elucidate the different activities of CGL and MTL against tumor cells, we compared the binding

energy of these lectins with Gb3; for this, the crystal structure of CGL complex with globotriose (PDB ID 5F90_B) was chosen as a prototype. A model of the spatial structure of MTL and its 3D superposition with the prototype CGL lectin in combination with Gb3 is shown in Figure 1.

The analysis of contacts in carbohydrate-binding sites of lectins was carried out using the Ligand Interaction module of the MOE program. Our results showed that CGL binds, besides the terminal monosaccharide residue of Gb3, with the adjacent monosaccharide via hydrogen bonds formed with asparagine in the first and second binding sites and glutamic acid in the third site (Figure 2A). MTL, unlike CGL, binds only to the terminal monosaccharide residue of Gb3 (Figure 2B). The binding energy of Gb3 with lectins MTL and CGL, calculated using the GBVI/WSA ΔG function, was −56.456 and −76.942 kcal/mol, respectively. This indicates a more efficient binding of CGL to the ligand. The model structure was optimized using the force potential of Amber10:EHT.

Alignment of the amino acid sequences of lectins (Figure 3) showed that GCL possesses asparagine and glutamic acid residues (marked in yellow) next to the carbohydrate-binding sites (marked in blue). These residues form hydrogen bonds with the second monosaccharide residue of Gb3 in the case of CGL but are absent in MTL. This makes the interaction between CGL and Gb3 stronger when compared to MTL. Thus, we speculate that CGL has more pronounced biological activity in cells expressing Gb3.

3.2 | Antiproliferative activity of lectins

To check our hypothesis, we studied the antiproliferative effect of lectins on Burkitt's lymphoma cells Raji, EB-1, and Daudi using the MTS method. Both lectins exhibited high activity on lymphoma cells (Figure 4). However, the antiproliferative effect of MTL was much weaker. The observed difference in antiproliferative activity was in line with our hypothesis of stronger CGL binding to the cellular surface. Raji cells were most sensitive to the antiproliferative actions of lectins and therefore were selected for further experiments.

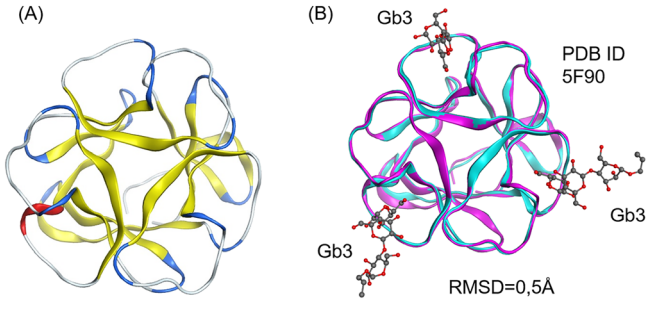

FIGURE 1 Computer modeling of lectin structure. (A) Theoretical model of the structure of MTL. (B) Superposition with the prototype crystal structure of CGL with Gb3. Protein structure is shown as a strip diagram in pink for MTL and turquoise for CGL. The standard deviation of all C-α atoms is 0.5 Å.

3.3 | Cell agglutination and internalization of lectins

Agglutination of Raji Burkitt's lymphoma cells by lectins was studied. For this, CGL and MTL at a concentration of 100 μg/mL were mixed with a suspension of lymphoma cells and the formation of agglutinates was assessed using a light microscope.

CGL strongly agglutinated Raji cells (Figure 5), which are known to have a high surface expression of terminal residues of α-Gal.[30] Thus, lectin-induced formation of agglutinates (clusters of cells glued together) was observed (Figure 5). Agglutination of tumor cells treated with MTL was significantly weaker, which is consistent with the results of molecular modeling.

Next, the internalization of lectins into Raji Burkitt's lymphoma cells has been studied. Cells were incubated in the presence of fluorescein-conjugated lectin and Hoechst 33342 nuclear dye. Cells were analyzed using LSM 710 LIVE Axio Observer confocal microscope (Carl Zeiss GmbH, Germany). After 24 h of incubation cell surface fluorescence was detected at a CGL concentration of 1.5 μg/mL (Figure 6). A strong dose-dependent intracellular fluorescence was observed in the cells exposed to CGL-FITC due to conjugate uptake, but at high concentrations almost all lectin was localized on the surface of the cell and agglutination of all cells was observed (Figure 6). In the case of MTL minimal internalization was found, as only a weak fluorescence was detected at a concentration of 12.5 μg/mL. Even at the highest concentrations tested, no agglutination of lymphoma cells was observed.

3.4 | Effect of lectins on the cell cycle of tumor cells

The effect of lectins on the cell cycle of Raji cells was also investigated using flow cytometry. Previously we have shown that treatment of Raji cells with CGL at a concentration of 20 μg/mL leads to arrest of the cell cycle in the G2/M phase.[25] Here, we examined the effect of MTL and incubated the cells with MTL at various concentrations in comparison with CGL. Then, cells were fixed, labeled

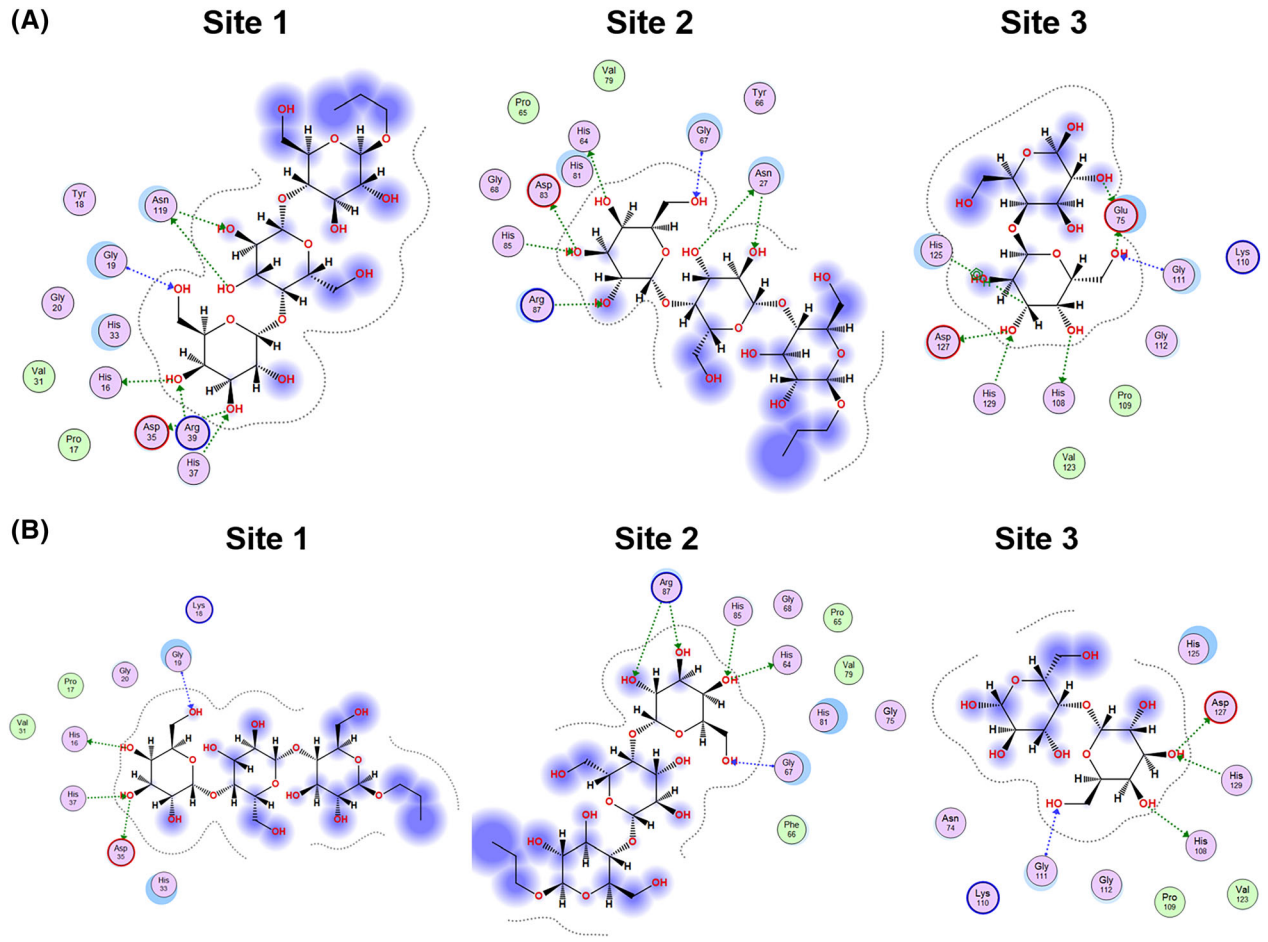

FIGURE 2 2D diagram of lectin contacts with ligand: (A) CGL contacts with Gb3. The total energy of binding of three sites with Gb3 is −76.942 kcal/mol; (B) MTL contacts with Gb3. The total energy of binding of three sites with Gb3 is −56.456 kcal/mol.

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Mytilus trossulus      AKI29293.1    MTTFLIKHKASGKYFHPKGGTSNPPNGTNLVLHSDIHERMYFQFEVVNERWRYIKHVASE 60
Crenomytilus graynus   AEY80387.1    MTTFLIKHKASGKFLHPYGGSSNPANNTKLVLHSDIHERMYFQFDVVDERWGYIKHVASG 60

                       AKI29293.1    KIVHPFGGKADPLNGTNMVLHQDRHDRALFAMDFFNDNIRHKGGKYIHPKGGSKNPSNGN 120
                       AEY80387.1    KIVHPYGGQANPPNETNMVLHQDRHDRALFAMDFFNDNIMHKGGKYIHPKGGSPNPPNNT 120

                       AKI29293.1    LTVMHGDEHGAMEFIFVSPKNKDKRVLVYA   150
                       AEY80387.1    ETVIHGDKHAAMEFIFVSPKNKDKRVLVYA   150
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FIGURE 3 Alignment of amino acid sequences of MTL and CGL. Carbohydrate-binding sites are marked in blue, asparagine and glutamic acid residues, which determine a stronger interaction with Gb3—in yellow.

with propidium iodide, and further analyzed with flow cytometry technique. Our results indicated that MTL is also able to inhibit the progression of the cell cycle in the phase of preparation for mitosis (G2 phase) and/or mitosis (M phase), however, CGL was more effective (Figure 7A,B).

Moreover, using western blotting analysis we could show that both lectins inhibited key proteins involved in cell cycle progression in the G2/M phase. Thus, treatment of Raji cells with CGL decreased the total expression of cdc2, while increasing the level of its phosphorylated form (p-cdc2) at Tyr15, which is known to result in the inactivation of this protein.[31] Simultaneously, cyclin B1 synthesis was inhibited. All this together resulted in the blocking of the cyclin B1/cdc2 complex formation and cell cycle arrest in the G2/M phase. In addition, MTL induced the accumulation of p-cdc2, but this effect was weaker when compared to CGL (Figure 7C–F). Moreover, MTL did not affect the expression of total cdc2 and cyclin B1. Hence, the effect of MTL on the cell cycle of Raji cells was less pronounced when compared to CGL. These results are in line with the suggestions done by

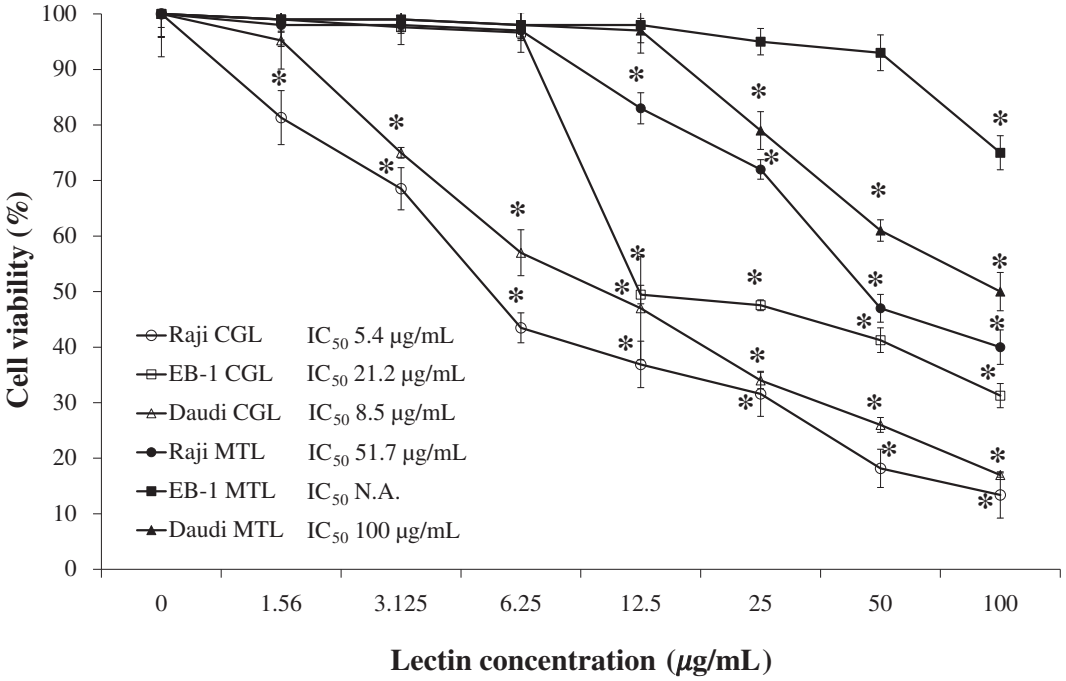

FIGURE 4 Antiproliferative effect of CGL and MTL on Burkitt's lymphoma cells was established using the MTS method. N.A.—not achieved. Data are represented as means ± SD as determined from triplicate experiments. Student's *t*-test was used to evaluate the data with the significance level $*p \leq .05$.

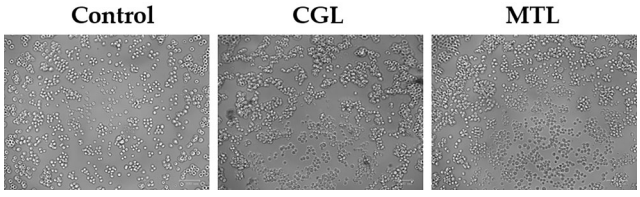

FIGURE 5 Agglutination of Raji cells. The cell suspension was incubated in the presence (100 μg/mL) or absence (control) of lectins. The results of agglutination were detected using a light microscope after 1 h.

computer modeling, which predicted a weaker binding of MTL to Gb3 resulting in a less pronounced biological effect of this lectin.

3.5 | Effect of lectins on mitochondria damage

We further investigated the effects of the lectins on mitochondria-associated proteins and could show that CGL induced a release of apoptosis-inducing factor (AIF) and cytochrome C from mitochondria to the cytoplasm as well as activation of caspase-3 (Figure 8A,B). The effects of MTL were less pronounced and no activation of caspase-3 was observed (Figure 8C,D).

The effect of lectins on the mitochondria damage was further confirmed by analysis of the mitochondrial membrane potential (Δψm) of the exposed cells. Lymphoma cells were incubated with lectins for 24 h and Δψm was evaluated (Figure 8E). Both lectins induced a drop-down of Δψm in a concentration-dependent manner, however CGL was more effective (Figure 8F).

3.6 | Effect of lectins on ROS production

Reactive oxygen species (ROS) are one of the important secondary messengers in the transmission of signals in the cell, which regulate many biological processes. An increase in the intracellular ROS level to highly toxic values is the mechanism of cell death induction. The effect of lectins on the intracellular ROS level was studied. For this, cells treated with lectins at various concentrations were stained with 2′,7′-dichlorofluorescein diacetate (DCFDA) and the level of fluorescence was determined using a fluorescence microplate reader. In this experiment, both lectins insignificantly increased the concentration of ROS in Raji cells (Figure 9). These data suggest that the effect of lectins on the proliferation of tumor cells is not mediated by ROS.

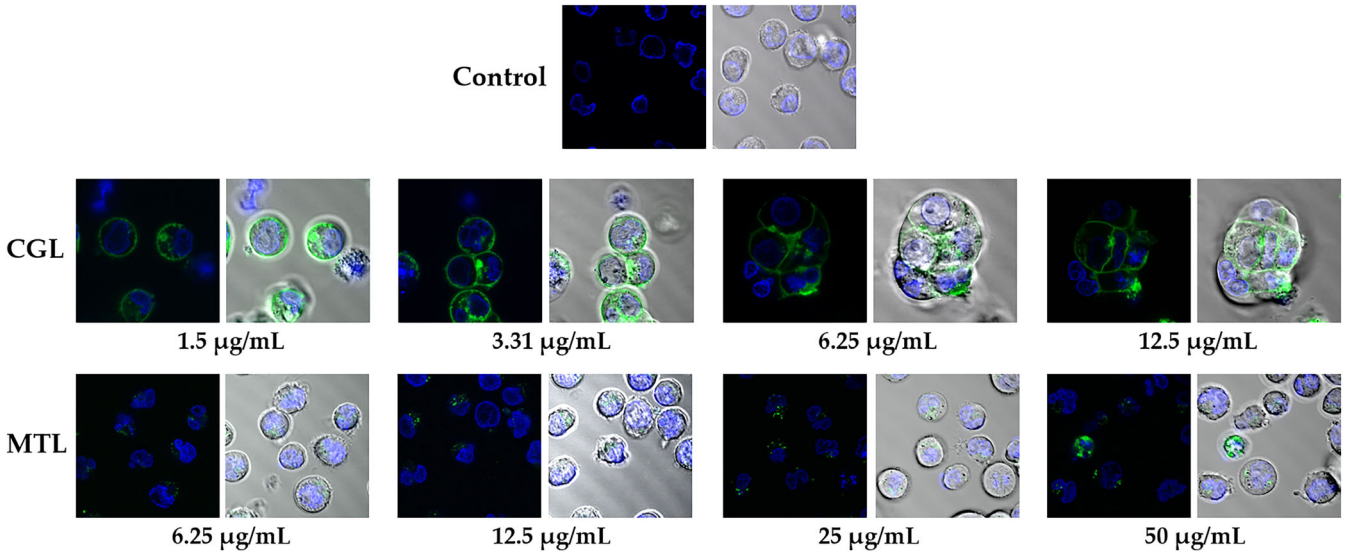

FIGURE 6 Internalization of lectins into Raji cells. Cells were incubated in the presence or absence (control) of FITC-conjugated lectins (green). Hoechst 33342 was used as a nuclear dye (blue). The results of internalization were detected using a confocal microscope after 24 h.

3.7 | Activation of MAPK pathway by lectins

MAPK (mitogen-activated protein kinases) signaling pathways play an important role in cell growth and differentiation, cell cycle progression, as well as cell death. Therefore, we studied a lectin-mediated expression and activation of the major kinases of the MAPK signaling pathway and could show that CGL activates several of them. Thus, at the highest concentration, CGL activated the key ERK1/2 kinase of the classical MAPK pathway regulated by extracellular signals in Raji cells (Figure 10A,B). In addition to ERK1/2, CGL induced phosphorylation of stress-activated kinase p38. In this experiment, MTL showed a similar effect (Figure 10C,D), inducing phosphorylation of ERK1/2, but less active compared to CGL. Unlike CGL, MTL did not induce phosphorylation of stress-activated kinase p38 but activated JNK1/2.

3.8 | Spontaneous formation and growth of cell colonies

The effect of CGL and MTL on the spontaneous formation and growth of colonies of human tumor Raji cells was studied using the soft agar assay. Lectins inhibited the spontaneous formation and growth of human lymphoma cell colonies with different efficacy. Thus, CGL was the most effective as significantly inhibited the formation and growth of the colonies with INCC50 2.9 μg/mL (Figure 11A,B). MTL was less effective and 50% inhibition of colony formation was achieved at a concentration of 29.6 μg/mL (Figure 11C,D).

4 | DISCUSSION

The medical potential of lectins lies in the area of diagnostics and treatment of cancer,[32] as well as modulators of immune processes[33] and prevention of viral infections.[34] A number of plant-derived lectins are able to induce autophagy in various tumor cells by modulating the BNIP-3, ROS-p38-p53, and Ras-Raf, as well as PI3K-Akt signaling pathways.[32] Exogenous expression of some lectins from marine sources induces tumor cell apoptosis via the activation of PRMT5-E2F-1 pathway,[35] on the other hand, the cytotoxicity of many lectins reduces their applicability in clinical practice. For this reason, lectins with reduced unspecific cytotoxicity are of particular interest. Thus, an ABL lectin isolated from the edible mushroom *Agaricus bisporus* exhibits potent antiproliferative activity against epithelial tumor cells, however, it does not exhibit cytotoxic activity against normal cells.[36] This lectin recognizes TF antigen (Thomsen–Friedenreich antigen), a disaccharide derived from Galβ1-3GalNAc which is a well-known cell surface marker of neoplastic cells. BEL, a lectin with similar properties, was isolated from another edible mushroom, *Boletus edulis*.[37] Most lectins, which are considered as candidates for medical use, including ABL and BEL, exhibit specificity for rather common β-linked carbohydrates. Therefore, lectins that have unusual ligand specificity and exhibit a cytotoxic effect against tumor cells are of particular interest for research. For example, lectins MytiLec and CGL

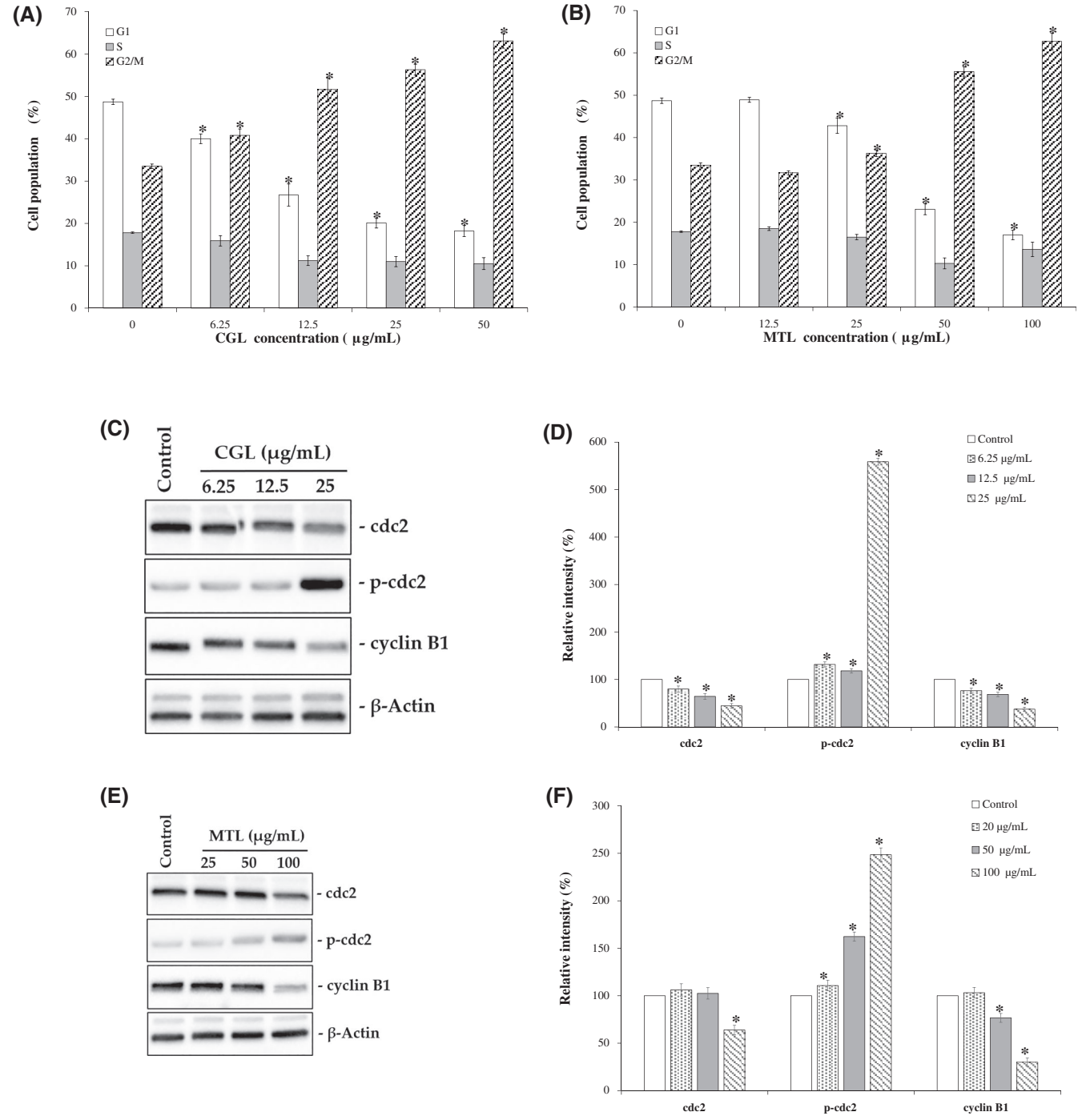

FIGURE 7 Influence on the cell cycle of Raji Burkitt's lymphoma cells. (A, B) Effect of CGL and MTL established by flow cytometry after PI staining. Effect of (C, D) CGL and (E, F) MTL on the cell cycle checkpoints evaluated by western blotting. Data are represented as means ± SD as determined from triplicate experiments. Student's *t*-test was used to evaluate the data with the significance level $*p \leq .05$.

bind to Gb3, a cell surface marker of some tumor cells, such as Raji Burkitt's lymphoma cells, and exhibit anti-proliferative activity.[20,25] Another representative of Gb3-specific lectin is SAL originally isolated from *Silurus asotus*. This lectin binds to Gb3 on the surface of lymphoma cells, inducting their shrinkage by 10%.[38] Lectin activity was in all cases inhibited by the addition of α-galactosides. This proves that the interaction of lectins with Gb3 plays a key role in their cytotoxic effect on tumor cell lines.

In silico modeling of proteins helps make predictions and paves the way to the creation of new, more advanced protein molecules with the required properties.[39] Protein 3D structure can be predicted based on amino acid sequence homology. The goal of homology

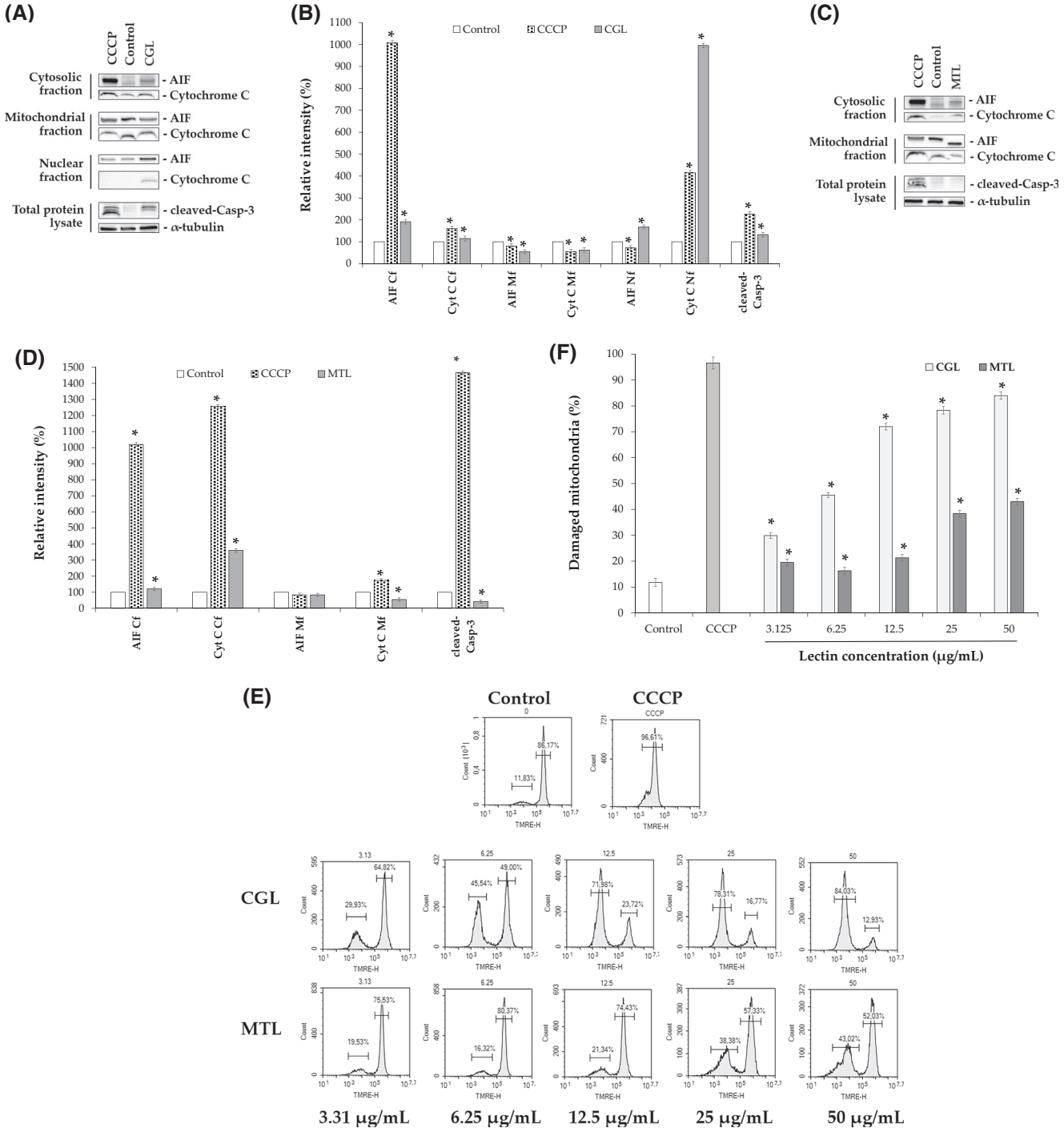

FIGURE 8 Effect of lectins on the mitochondria of Raji tumor cells established by (A–D) western blotting and (E, F) flow cytometry. CCCP (carbonyl cyanide 3-chlorophenylhydrazone) is a positive control that suppresses the ability of mitochondria to carry out oxidative phosphorylation. Data are represented as means ± SD as determined from triplicate experiments. Student's *t*-test was used to evaluate the data with the significance level $*p \leq .05$.

or comparative protein modeling is to generate a suitable three-dimensional model for a protein of unknown experimental structure (i.e., target) through sequence similarity to known structure (i.e., templates) present in databases. To date, this approach is one of the most accurate methods available. The overall range of accuracy achieved by comparative models is similar to nuclear magnetic resonance spectroscopy or X-ray crystallography techniques.[39]

Primary structure analysis showed that MTL exhibits a high degree of homology with CGL.[19] The high identity of the amino acid sequences indicates the possibility of

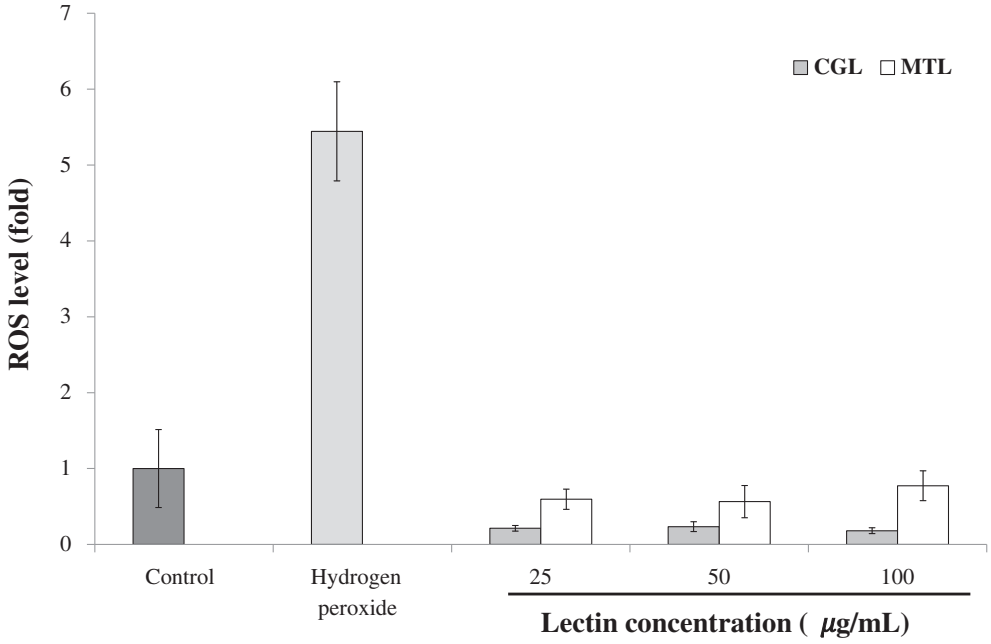

FIGURE 9 Effect of lectins on ROS production in Raji tumor cells. Cells were treated with various concentrations of lectins and stained with DCFDA. The level of fluorescence was determined using a fluorescence microplate reader.

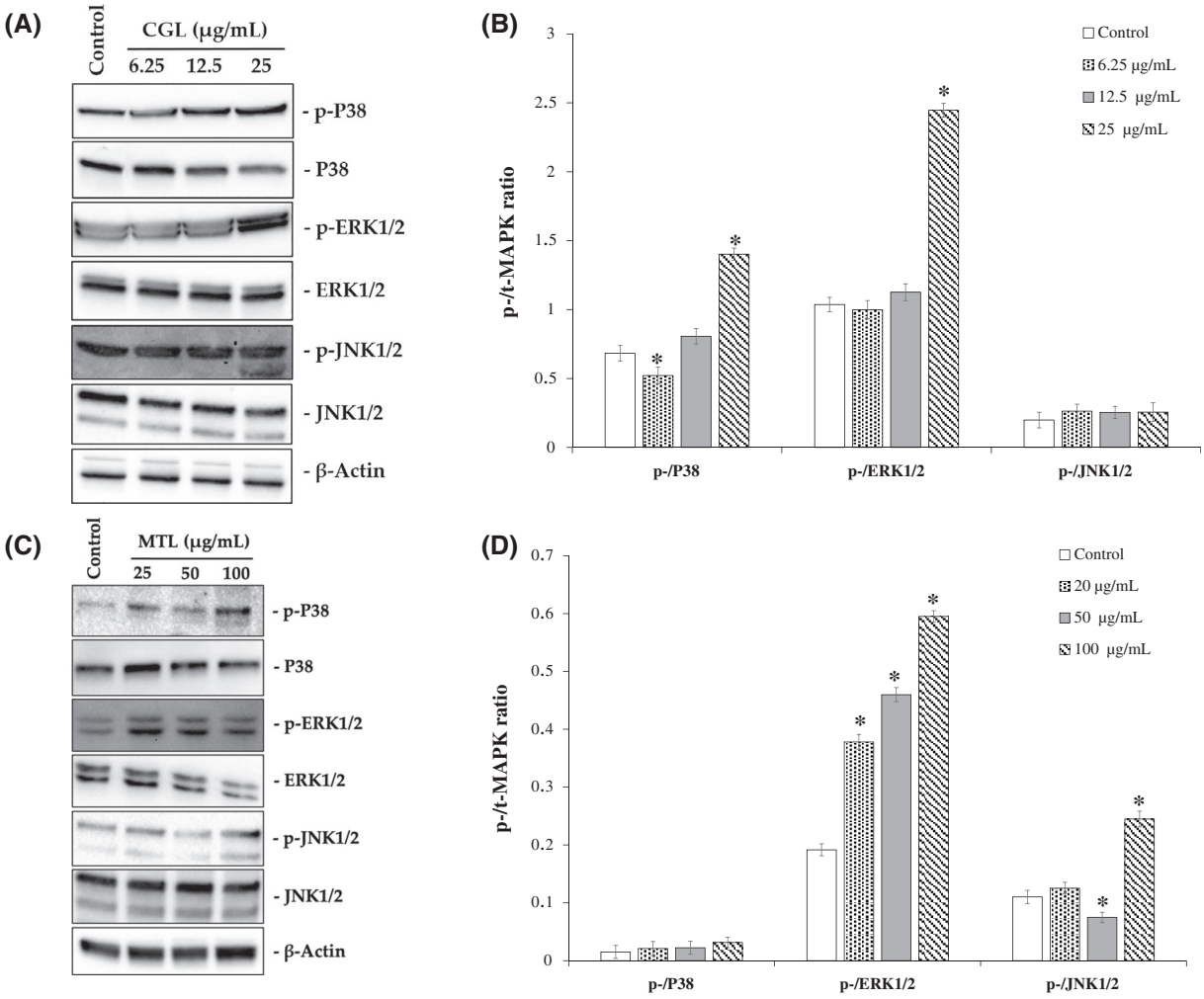

FIGURE 10 Effect of (A, B) CGL and (C, D) MTL on the MAPK signaling pathway in Raji tumor cells evaluated by western blotting. Data are represented as means ± SD as determined from triplicate experiments. Student's *t*-test was used to evaluate the data with the significance level $*p \leq .05$.

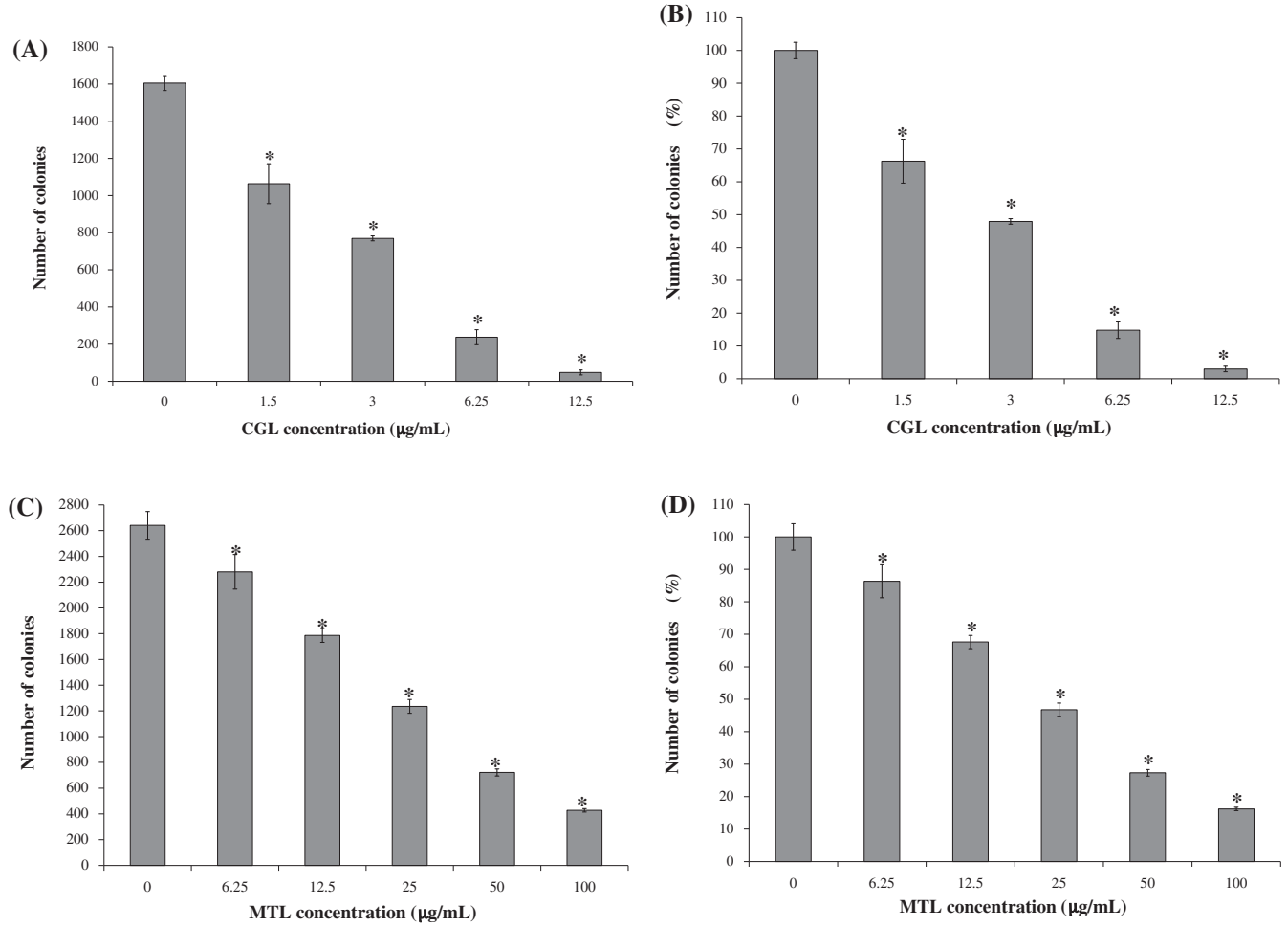

FIGURE 11 Effect of (A, B) CGL and (C, D) MTL on colony formation of Raji tumor cells presented as (A, C) absolute count and (B, D) the percentage of control. Data are represented as means ± SD as determined from triplicate experiments. Student's *t*-test was used to evaluate the data with the significance level $*p \leq .05$.

constructing a theoretical model of high accuracy. Therefore, the crystal structure of the CGL complex with Gb3 (PDB ID 5F90_B) was chosen as a prototype (Figure 1). Analysis of contacts in the carbohydrate-binding sites of lectins showed that in the case of CGL, Gb3 binds to the lectin not only with the terminal monosaccharide residue but also with the next monosaccharide via hydrogen bonds due to asparagine residues in the first and second binding sites and a glutamic acid residue in the third site (Figure 2A). These data correspond well with previous experiments on establishing CGL structure by X-ray crystallography.[29] At the same time, MTL binds exclusively to the terminal monosaccharide residue of Gb3 (Figure 2B). The binding energy of MTL and CGL is −56.456 and −76.942 kcal/mol, respectively, indicating more efficient binding of CGL to the ligand. Since molecular recognition of terminal carbohydrates on the surface of tumor cells is a key point for the manifestation of the biological effects of lectins, we assumed that CGL and MTL may possess different activities against tumor cells which should correlate with the binding energy of these lectins to carbohydrate ligands.

Our assumption was confirmed by a series of experiments. We compared the effect of CGL and MTL on various cell lines of Burkitt's lymphoma, which highly express Gb3 on their surface.[40] Both lectins were able to inhibit the proliferation of lymphoma cells, however, CGL activity was much stronger confirming a correlation of the biological effect with the structure and binding energy of the carbohydrate-binding site.

One of the common features of lectins is the agglutination of cells when lectin binds carbohydrate receptors on the surface of two adjacent cells. Moreover, it can lead to internalization of lectin and thus trigger different cell signals. We observed that both lectins were able to agglutinate (Figure 5) and internalize (Figure 6) Raji cells, but the effect in the presence of CGL was stronger which was in line with molecular modeling results. It is possible that

by entering the cell, CGL activates a number of signaling pathways such as caspase activation, MAPK, and G2M checkpoints regulation leading to cell cycle arrest and apoptosis. Another member of mytilectin family—MytiLec, agglutinated and internalized Ramos Burkitt's lymphoma cells, which also highly express Gb3.[41] Sunflower mannose-binding lectin Helja was able to internalize into the cell of fungi *Sclerotinia sclerotiorum* leading to cell death,[42] and internalization of lectin from *Ibacus novemdentatus*-induced apoptosis of mammalian cancer cells.[43]

Previously we showed that CGL was able to arrest the cell cycle of Raji cells in the G2/M phase.[25] The same effect we observed for MTL in the present study. Inhibition of cycle progression in the same phase is also observed in the case of a number of chemotherapy drugs used to combat various types of cancer.[44–46] This effect may indicate the ability to stop or inhibit the growth of tumor cells without a pronounced cytotoxic effect. To analyze the details of lectin-induced cell cycle arrest we evaluated the activation of main checkpoints. Indeed, both lectins were able to block the cyclin B1/cdc2 complex formation (Figure 7), which is one of the checkpoints in the progression of the G2/M phase. Cyclin B1 is a member of a family of proteins that activate specific cyclin-dependent kinases required for cell cycle progression. The entry of all eukaryotic cells into mitosis is regulated by cdc2 activation during the G2/M transition. This process is controlled in several steps, including cyclin B1 binding and cdc2 phosphorylation at Thr161.[47] However, a critical regulatory step in cdc2 activation during progression to mitosis is the dephosphorylation of cdc2 at Thr14 and Tyr15 by cdc25C.[48] Phosphorylation at Thr14 and Tyr15 leads to inhibition of cdc2.[31] Cdc25C is a protein phosphatase responsible for dephosphorylation and activation of cdc2.[49] Checkpoint kinase Chk1 phosphorylates cdc25C at Ser216 in response to DNA damage.[50] Chk1 activation involves phosphorylation at Ser317 and Ser345 by ATM/ATR followed by autophosphorylation of Ser296.[51] Activated Chk1 can inactivate cdc25C via phosphorylation at Ser216, blocking cdc2 activation and transition to mitosis.[52]

One of the key mechanisms of antiproliferative action of substances in human tumor cells is the damage of mitochondria, which leads to the release of apoptotic factors.[53] Mitochondrial dysfunction is involved in the induction of apoptosis and is even considered as a major event in the apoptotic pathway.[53] Indeed, the discovery of mitochondrial transitory permeability has been shown to induce depolarization of the transmembrane potential, release of apoptogenic factors, and loss of oxidative phosphorylation.[54] This is one of the known mechanisms of caspase-independent cell death. Cytochrome C and AIF are the most studied mediators of caspase-independent cell death.[55] Following leakage from mitochondria into the cytoplasm, AIF localizes in the nucleus, where it can mediate chromatin condensation and, as a result, cell death. This process does not require active caspases.[56] Cytochrome C is an important factor in the activation of caspase-3 and its increase in the cytosol may indicate mitochondrial damage. Exposure to CGL resulted in a release of AIF and cytochrome C from mitochondria into the cytoplasm (Figure 8A). In addition, there was an increase in the amount of AIF and cytochrome C in the nuclear fraction. Activation of caspase-3 was also shown. For MTL, the effect was less pronounced (Figure 8C). Here, the release of AIF and cytochrome C from mitochondria into the cytoplasm was also found, while no decrease of the AIF level in mitochondrial fraction was observed. No changes in the level of AIF and cytochrome C in the nuclear fraction were found in the cells exposed to MTL and no activation of caspase-3 was detected. Mitochondrial damage is also accompanied by a change in the mitochondrial membrane potential ($\Delta\psi m$). The effect of lectins on $\Delta\psi m$ was confirmed by flow cytometry using the mitochondrial-specific cationic dye TMRE. TMRE fluorescence depends on the depolarization of the mitochondrial membrane and the latter can be detected as a decrease in TMRE signal intensity. Remarkably, treatment of lymphoma cells with CGL leads to mitochondrial damage (Figure 8E), accompanied by a change in $\Delta\psi m$ and the release of apoptotic factors. MTL induced drop-down of $\Delta\psi m$ in Raji cells less effectively. Thus, CGL was more toxic to Raji cells and induced apoptosis, while MTL did so to a lesser extent. Similar effects were observed in the human monocytic leukemia cell line THP-1 treated with *Olneya tesota* PF2 lectin, which is known to trigger apoptosis and change the $\Delta\psi m$.[57] Other examples are *Viscum album* agglutinin which in hepatocellular carcinoma Hep3B cells resulted in a loss of $\Delta\psi m$,[58] as well as sialic acid-binding lectin from *Rana catesbeiana* oocytes which induced a caspase-dependent apoptosis in Jurkat cells following mitochondrial perturbation.[59]

Recent research suggests that the ERK1/2 pathway alone can activate both JNK1/2 and p38.[60] Some mannose-binding proteins inhibit cell proliferation by activating these pathways.[61,62] These stress-activated kinases play a role in tumor suppression, apoptosis, termination of cell differentiation, and autophagy.[63] Thus, CGL binding to Gb3 of Raji cells promotes both the classical MAPK pathway (ERK1/2) and the stress-activated MAPK pathway p38. MTL also induced ERK1/2 activation, but less effectively, and activated JNK1/2 rather than the p38 pathway. CGL-induced phosphorylation of the ERK1/2 pathway can lead to cell cycle arrest. It is known that ERK1/2 overexpression can lead to arrest of

the cell cycle in the G2/M phase.[64] Significantly increased ERK1/2 activity in the G2/M phase blocks the activity of cyclin B/cdc2 complex formation in three ways. First, ERK1/2 activity increases the activity of Wee1 kinase, which phosphorylates cdc2 at Tyr15, inactivating the cyclin B/cdc2 complex. Second, ERK activity increases the activity of Chk1 kinase, which induces cytoplasmic retention and degradation of cdc25. When cdc25 phosphatase is not translocated into the nucleus, it cannot dephosphorylate and therefore activate cdc2. Finally, ERK1/2 activation leads to a decrease in cdc25 phosphatase expression due to its degradation in the cytoplasm, and the expression of cdc25 is known to reverse upon inhibition of ERK1/2 activity.[63,64] Perhaps, we are seeing a similar pattern but this requires further evaluation.

The main properties of all malignant tumors include an increased proliferation rate, loss of the ability to fully differentiate, and undergo apoptotic death, as well as invasive growth and metastatic potential. Inhibition of the clonogenicity can serve as an indicator for the potential in vivo efficacy of the anticancer drug.[65] Spontaneous colony formation and growth is a cell survival assay, based on the capacity of a single cell to grow into colonies after treatment with compounds tested. The ability of lectin to block colony formation may be due to its binding with surface glycans of tumor cells leading to agglutination resulting in the loss of cells' ability to form colonies.[66] In line with this, our data showed that both lectins inhibited the formation and growth of Raji cells colonies compared to the untreated control cells in a dose-dependent manner (Figure 10). Similar activity was observed for *Musa acuminata* lectin, which mitigated the cell proliferation, colony formation, cell migration, arrested the cell cycle in the G2/M phase, and induced apoptosis.[67]

5 | CONCLUSION

In conclusion, CGL and MTL are capable of inhibiting the proliferation of human lymphoma cells to a varying degree. Both lectins suppress the formation of colonies and affect the cell cycle of tumor cells. The effect is primarily mediated by damaging mitochondria and blocking key points of the cell cycle of tumor cells. It has been suggested by computer modeling that the different activities of CGL and MTL in relation to the lymphoma cells may be due to the energy value of the binding of these lectins to carbohydrate ligands on the cell surface. This indicates that molecular recognition of residues of terminal carbohydrates on the surface of tumor cells is a key moment for the manifestation of the biological activity of lectins.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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