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Apoptotic Effects Sulfated Polysaccharides of *Caulerpa racemosa* Extract on Colorectal Cancer Cells through Caspase-3

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Background: Colorectal cancer originates from progressive genetic alterations in colorectal epithelial cells. While current therapies (surgery, radiotherapy, and chemotherapy) remain cornerstone treatments, chemotherapy often induces systemic toxicity, adverse effects, and acquired resistance. Sulfated polysaccharides (SPs) from the green alga *Caulerpa racemosa* demonstrate higher sulfate content than red algal derivatives, correlating with enhanced bioactivity. Despite their potential, SPs from green algae remain understudied compared to brown and red algal counterparts. This study evaluated the anticancer potential of *C. racemosa* SPs against colorectal cancer through viability inhibition and apoptosis induction.

Materials and methods: SPs were extracted via microwave-assisted extraction (MAE) and characterized using iodine testing and FTIR spectroscopy. Cytotoxicity was evaluated in WiDr colorectal cancer cells using MTT assay after 24-hour exposure. Apoptotic mechanisms were investigated through *in silico* molecular docking targeting Caspase-3 activation.

Results: SPs were confirmed by a blue color change and FTIR absorption at 1232 cm^{-1}. At 100 µg/mL, low toxicity was observed based on abundant formazan crystals. Concentrations of 200-400 µg/mL showed predominant viable cells, whereas 500 µg/mL caused significant growth inhibition and cell death. *In silico* analysis demonstrated that SPs may induce apoptosis by Caspase-3 activation.

Conclusion: SPs of *C. racemosa* inhibit colorectal cancer cell viability at a concentration of 500 µg/mL and may induce apoptosis via Caspase-3 activation.

Keywords: *apoptosis, Caulerpa racemosa, colorectal cancer, sulfated polysaccharides*

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Introduction

Colorectal cancer, characterized by the progressive accumulation of genetic mutations in colorectal epithelial cells, represents a significant global health burden.[1] Recent 2024 data from the International Agency for Research on Cancer (IARC) ranks colorectal cancer as the third most prevalent malignancy worldwide, with Indonesia reporting 34.189 cases (8.6% of national cancer incidence) in 2020.[2,3] Current treatment modalities include surgery, radiotherapy, and chemotherapy.[4] Current therapeutic approaches, including surgical resection, radiotherapy, and chemotherapy, face substantial limitations: surgical interventions show reduced efficacy in metastatic disease, radiotherapy demonstrates diminished effectiveness with increasing tumor volume, while chemotherapy suffers from nonspecific cytotoxicity, financial constraints, and variable induction of programmed cell death.[4-6]

The apoptotic pathway, particularly through Caspase-3 (cysteine-aspartic acid protease-3) activation, presents a critical therapeutic target. As the primary executioner protease in apoptosis, Caspase-3 mediates proteolytic cleavage of essential cellular components, initiating controlled cell dismantling.[7-9] This mechanism has spurred interest in natural compounds with apoptotic potential, particularly marine-derived bioactive molecules that offer enhanced specificity and reduced toxicity compared to conventional chemotherapeutics.[10] Marine algae, especially under environmental stress conditions, produce unique secondary metabolites absent in terrestrial flora, with *Caulerpa racemosa* emerging as a promising but underexplored source.[11] This green alga contains distinctive bioactive compounds including siphonaxanthin, phenolics, and sulfated polysaccharides (SPs) with demonstrated anticancer properties.[12]

While terrestrial plant-derived compounds (flavonoids, alkaloids, terpenoids) have shown anticancer potential, their clinical translation is often limited by poor aqueous solubility and bioavailability.[13-14] In contrast, *Caulerpa*-derived SPs exhibit superior physicochemical properties and bioactivity. Notably, *C. racemosa* SPs contain higher sulfate content than red algal counterparts, with sulfate enrichment correlating with enhanced biological activity.[12,15] The structural complexity and underexplored nature present unique opportunities for novel anticancer development. Thus, this study investigated the potential of *C. racemosa* SPs to inhibit the viability of colorectal cancer cell and induce Caspase-3-mediated apoptosis, addressing the critical need for more effective therapies against this globally prevalent malignancy.

Materials and methods

C. racemosa Samples Collection and Preparation

Fresh *C. racemosa*, specimens (including stolons and fronds) were collected from Laikang Village, Takalar Regency, Sulawesi Selatan Province. After thorough washing with seawater followed by distilled water, samples were shade-dried at 25±2°C for 14 days until constant weight was achieved. The dried material was pulverized using a sterile blender and stored in airtight containers at -20°C until extraction.[16]

C. racemosa Extraction with Microwave Assisted Extraction (MAE) Method

As much as 33.35 g powdered algal material was homogenized with 0.1 N HCl with 1:20 w/v ratio using continuous magnetic stirring at 500 rpm for 20 minutes. The mixture was subjected to microwave irradiation using a closed-vessel microwave extraction system (Mars 6, CEM Corporation, Charlotte, NC, USA) operating at 450W for 8 minutes with temperature maintained at 60±5°C through infrared sensors. The resulting suspension was immediately filtered through Whatman No. 1 filter paper under vacuum. The filtrate underwent sequential purification beginning with 2% $CaCl_2$ precipitation at 4°C for 24 hours to remove proteins, followed by centrifugation at 6,000×g for 15 minutes at 4°C using a refrigerated centrifuge. The supernatant was then mixed with 96% ethanol in a 1:2 v/v ratio and stored at -20°C for 12 hours to precipitate polysaccharides. The precipitate was collected by centrifugation 4,000×g for 10 minutes), washed twice with 70% ethanol, and dried in a vacuum oven at 42°C for 48 hours to obtain the final SPs extract. The extraction yield was determined gravimetrically and expressed as percentage of initial algal dry weight.[17-20]

Qualitative Test of Polysaccharide Content

Ten mg *C. racemosa* extract was dissolved in 5 mL distilled water, mixed with 2 drops of Lugol's iodine (Cat. No. I7011; Sigma-Aldrich, St. Louis, Missouri, USA). The SPs presence was confirmed by blue chromogen formation.[20]

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Fourier Transform Infrared (FTIR) Analysis of C. racemosa Extract

Dried extract (1 mg) was mixed with KBr (100 mg, FTIR grade) and pressed into pellets. Spectra were acquired (32 scans, 4 cm^{-1} resolution) from 4000-400 cm^{-1} using FTIR (Shimadzu IRPrestige-21, Kyoto, Japan) equipped with deuterated L-alanine doped triglycine sulfate (DLATGS) detector. Sulfate ester bonds were identified by S=O stretching at 1232 cm^{-1} and C-O-S bending at 820 cm^{-1}.[21]

in vitro Cytoxicity and Selectivity Assay

Complete culture medium was prepared by supplementing Dulbecco's modified eagle medium (DMEM) (Cat. No. 11965092; Gibco™, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Cat. No. 16000044; Gibco™), 1% penicillin-streptomycin (Cat. No. 15140122; Gibco™), and 1% amphotericin B (Cat. No. 15290018; Gibco™). WiDr colorectal cancer cells were thawed, cultured in complete medium, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 until reaching confluency. Cells were then detached using 0.25% trypsin-EDTA, counted by trypan blue exclusion, and seeded into 96-well plates at a density of 5×10^3 cells per well.[22-24]

C. racemosa extract powder was dissolved in Tris-HCl buffer (Cat. No. T5941; Sigma-Aldrich) to prepare a 1000 µg/mL stock solution, which was serially diluted with complete medium to obtain final concentrations of 100, 200, 300, 400, and 500 µg/mL. Untreated cells cultured in complete medium served as the negative control. Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by measuring the reduction of MTT to formazan crystals in metabolically active cells. After incubation with the extract, formazan crystals were solubilized in dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using an ELISA reader (Bio-Rad Model 680, Hercules, CA, USA) to determine cell viability.[25-27]

Apoptosis Analysis: in silico Molecular Docking

Molecular docking analysis was conducted to evaluate the potential interaction between SPs from *C. racemosa* and Caspase-3 (PDB ID: 1PAU), a key enzyme in the apoptosis pathway. The 3D structures of D-galactose-6-sulfate (PubChem CID: 42628615), D-glucose-6-sulfate (PubChem CID: 21123007), and D-mannose-6-sulfate (PubChem CID: 101110136) were obtained from the PubChem database and prepared for docking using Open Babel.

The structure of Caspase-3 was retrieved from the Protein Data Bank and prepared by removing water molecules and ligands. The docking procedure was carried out using AutoDock Vina via PyRx, with the grid centered on the native ligand binding site. The native ligand Ac-DEVD-CHO (Acetyl-Aspartic acid-Glutamic acid-Valine-Aspartic acid-Aldehyde), a synthetic peptide inhibitor of Caspase-3, was used as a native ligand in the molecular docking study and was used as a reference control for comparison. Docking results were analyzed and visualized using BIOVIA Discovery Studio to identify interaction types and binding sites.[28]

Results

Sulfated Polysaccharides Were Isolated and C. racemosa Extract Inhibited WiDr Cell

The MAE of *C. racemosa* yielded a white powdered extract containing SPs, as confirmed by qualitative analysis. The characteristic colorimetric transition from clear to blue upon iodine addition demonstrated the presence of polysaccharide components. FTIR spectroscopic analysis revealed distinct vibrational signatures of sulfate groups, with a prominent absorption band at 1232 cm^{-1} corresponding to S=O stretching vibrations (Figure 1). Comparative analysis with fucoidan standard (1265 cm^{-1}) confirmed the sulfated nature of the extracted polysaccharides. Additional absorption bands in the 1260-1220 cm^{-1} and 1200-1050 cm^{-1} regions indicated the presence of fucose residues, characteristic

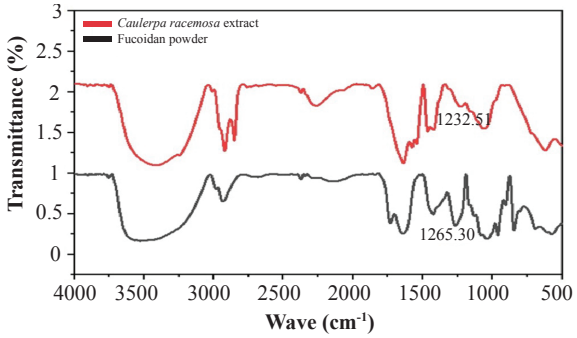

Figure 1. Comparative FTIR spectral analysis of fucoidan standard and *C. racemosa* SPs extract. Characteristic absorption bands at 1265.30 cm-1 (fucoidan) and 1232.51 cm-1 (*C. racemosa* extract) correspond to S=O stretching vibrations of sulfate esters, confirming the presence of SPs in both samples. Additional peaks between 1200-1050 cm-1 indicate fucose-containing polysaccharide structures.

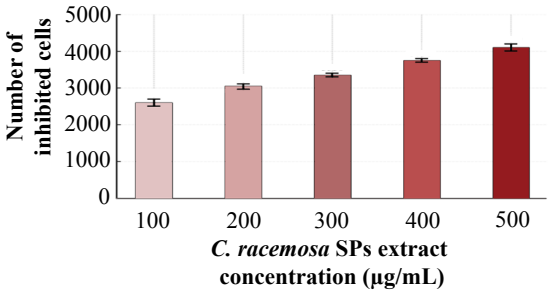

Figure 2. *C. racemosa* SPs extract inhibited the proliferation of WiDr colorectal cancer cells in a concentration-dependent manner. Cells were treated with SPs at concentrations ranging from 100-500 µg/mL for 24 hours, and cell viability was assessed using the MTT assay. Data are presented as mean ± SD.

of SPs structures. These findings collectively verify the successful isolation of SPs from *C. racemosa*, which are structurally composed of sulfated monosaccharides including D-galactose-6-sulfate, D-glucose-6-sulfate, and D-mannose-6-sulfate.[29-30]

The SPs extract exhibited concentration-dependent growth inhibition of human colorectal adenocarcinoma WiDr cells (Figure 2). Treatment with 100 µg/mL extract resulted in 2600±100 inhibited cells, while 200 µg/mL led to 3050±86.6 inhibited cells. At 300 µg/mL and 400 µg/mL concentrations, the number of inhibited cells increased to 3350±50 and 3750±50, respectively. The highest inhibition was observed at 500 µg/mL, with 4100±100 inhibited cells out of a total of 5000. Dose-response analysis calculated an IC_{50} value of 32.46 µg/mL, demonstrating potent cytotoxic activity at relatively low concentrations. These results indicate that *C. racemosa* SPs significantly impair cell viability in a dose-responsive manner.

C. racemosa Extract Induced Morphological Changes and Apoptosis in WiDr Cells

Microscopic evaluation revealed distinct concentration-dependent morphological alterations in WiDr cells following treatment with *C. racemosa* extract (Figure 3). At 100 µg/mL (Figure 3A), abundant purple formazan crystals (indicative of metabolic activity) were observed alongside distinct pale regions, suggesting initial cytotoxic effects. This pattern persisted at intermediate concentrations (200-400 µg/mL; Figures 3B-D), where viable cells remained predominant but showed progressive reduction in formazan deposition. The most pronounced effects occurred at 500 µg/mL (Figure 3E), where extensive cell paleing and dramatic reduction in formazan crystals indicated significant loss of viability, consistent with either apoptosis or necrosis.[31-32]

Sulfated Polysaccharides from C. racemosa Modulated Caspase-3 Binding Affinity In Silico

The docking analysis revealed strong binding affinities, with binding energies of -5.0 kcal/mol, -4.8 kcal/mol, and -4.8 kcal/mol, respectively. In comparison, the native ligand Ac-DEVD-CHO, which is designed to occupy the active site of Caspase-3 and inhibit its enzymatic activity, exhibited a less favorable binding energy of -3.6 kcal/mol (Table 1). Ac-DEVD-CHO mimics the natural substrate sequence and functions as an apoptosis inhibitor by preventing Caspase-3 from cleaving its substrate.[33] It interacts through hydrogen bonding with key active-site residues such as Arg271, Ser272, Glu221, and Ser219 (Figure 4D).

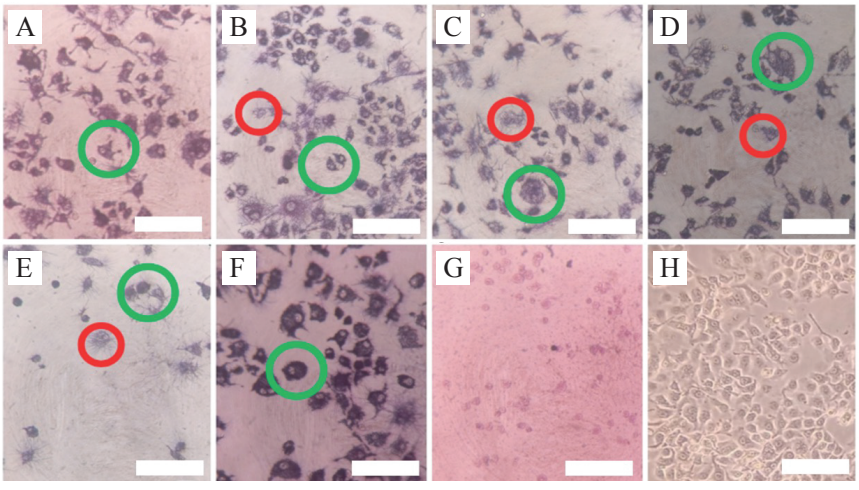

Figure 3. Morphological changes in WiDr colorectal cancer cells after 24-hour treatment with *C. racemosa* SPs extract. A-E: 100-500 µg/mL, ranging from predominantly viable cells (A) to extensive apoptotic bodies (E). F: Untreated control, normal epithelial morphology. G: Media control, absence of contamination. H: Reference, normal fibroblast-like morphology. Red circles: apoptotic cells (nuclear condensation); Green circles: viable cells with intact morphology. White bar: 50 µm.

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Table 1. Docking results of test compounds and native ligands with Caspase-3.

Compound	Docking Score (kCal/mol)	Hydrogen bonding interactions	Other interactions
D-galactose-6-sulfate	-5.0	Glu381, Asn342, Asp502	Asp345, Phe380, Gln351, Ser381A (van der Waals)
D-glucose-6-sulfate	-4.8	Trp348, Asp502, Asn342	Phe381B, Glu381, Ser381A, Asp345, Phe380, Gln351 (van der Waals)
D-mannose-6-sulfate	-4.8	Glu381, Asn342, Trp348	Glu379, Phe380, Asp345, Asp502 (van der Waals)
Ligand native Ac-DEVD-CHO	-3.6	Arg271, Ser272, Glu221, Ser219	Asp222, His224, Cys270, Lys220, Arg269 (van der Waals)

Among the tested compounds, D-galactose-6-sulfate exhibited the strongest interaction with a docking score of -5.0 kcal/mol and formed three hydrogen bonds (Figure 4A). Both D-glucose-6-sulfate and D-mannose-6-sulfate also demonstrated favorable binding energies, forming hydrogen bonds with different residues (Figures 4B and 4C). Interestingly, the test compounds did not bind to the same active site as Ac-DEVD-CHO but instead interacted with different regions of the Caspase-3 receptor. This suggests that they may act as allosteric activators of Caspase-3, potentially enhancing apoptotic activity via a non-competitive mechanism.

Discussion

This study successfully identified SPs in *C. racemosa* extract through two complementary analytical approaches. The characteristic blue chromogen formation with iodine staining and the distinct FTIR absorption band at 1232 cm^{-1} (corresponding to S=O stretching vibrations of sulfate esters) provide conclusive evidence of SPs. These findings align with established literature reporting sulfate group signatures within the 1200-1265 cm^{-1} spectral range.[29] Notably, the bioactivity of SPs - particularly their anticancer properties - is critically dependent on structural characteristics including sulfation pattern, degree of substitution, and molecular weight distribution.

The observed inhibition of WiDr cell viability by *C. racemosa* SPs extract demonstrated a clear concentration-dependent response. At 100 µg/mL, the extract inhibited 2600±100 cells, with a steady increase in the number of inhibited cells as the concentration increased, reaching 4100±100 inhibited cells at 500 µg/mL. This trend suggests that *C. racemosa* SPs possess potent antiproliferative properties, particularly at higher concentrations.

The extract demonstrated significant cytotoxic activity against WiDr colorectal cancer cells, with an IC_{50} of 32.46 µg/mL. According to United States National Cancer Institute (NCI) guidelines, this value positions the extract within the moderate-to-high cytotoxic potency range (30-100 µg/mL = moderate; <30 µg/mL = strong). The observed activity likely stems from multiple structural features of the SPs: (i) the high sulfate content facilitating electrostatic interactions with cell membranes, (ii) optimal molecular weight enabling cellular uptake, and (iii) specific monosaccharide composition influencing receptor binding. These structural attributes may collectively contribute to anticancer effects through mitochondrial depolarization, reactive oxygen species generation, and caspase cascade activation, as reported in other algal SP studies.[12]

Microscopic analysis revealed a clear concentration-dependent response. The progressive reduction in formazan crystal formation (from 100 µg/mL to 500 µg/mL) coupled with increasing pale cellular regions strongly suggests metabolic inhibition and cell death induction. At 500 µg/mL, we observed hallmark apoptotic morphology, including cell shrinkage and rounding, membrane blebbing, apoptotic body formation, nuclear condensation. These morphological changes align with classical features of caspase-mediated apoptosis.[34-36]

In silico results also showed apoptotic features against caspase-3 from *C. racemosa* SPs (D-Galactose-6-Sulfate, D-Glucose-6-sulfate, and D-Mannose-6-Sulfate). These findings are consistent with previous studies on SPs derived from marine algae, which demonstrated anticancer effects through mechanisms such as reactive oxygen species formation, mitochondrial membrane disruption, and caspase activation.[37] SPs from *C. lentillifera* have also shown inhibition of colorectal cancer cell proliferation and modulation of the PI3K/Akt signaling pathway. It has been reported that SPs from marine algae induce apoptosis in human cancer cells via the mitochondrial pathway.[38]

Although the results are promising, there are limitations. This study was conducted *in vitro* using a single

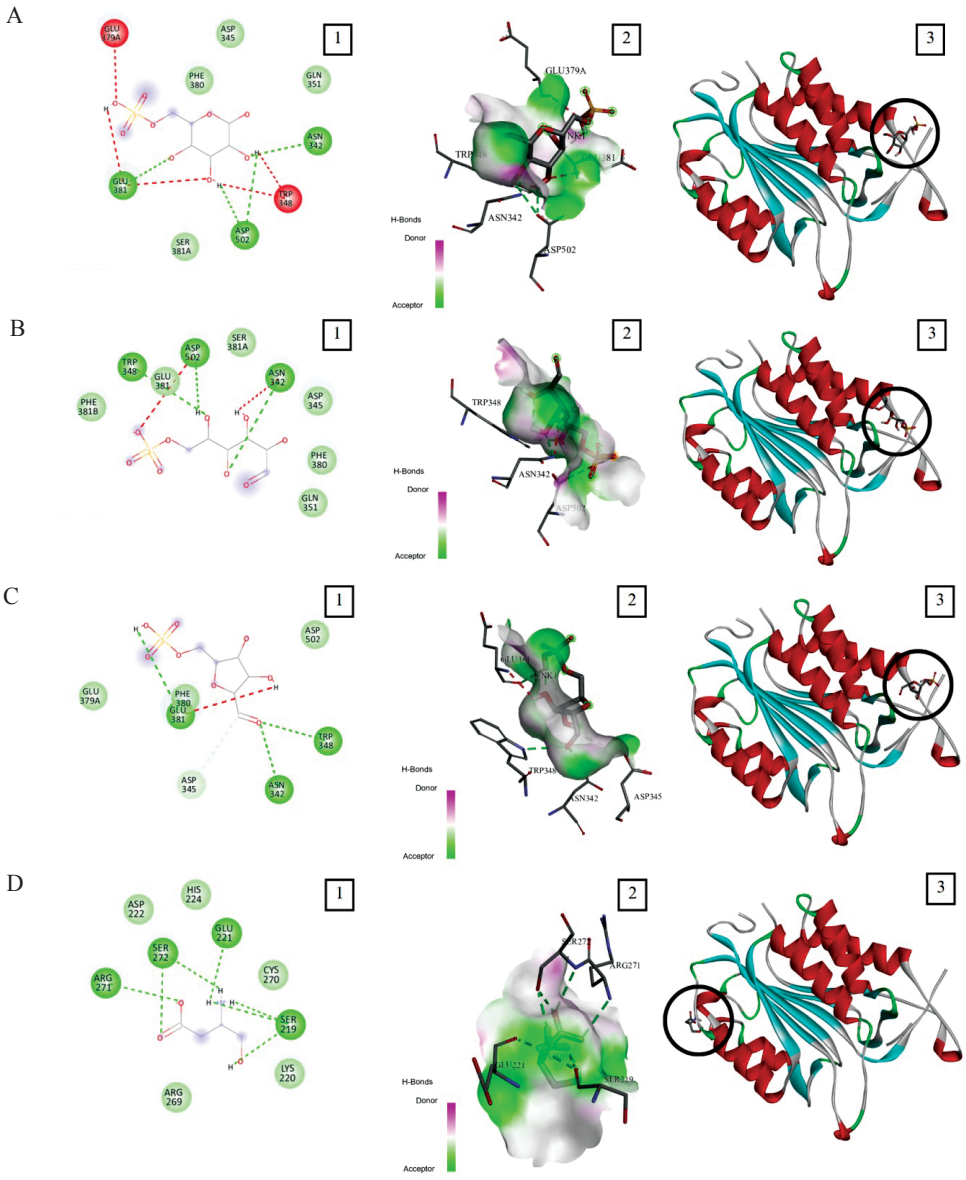

Figure 4. *In silico* interaction analysis of D-Galactose-6-Sulfate (A), D-Glucose-6-Sulfate (B), D-Mannose-6-Sulfate (C), and native ligand Ac-DEVD-CHO (D) with Caspase-3 (PDB ID: 1PAU). 1: 2D visualization showing interaction types, number of bonds, and involved amino acids (e.g., van der Waals, hydrogen bonds, unfavorable contacts); 2: Binding distance and receptor surface representation; hydrogen bonds indicated by dashed lines with green/pink surfaces as donors/acceptors; 3: 3D visualization (highlighted by black circle) shows test ligands bind at different sites than Ac-DEVD-CHO.

cell line, limiting generalizability. Additionally, molecular assays such as Annexin V/PI staining or gene expression profiling were not included and are necessary to confirm the apoptosis pathways involved. Although apoptotic morphology was observed under microscopy and supported by molecular docking on Caspase-3, this study did not quantify apoptotic cells through standard apoptosis assays. Future research should investigate intrinsic and extrinsic apoptosis mechanisms. Overall, SPs from *C. racemosa* exhibit strong cytotoxic effects on WiDr colorectal cancer cells and, based on *in silico* results, can induce apoptosis through Caspase-3 activation. These findings support their potential as natural anticancer cells. Further studies are needed to evaluate their efficacy and safety *in vivo*.

Conclusion

C. racemosa SPs exhibit dose-dependent anticancer activity against WiDr cells, with 500 µg/mL demonstrating significant viability reduction. The observed effects may be mediated through Caspase-3-dependent apoptosis, positioning these SPs as promising candidates for further colorectal cancer cells research.

Authors' Contributions

TW prepared the manuscript and conducted the stages of determining apoptosis patterns using the MTT assay method. AAMA carried out the extraction of *C. racemosa* and

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performed qualitative tests on sulfated polysaccharides. NH conducted FTIR analysis of sulfated polysaccharides and prepared the test compounds. AAH prepared the complete culture media and harvested Widr cells. AH prepared tools and materials, and carried out the thawing and incubation of Widr cells. HW provided research direction and designed the experiments.

Conflict of Interest

The authors declare that they have no conflicts of interest or competing interests related to the content of this manuscript.

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