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Phytochemical analysis, antioxidant activity, and cytotoxic effects of *Caulerpa lentillifera* extracts inducing cell apoptosis and sub-G/G0-G1 cell cycle arrest in KON oral cancer cells

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Abstract

Background Marine algae have excellent phytoconstituents with notable biological activity and bioactive therapeutic benefits, but the anti-oral cancer activity of *Caulerpa lentillifera* (*C. lentillifera*) has not been widely studied. This study aimed to explore the anti-cancer properties of *C. lentillifera* to gain insights into possible treatment approaches.

Methods The three *C. lentillifera* extracts were prepared using the maceration method with methanol (CLM), ethanol (CLE), and acetone (CLA). The chemical composition of extracts of *C. lentillifera* was investigated. Its metabolite profiles were selectively further investigated using the LC-QTOF MS/MS technique and their antioxidative activity was evaluated. The cytotoxic effect on KON cells and MRC-5 cells was assessed using the MTT test. Morphological changes and apoptosis were examined through Hoechst 33,258 and AO double staining, while DAPI and FDA double labeling were used to observe the nucleus and cytoplasm. Using a flow cytometer, the percentage of cell cycle arrest was calculated and the fraction of cell death was examined.

Results The CLA exhibited higher quantities of TPC, TFC, chlorophyll a, and chlorophyll b compared to the CLM and CLE. The LC-QTOF MS/MS analysis revealed ten major phytochemicals in the CLA. The three *C. lentillifera* extracts exhibited antioxidative activity, with the CLE demonstrating significantly higher antioxidant activity compared to the CLM and CLA. *In-vitro*, the KON oral cancer cells exhibited sensitivity to CLA, CLE, and CLM in that order. The three extracts induced ROS-mediated cell death as well as disruption of mitochondrial membrane potential, with concentrations at IC_{40}, IC_{60}, and IC_{80} leading to apoptosis within 24 h. Furthermore, the cell cycle of KON cells was blocked in sub-G and G0-G1 by all three extracts. Notably, the extracts significantly impeded colony growth, migration, and invasion. The increase in cellular uptake was measured using the TEER test.

Conclusion The findings showed that *C. lentillifera* has several functional metabolites, antioxidative activity, and strong anti-tumor properties. According to these results, *C. lentillifera* extracts may be utilized to treat oral cancer.

Keywords *Caulerpa lentillifera* extracts, LC-QTOF MS/MS analysis, OSCC, Antioxidant activity, Cytotoxic activity

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Introduction

One common type of head and neck cancer is oral squamous cell carcinoma (OSCC), which begins in the oral mucosa. Ninety percent of malignancies that develop in the oral cavity are histologically squamous cell tumors [1]. Global Cancer Observatory (GCO) statistics show that in 2020, there were 377,713 instances of OSCC worldwide, with Asia accounting for the majority of these cases [2]. With a high rate of morbidity and malignancy and a dismal prognosis, OSCC is extremely threatening. Because of its high recurrence incidence and propensity for lymph node metastasis, it is difficult to treat OSCC [3]. Radiation and chemotherapy can kill the majority of cancer cells, but any remaining tumors may relapse and become resistant to treatment. The need for further therapies is increased when oral cancers become resistant to chemotherapy and radiation [4]. Radiation, chemoradiotherapy, and immunotherapy comprise the majority of non-surgical treatment options for OSCC [5]. The prognosis for most patients with OSCC remains poor, with the typical 5-year survival rate ranging from 50 to 60%. Despite significant advancements in treatment, one of the main factors contributing to this outcome is the growing resistance of OSCC cells to conventional chemotherapeutic drugs [6]. Research conducted to identify novel therapeutic compounds with improved pharmacotoxicological characteristics is still in progress. An effective way to discover new medications, including ones for cancer treatment, is through natural constituents [7]. A new strategy for treating and preventing OSCC is the use of natural products. Natural treatment options are more affordable, less toxic, and less dangerous when compared to modern chemotherapeutic approaches [8, 9]. Natural products are extremely important because of their low toxicity, safety, and accessibility [10, 11]. As a low-cost, readily available, manageable, and palatable cancer treatment option, the use of phytochemicals derived from plant and marine natural products for chemoprevention is becoming increasingly popular [12]. The importance of natural agents, such as plant secondary metabolites, or phytochemicals, in preventing the development of cancer and serving as a substantial source of anti-cancer medications has been demonstrated [13]. It is well known that seaweed is a good source of biopolymers, or molecules with distinct chemical properties, which are useful for creating new treatments with higher potencies and specificities [14].

The marine environment is where algae are primarily found. Algae produce a wide variety of metabolites. Recently, there has been significant interest in these compounds for the identification of potentially useful pharmaceutical drugs, such as those with anti-cancer properties. Several chemicals derived from marine algae have demonstrated significant anti-cancer properties against various cancer cell lines [15]. A large number of experts are interested in algae because of their high quantity of bioactive components, and they have suggested possible uses in the industrial and medicinal domains. Numerous studies conducted in vivo and in vitro over the past few decades have shown that algae have a wide range of possibilities in cancer therapy [16, 17]. Green seaweed, notably *C. lentillifera*, is commonly eaten as a raw vegetable in Southeast Asia. It is interesting to note that the food industry has recently become more aware of this green seaweed. There have been reports on the biological activities such as anti-hypertensive, anti-inflammation, anti-pyretic, anti-bacterial, anti-cancer, and anti-coagulant effects [18]. Due to changes in global temperatures, *C. lentillifera* is now present on the Korean Peninsula, while it originated in tropical areas near the Indo-Pacific region, such as Southeast Asia. Known for being edible and abundant in minerals, dietary fiber, vitamin A, vitamin C, and various important unsaturated fatty acids, within the genus Caulerpa [19, 20], *C. lentillifera* is an ulvophyte green alga with several functional metabolites, antioxidant capacity, and anti-obesity properties. These attributes have been shown in previous studies and are supported by scientific evidence. Thus, they may be used as precursors for novel therapeutic approaches aimed at treating diseases related to obesity. It can be inferred from the results that *C. lentillifera* possesses several bioactive metabolites, antioxidant capacity, and anti-obesity properties. A previous study prepared *C. lentillifera* extracts using two different ethanol extraction methods (Maceration and Soxhlet), and the highest abundance of compounds was obtained from *C. lentillifera* ethanolic macerated-extract fraction (ME). A more intriguing combination of bioresources include ME (Maceration—Ethanol), MH (Maceration—n-Hexane), and SE (Soxhletation—Ethanol) as anti-cancer drugs, particularly for hepatoma, breast, and colorectal malignancies, and even leukemia. Therefore, *C. lentillifera* has the potential to be developed as a promising medication candidate in the future and to be a functional food source with substantial therapeutic advantages [21]. An in vitro investigation revealed that ß−1,3-Xylan isolated from *C. lentillifera* suppressed the growth of MCF-7 human breast cancer cells and instigated chromatin condensation, poly ADP-ribose polymerase (PARP) degradation, and caspase-3/7 activation, suggesting that it encouraged cell death in these MCF-7 cells [22]. Researchers found that ethanol-hexane seaweed from *C. lentillifera* greatly decreased the viability of A147 glioblastoma cells and strongly slowed down the growth of the glioblastoma cell cycle. Also, the seaweed extracts accelerated the A147 cells' programmed cell death [23]. In this study, to elucidate the distinct

compounds based on the degree of polarity of the compounds, maceration extraction methods were used in this work. The extraction used three solvents, namely methanol, ethanol, and acetone, to prepare *C. lentillifera* methanol extract (CLM), *C. lentillifera* ethanol extract (CLE), and *C. lentillifera* acetone extract (CLA). Chemical constituents, metabolite profile and antioxidative activity of *C. lentillifera*, were characterized. Moreover, there has not been any information released regarding *C. lentillifera*'s anti-cancer effects on survival, induction of apoptosis, cell cycle inhibition, and life cycle development of oral cancer KON cells. This study aims to investigate the anti-cancer effects of *C. lentillifera* extracts on KON oral cancer cells, focusing on cell survival, apoptosis, and cell cycle inhibition.

Materials and methods

Chemical and reagents

The algae were cleaned using distilled water and stored at 4 °C until the start of the experiment. Methanol, ethanol, acetone, and DMSO were analytical grades purchased from Unitywewell Co., Ltd. (Bangkok, Thailand). Folin-Ciocalteu's phenol reagent and potassium persulfate ($K_2S_2O_8$) were purchased from Merck (Darmstadt, Germany). Gallic acid was purchased from TCI (Tokyo, Japan). Sodium bicarbonate was purchased from Ajax Finechem (New South Wales, Australia). The standard quercetin was purchased from ChemFaces (Hubei, China). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Sigma-Aldrich (Wisconsin, USA). Hoechst 33,258 (SolarBio®, China), acridine orange (AO) (SolarBio®, China), propidium iodide (PI) (SolarBio®, China), 3-(4,5-dimethyl-2-thiazolyl)−2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, USA), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA), 4',6'-Diamidine-2'-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, USA), 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma-Aldrich, USA), and rhodamine 123 (SolarBio®, China) were also obtained.

Cell lines

The KON oral cancer cells and MRC-5 lung fibroblast cells were supplied by Asst. Prof. Dr. Sukannika Tubtimsri, Division of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Burapha University. KON oral cancer cells (passage number: 36) are a kind of oral cavity squamous cell carcinoma that was isolated from a 76-year-old man. A 14-week-old male embryo's lung tissue was used to identify MRC-5 lung fibroblast cells (passage number: 15). Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin solutions were used to cultivate both cell lines in T-75 culture flasks. The KON oral cancer cells and MRC-5 lung fibroblast cells were cultivated in an incubator (Shel Lab, USA) with 5% CO_2 at 37 °C. KON cells and MRC-5 cells were subcultured every three or four days. Cell viability was assessed using a 0.4% trypan blue exclusion assay to confirm 90–95% viability.

Preparation of *C. lentillifera* extract

The edible grade of algae, *C. lentillifera*, was obtained from Family Farm, Phetchaburi Province, Thailand. The *C. lentillifera* was identified based on distinct morphological feature according to the algae database (https://www.algaebase.org/search/species/detail/?species_id=3754). The herbarium was deposited at Faculty of Pharmaceutical Sciences, Burapha University with code AG22-001.The fresh algae was cleaned, freeze-dried, and pulverized. The dry algae were macerated with three different organic solvents, including methanol, ethanol, and acetone, with solvent to solid ratio 20 mL/1 g for 24 h at room temperature (25 °C). The containers were protected from light throughout the extraction period. The samples were filtered and macerated again for 4 times. The filtrates were pooled and evaporated using an evaporator (Buchi, Switzerland) to obtain the extracts, which were kept at 4 °C in a sealed container until further investigation.

Determination of extraction yield

The extraction yield of *C. lentillifera* was calculated by the following equation: Extraction yield (%) = (W1/W0) × 100, where W1 is the weight of dry extract (g) and W0 is the weight of dry *C. lentillifera* (g).

Total phenolic content

The TPC determination method was adapted from a procedure described by Petchsomrit et al. (2022). The extract was dissolved in DMSO to obtain a concentration of 10 mg/mL. Then, 20 µL of the sample was mixed with 50 µL of 10% Folin-Ciocalteu reagent and 80 µL of 7.5% (w/v) sodium bicarbonate solution in a 96-well plate. The mixture was kept in the dark for 30 min and the absorbance was measured at 765 nm (microplate reader FLUOstar Omega, BMG Labtech, Germany). The process was performed in triplicate, and the results were presented as mean ± SEM of mg gallic acid equivalent per g extract (mg GAE/g extract) [24].

Total flavonoid content

The total flavonoid content of the extracts was investigated according to Haile and Kang (2019), with some modifications. Briefly, the extract was dissolved in 50% DMSO. One hundred twenty microliters of algal extract

(5 mg/mL) and 10 µL of 5% $NaNO_3$ were mixed in. After 5 min, a 10% $AlCl_3$ solution (20 µL) was added and incubated for 6 min. Next, 1 M NaOH (60 µL) was added to the solution and incubated for 11 min. The absorbance of the extracted sample was measured at 510 nm against the blank sample, which substituted algal extract with 50% DMSO. The standard solution of quercetin was prepared (0.06–2 mg/mL). The amount of flavonoids was expressed as mg quercetin equivalent per gram of extract (mg QE/g extract) [25].

Total chlorophyll contents

Total chlorophyll content was determined using UV absorption and calculated according to Lichtenthaler et al. (1983). Briefly, the extract was dissolved in methanol to obtain UV absorbance in the range of 0.2 – 0.8. The absorption was recorded at wavelengths 653 and 666 nm [26]. The chlorophylls a and b contents were calculated according to the following equations:

$$C_a = 13.95A_{666} - 6.88A_{653}$$

$$C_a = 29.96A_{653} - 7.32A_{666}$$

where C_a and C_b, are chlorophylls a and b (µg/mL), respectively. The A_{653} and A_{666} are the absorbance at wavelengths 653, 666 nm, respectively. The contents were calculated and reported as mg/g extract.

LC-QTOF MS/MS analysis

The LC-QTOF MS/MS analysis was conducted using the Agilent 1260 Infinity II system coupled with a 6545 QTOF mass spectrometer. Two microliters of the extract (1 mg/mL) were chromatographed on an InfinityLab Poroshell 120 SB-C18 column (2.7 µM, 2.1 mm × 100 mm). The column temperature was maintained at 35 °C. The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient elution was started with 5% of mobile phase B at a flow rate of 0.3 mL/min for 2 min. The concentration of mobile phase B was increased from 5 to 99% within 18 min. After the concentration reached 99% of mobile phase B, the condition was maintained for 5 min and later equilibrated with 5% of mobile phase B for 5 min. The mass spectrometric analysis was performed with QTOF equipped with an ESI source with the following parameters: drying gas (N_2) temperature 320 °C; flow rate 8 L/min, nebulizer 45 psi; sheath gas temperature 350 °C; sheath gas (N_2) flow rate 11 L/min; capillary voltage 3500 V and nozzle voltage 1,000 V. The mass detection was performed in positive electrospray mode. The mass scan was performed in the range of 100–1000 m/z. The data was analyzed using the METLIN (PCDL B.08.00) metabolites database.

ABTS assay

The antioxidant activity of *C. lentillifera* extracts was examined using the ABTS assay (Petchsomrit et al., 2022.). In brief, the stock solution of 14 mM ABTS + •solution and 4.9 mM potassium persulfate solution (1:1 v/v) was prepared and incubated in the dark for 16 h before use. The stock solution was diluted with DMSO to acquire an absorbance of 0.7–0.9 units at 734 nm. The diluted stock solution was mixed with each sample (10 mg/mL) at a ratio of 1:1 v/v in a 96-well plate and incubated for 6 min. Trolox was used as standard. The absorbance was measured at 734 nm [24]. The investigation was performed in triplicate. The radical scavenging activity of ABTS was calculated using the following equation: %Inhibition = [(A0—As)/A0] × 100, where A0 is the absorbance of ABTS solution without the sample, and As is the absorbance of the sample.

In vitro cytotoxicity on oral squamous cell carcinoma cells (KON)

The cell viability of the KON cells was determined using a 3-(4,5-Dimethylthiazol-2-yl)−2,5-Diphenyltetrazolium Bromide (MTT) assay (Manmuan et al., 2024.). KON cells at a density of 1×10^4 cells/well were seeded into a 96-well plate (Corning Inc., Tewksbury, MA, USA) for 24 h in a CO_2 incubator. Plates were incubated overnight, and the KON cells were exposed to the CLM, CLE, and CLA in different concentrations (7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL) for 24 h. Ten microliters of MTT (10 mg/mL) in PBS solution were added to each well. Plates were incubated at 37 °C for 2 h until the formation of formazan crystals was achieved. The supernatant was removed, and 100 µL of DMSO was added to the formazan crystal precipitate in each well for 15 min. The absorbance values were measured at 570 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Germany). Untreated KON cells were used as the negative control [27]. Percent cytotoxicity was calculated according to the following equation: % Cytotoxicity = 100-[Cell viability = ($O.D._{sample}$-$O.D._{blank}$/$O.D._{control}$-$O.D._{blank}$ × 100%)]. The IC_{40}, IC_{60}, and IC_{80} values (inhibitory concentrations at 40%, 60%, and 80%) were used to calculate the specific concentration of *C. lentillifera* required to lower the viable cell population by 40%, 60%, and 80%, respectively. Linear regression analysis was utilized to calculate the IC_{40}, IC_{60}, and IC_{80} values, which were then selected for use in further trials.

Nuclear and cytoplasm analysis by 4′,6-diamidino-2-phenylindole (DAPI) and fluorescein diacetate (FDA) double staining

KON cells were grown at a density of 5×10^4 cells/well on sterilized glass coverslips in a 6-well plate and treated with IC_{40}, IC_{60}, and IC_{80} concentrations of CLM,

CLE, and CLA for 24 h. Each well had its aspiration. To eliminate any floating cells, cells were washed twice with 1X PBS solution. Cells were then fixed with 4% paraformaldehyde (PF) for 15 min and permeabilized with 0.2% Triton-X-100. Cells were stained with 2.5 µg/mL DAPI (Molecular Probes, USA) and 0.5% FDA for 30 min in a dark environment. PBS was used to wash the cells before sealing the coverslips onto the slides. The cytomorphology of the KON cells was studied using an inverted fluorescence microscope (ECLIPSE Ts2R, Nikon, Tokyo, Japan) at 20×objective magnification.

Characterization of cell death and apoptosis by Hoechst 33,258 and Acridine orange (AO) double staining

KON cells at a density of 5×10^4 cells/well were grown on sterile glass coverslips in a 6-well plate for 24 h. The KON cells were treated with IC_{40}, IC_{60}, and IC_{80} concentrations of CLM, CLE, and CLA for 24 h. Then, nuclear staining was achieved using Hoechst 33,258, and cytoplasm staining was performed using AO dye. After the incubation period, the KON cells were washed twice with a 1X PBS solution to remove debris and floating cells. The KON cells were then fixed with 4% paraformaldehyde (PF) for 15 min and permeabilized with 0.2% Triton-X-100. Apoptotic nuclei were visualized using a fluorescent microscope (ECLIPSE Ts2R, Nikon, Tokyo, Japan). NIS-Elements imaging software was used for image analysis.

Determination of cell death by propidium iodide (PI) with flow cytometer analysis

KON cells were cultivated in a 6-well plate for 24 h at a density of 5×10^4 cells per well. Throughout 24 h, IC_{40}, IC_{60}, and IC_{80} concentrations of CLM, CLE, and CLA were used to treat the KON cells. Trypsin/EDTA was used to harvest the cells, and 1X PBS was used twice for washing. The pellets were then painstakingly collected using centrifugation and reconstituted in a 1X cold PBS solution. Following the manufacturer's instructions, the KON cells were stained for 15 min at room temperature in the dark using PI color. Using a flow cytometer (CytoFLEX; Beckman Coulter, Inc.), the KON cells were subjected to flow cytometry analysis. The PI channel revealed the red fluorescence of PI (Ex=535 nm, and Em=615 nm). The data acquisition and analysis were performed using CytExpert software version 2.4.0.28 and a minimum of 10,000 cells was analyzed in each group. The ratio of PI^+ to KON cells was measured by flow cytometry as described by the manufacturer's instructions.

Determination of intracellular reactive oxygen species (ROS) by 2′, 7′dichlorofluorescein (DCF)

In a 6-well plate, 5×10^4 KON cells were seeded per well for a duration of 24 h. The IC_{40}, IC_{60}, and IC_{80} concentrations of CLM, CLE, and CLA were applied to the KON cells for 24 h. The cells underwent two rounds of washing with a 1X PBS buffer after being stained for 45 min at 37 °C with 25 µM of dichlorofluorescein diacetate (DCF-DA) in a PBS buffer. A fluorescent microscope (ECLIPSE Ts2R, Nikon, Tokyo, Japan) was used to quantify the amount of green fluorescence present inside the KON cells.

Identification of reactive oxygen species (ROS) formation using oxidized DCF-DA

The effects of CLM, CLE, and CLA on ROS production were assessed using flow cytometry. The KON cells (density 5×10^4 cells/well) were grown on a 6-well plates for 24 h and then treated with the various extracts at doses of IC_{40}, IC_{60}, and IC_{80}. Cell pellets were collected and resuspended in PBS solution. The KON cells were treated with 25 µM of DCF-DA for 40 min in the dark. The cells were centrifuged at 2,000 g for 2 min and then resuspended in 500 µL of PBS solution. The levels of ROS production were evaluated using a CytoFLEX flow cytometer (Beckman Coulter, Inc). The DCF fluorescence intensity was measured at an excitation wavelength of 485–495 nm, whereas the fluorescence emission was measured in the green channel at 525–530 nm.

Evaluation of the potential of the mitochondrial membrane (ΔΨ m) by flow cytometer

Rhodamine 123 (Rho123) was used in the flow cytometry to measure the integrity of the mitochondrial membrane potential. KON cells (density 5×10^4 cells/well) were briefly cultivated in 6-well plates and left for 24 h, during which the KON cells were exposed to the CLM, CLE, and CLA at doses of IC_{40}, IC_{60}, and IC_{80}. The cell pellets were trypsinized using a 0.25% trypsin–EDTA solution following the incubation period. Five hundred microliters of PBS solution containing 10 µM Rho123 was used to resuspend the cell pellets, which then underwent a 20-min incubation period in a dark environment. Following a 2-min centrifugation at 2,000 g, the cell pellets were resuspended in 500 µL of PBS solution. A CytoFLEX flow cytometer (Beckman Coulter, Inc.) was used to measure the fluorescence intensity of Rho123 to confirm the loss of mitochondrial membrane potential. The reduction in green Rho123 fluorescence indicated reduced $\Delta\Psi m$.

Cell cycle analysis with flow cytometer analysis

A 6-well plate was seeded with 5×10^4 KON cells per well, and the cells were cultured for an entire night. Three different strengths of CLM, CLE, and CLA were used to treat the KON cells over the course of 24 h. After trypsinizing the cells with trypsin/EDTA, they were fixed for the night in 100% ethanol. The cell pellets were harvested

using centrifugation for 5 min at 1,000 rpm. Then, PI dye was used to stain the KON cells for 30 min. A flow cytometer (CytoFLEX; Beckman Coulter, Inc.) operating at an excitation wavelength of 488 nm was used to detect red fluorescence.

Colony formation assay

In a 6-well plate, KON cells were cultivated for 24 h at a density of 1.5×10^3 cells per well. For a duration of 24 h, cells were cultivated in DMEM medium with or without IC_{40}, IC_{60}, and IC_{80} doses of CLM, CLE, and CLA. The culture was incubated for an additional 7 days, with a new medium added following the removal of the old one. The culture media were taken out of every well. To eliminate floating cells, 1X PBS was used to wash the cells. Acetic acid (3) and methanol (7) were used to fix the development of cell colonies for 15 min. After thirty minutes, the cells were stained with 0.5% crystal violet. Five random fields per well were used to photograph cells using a 10× inverted microscope (Olympus, Japan). The data were presented as a percentage of colonies formation.

In vitro wound healing assay

The in vitro scratch assay was used to investigate the impact of the CLM, CLE, and CLA on KON cell migration. KON cells at a density of 1×10^6 cells/well were plated into a 6-well plate and allowed to grow to 100% confluence. Subsequently, the culture was incised using a 200 µL yellow pipette tip to generate a straight line, and all three extracts of *C. lentillifera* at IC_{40} concentrations in medium containing 5% FBS were added immediately. Within 0, 24, and 48 h, the wound scratch in the well was assessed. Using an inverted microscope set to 4x, the injured area was serially photographed in three different fields of view for every well. Cell migration was measured by calculating the area covered by migrating cells to the wounded area and the cell-free area. ImageJ software (version 1.47, NIH, USA) facilitated the area measurement. The data were presented as a % inhibition of cell migration. Inhibition of cell migration (%) = (total area of wound scratch in treated cells / total area of wound scratch in untreated cells × 100) −100%.

Transwell migration assay

A transwell insert was used to examine any possible inhibitory effects of the CLM, CLE, and CLA at IC_{40} concentrations on KON cancer cell migration. The migration assay was carried out in transwell Corning® chambers (Corning, USA) with a membrane pore size of 8 µm. In the lower chamber, a medium with 10% FBS was used as a chemoattractant, and cells (density: 1.5×10^3 cells/well) were plated on an insert that also had the full medium with the CLM, CLE, and CLA at IC_{40} concentrations. After 2 days, the inserts were stained with 0.1% crystal violet solution (Sigma, USA) and fixed in 4% paraformaldehyde. The migrated cells on the inserts' lower surface were counted and photographed. The inhibition of cell migration was quantified with the equation: Inhibition of cell migration (%) = 100—number of migrated cells in treated cells/number of migrated cells in untreated cells × 100%.

Matrigel invasion assay

To evaluate the anti-invasive properties of the CLM, CLE, and CLA at IC_{40} concentrations against oral cancer cells, the experiment was conducted in an in vitro model of the extracellular environment using a BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Bedford, MA, USA). The chamber included an 8-µm PET membrane and a thin layer of matrigel basement matrix, and the invasion chamber surface of a 24-well plate was coated with BD Matrigel Matrix that had been diluted with serum-free DMEM medium. Trypsin/EDTA was used to trypsinize the KON cells, and then they were resuspended in DMEM medium without any serum. For 48 h, the KON cells (density: 1.5×10^3 cells/well) were pipetted into the upper chamber of the invasion chamber (pore size: 8 µM; Corning®, Cambridge, MA) with or without the CLM, CLE, and CLA at IC_{40} concentrations. The system's lower compartment was fully loaded with 500 µL of DMEM that was enhanced with 10% FBS to act as a chemoattractant. The media were removed from the 24-well plate, and the cells were fixed in ice-cold methanol (AMRESCO, USA) for 15 min. Using a cotton swab, non-invading cells were removed from the invasion chamber's upper surface. For thirty minutes, the attached cells were stained with 0.5% crystal violet. Three times, 1X PBS was used to wash the upper chambers. An inverted microscope (Olympus, Japan) was used to photograph the invading cells at 10× magnification in 5 random fields per well. The information was displayed using the following formula: Inhibition of cell invasion (%) = 100—number of invaded cells in treated cells / number of invaded cells in untreated cells × 100%.

Transepithelial electrical resistance measurements

The cellular uptake of the CLM, CLE, and CLA in a monolayer cell culture is due to the barrier integrity, which is quantitatively determined by transepithelial electrical resistance (TEER) values. For 24 h, KON cells (2.5×10^4 cells/well) were cultured in a 6-well plate. The CLM, CLE, and CLA were added to DMEM media at IC_{40}, IC_{60}, and IC_{80} concentrations, and the cells were treated for 24 h. The TEER values were obtained using a Millicell® ERS-2 Voltohmmeter (Merck KGaA, Darmstadt, Germany), and they were reported as the mean of the millivolts from three independent experiments.

In vitro cytotoxicity on normal cells MRC-5

MRC-5 lung fibroblast cells were planted in DMEM media in 96-well plates at a density of 1×10^4 cells/well for 24 h. The cells were exposed to 7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL of the CLM, CLE, and CLA for a duration of 24 h. The proportion of cell cytotoxicity was determined using the MTT colorimetric test. The absorbance readings were determined using a microplate reader (FLUOstar Omega, BMG Labtech, Germany) that was operating at 570 nm. The data was plotted on a cytotoxicity graph, and the percentage of cytotoxicity was determined using GraphPad Prism software version 8.0 (GraphPad™ 8.0, San Diego, California).

Statistical analysis

GraphPad Prism™ software version 8.0 was used to analyze the data and plot the graphs. To analyze the data from the three independent studies, a one-way ANOVA was employed along with Tukey's Honestly Significant Difference (HSD) post hoc test. A p-value < 0.05 was regarded as statistically significant. The correlation between biological activity and chemical constituents was determined using Pearson's correlation. The strength correlation was classified as follows; values of $r = 0.01$ to 0.19 are considered very weak, values from 0.20 to 0.39 are weak, values from 0.40 to 0.59 are moderate, values from 0.60 to 0.79 are strong, and values from 0.80 to 1.0 represent a very strong correlation [28].

Results

Determination of %yields of algae *C. lentillifera* extracts

The extraction of the algae, *C. lentillifera*, was carried out using maceration using three different organic solvents. The physical appearance of the CLM, CLE, and CLA are shown in Fig. 1. The yields of CLE and CLM were notably higher at 44.06% and 33.70%, respectively, compared to the CLA, which yielded 6.39%.

Evaluation of the TPC, TFC, chlorophyll a, and chlorophyll b of the CLM, CLE, and CLA

As demonstrated in Table 1, *C. lentillifera* was extracted using a variety of organic solvents, including methanol, ethanol, and acetone. The CLA demonstrated higher TPC, TFC, chlorophyll a, and chlorophyll b levels than the CLM and CLE.

Metabolite profile of CLA via LC–MS

Because the CLA exhibited higher quantities of TPC, TFC, chlorophyll a, and chlorophyll b compared to the CLM and CLE, its metabolite profile was selectively subjected to further examination using the LC–MS approach. The metabolic profile of the extract was successfully obtained (Fig. 2) and was analyzed using non-targeted metabolic profiling. The analysis of the data was performed using the METLIN library metabolite match (> 80%). In Table 2, the ten compounds with the highest abundance are shown.

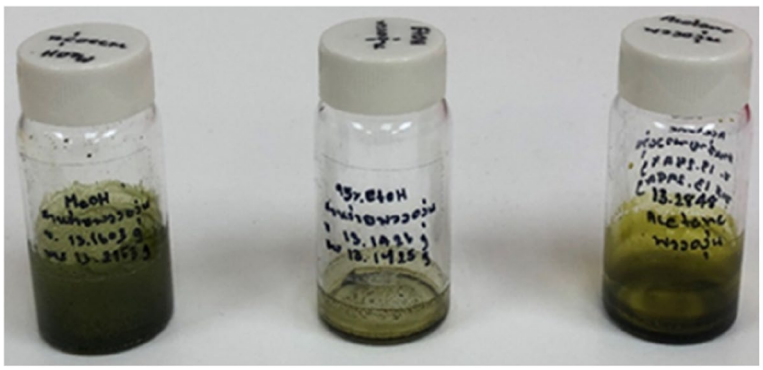


Fig. 1 The physical appearance of CLM, CLE, and CLA

Table 1 The concentrations of the TPC, TFC, chlorophyll a, and chlorophyll b of the CLM, CLE, and CLA

Solvent	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	Chlorophyll a (mg/g extract)	Chlorophyll b (mg/g extract)
CLM	2.95 ± 0.022	15.09 ± 0.282	4.45 ± 0.044	2.93 ± 0.071
CLE	2.29 ± 0.030	11.57 ± 0.465	2.92 ± 0.036	1.49 ± 0.043
CLA	4.37 ± 0.062	16.19 ± 1.251	35.22 ± 0.185	26.20 ± 0.268

Evaluation of the antioxidant activity of the CLM, CLE, and CLA

The antioxidant activity of the three *C. lentillifera* extracts was evaluated at a concentration of 10 mg/mL. All three extracts demonstrated antioxidative activity. The antioxidant (% inhibition) of CLM, CLE, and CLA were 5.41 ± 1.528, 28.48 ± 1.189, and 13.60 ± 0.856, respectively. The CLE showed significantly greater antioxidant activity than the others ($p < 0.01$).

Cytotoxicity of *C. lentillifera* extracts on KON cells

As shown in Fig. 3, KON cells were exposed to 7.8125–1,000 µg/mL of CLM, CLE, and CLA for 24 h and were subjected to the harmful effects of these three extracts. Incubation with a CLM for 24 h increased the cell mortality (100 ± 0.000% with the highest concentration, 1,000 µg/mL, $p < 0.001$) with an estimated 40%, 60%, and 80% of cell death (IC_{40}, IC_{60}, IC_{80}) values of 219.4 ± 40.03, 436.9 ± 34.10, and 649.1 ± 33.57 µg/mL. Incubation with the CLE for 24 h displayed a dramatic cell death (95.51 ± 4.494% with the highest concentration, 1,000 µg/mL, $p < 0.001$) with an estimated 40, 60, 80% of cell death (IC_{40}, IC_{60}, IC_{80}) values of 37.14 ± 193.8, 386.4 ± 108.50, and 735.6 ± 28.79 µg/mL. Incubation with the CLA for 24 h highly affected the cell viability of the KON cells (100 ± 0.000% with the highest concentration, 1,000 µg/mL, $p < 0.001$), and the KON cells were the most receptive to the CLA, with estimated 40%, 60%, and 80%

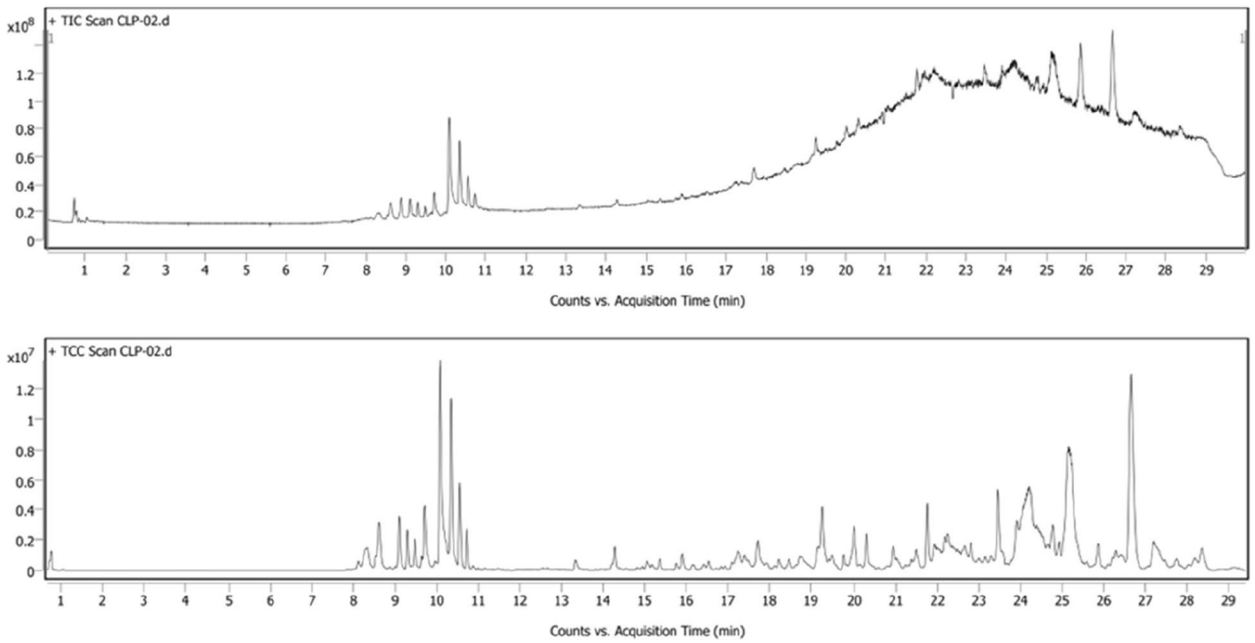


Fig. 2 The LC–MS chromatogram of CLA

Table 2 Metabolite profiles observed in CLA from LC–MS

No	% Abundance	Molecular formula	RT (min)	Mass (*m/z*)	Score	Proposed compound
1	11.994	$C_{34}H_{64}O_{13}$	10.082	680.43438	99.72	13-Sophorosyloxydocosanoic acid
2	7.938	$C_{39}H_{47}N_5O$	25.185	665.35751	99.87	Jubanine C
3	4.860	$C_{12}H_{16}N_2O_4S$	19.201	284.08306	100.00	Cysteinyl-Tyrosine
4	4.860	$C_{20}H_{12}O_2$	19.201	284.08306	96.78	11,12-Dihydroxybenzo[a]pyrene
5	4.857	$C_{13}H_{16}O_7$	19.201	284.08916	98.57	D-Vacciniin
6	4.856	$C_9H_9N_5O_5$	19.201	267.06154	90.05	2-Amino-4-oxo-6-(1,2-dioxoprolyl)−7,8-dihydroxypteridine
7	3.390	$C_{37}H_{60}O_9$	28.566	648.42372	100.00	Momordicoside K
8	3.165	$C_{21}H_{40}O_3$	17.534	340.29853	96.31	Polyoxyethylene (600) mono-ricinoleate
9	2.640	$C_{20}H_{18}N_2O_4$	24.852	350.12455	94.91	Na-p-Hydroxycoumaroyltryptophan
10	2.30	$C_{26}H_{42}O_3$	27.876	402.31166	99.78	Oryzarol

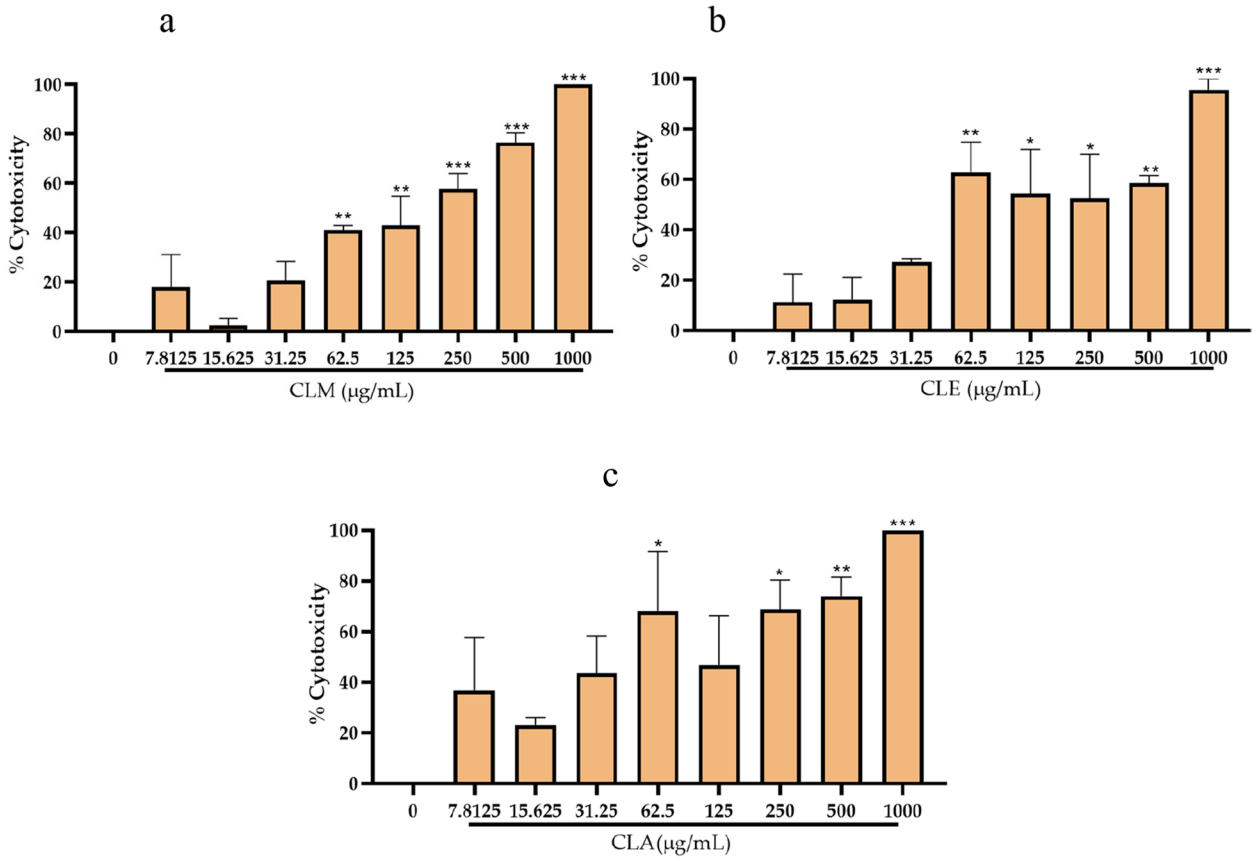


Fig. 3 Cytotoxicity of CLM, CLE, and CLA on KON cells. Cells were treated with 7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL of CLM (**a**), CLE (**b**), and CLA (**c**) for 24 h. The percent cytotoxicity was estimated by the MTT colorimetric assay. Data are expressed as mean ± SEM ($n = 3$) from the three independent experiments. *$p < 0.05$, significantly different from the control, **$p < 0.01$ significantly different from the control, ***$p < 0.001$ significantly different from the control

Table 3 The inhibitory concentrations (IC) values of CLM, CLE, and CLA at 40, 60, and 80% on KON cells after treatment for 24 h

C. lentillifera Extracts	Inhibitory concentrations (IC) at 24 h		
	IC_{40} (µg/mL)	IC_{60} (µg/mL)	IC_{80} (µg/mL)
CLM	219.4 ± 40.03	436.9 ± 34.10	649.1 ± 33.57
CLE	37.1 ± 193.80	386.4 ± 108.50	735.6 ± 28.79
CLA	4.06 ± 176.10	297.7 ± 128.90	591.3 ± 81.65

The values are expressed as mean ± SEM ($n = 3$)

of cell death (IC_{40}, IC_{60}, IC_{80}) values of 4.06 ± 176.10, 297.7 ± 128.90, and 591.3 ± 81.65 µg/mL (Table 3). These results demonstrate the negative effects of this extract on the viability of KON cells. However, it also implies that the effect is dependent on the solvent employed to extract the chemicals. As a result, additional trials would provide better knowledge of the long-term dynamics of cell and plant extract interactions.

Effects of the CLM, CLE, and CLA at IC_{40}, IC_{60}, and IC_{80} on cell morphology

As demonstrated in Fig. 4, after 24 h of exposure to IC_{40}, IC_{60}, and IC_{80} doses of CLM, CLE, CLA, fluorescent nuclei and cytoplasm staining were performed using DAPI and FDA dyes to assess the changes in cell morphology and membrane integrity in the KON cells. Images of the same field were captured with various filters. Our findings primarily demonstrated a concentration-dependent increase in cell mortality and fragmentation after 24 h, as well as a significant decrease in the ratio of viable intact cells. The proportion of KON cells adhering to the cover slip was reduced. These findings demonstrate the capabilities of *C. lentillifera* extracts on KON cells.

Characterization of cell death and morphological analysis

As shown in Fig. 5, the morphology of the nontreated KON cells showed typical monolayer growth, with cells

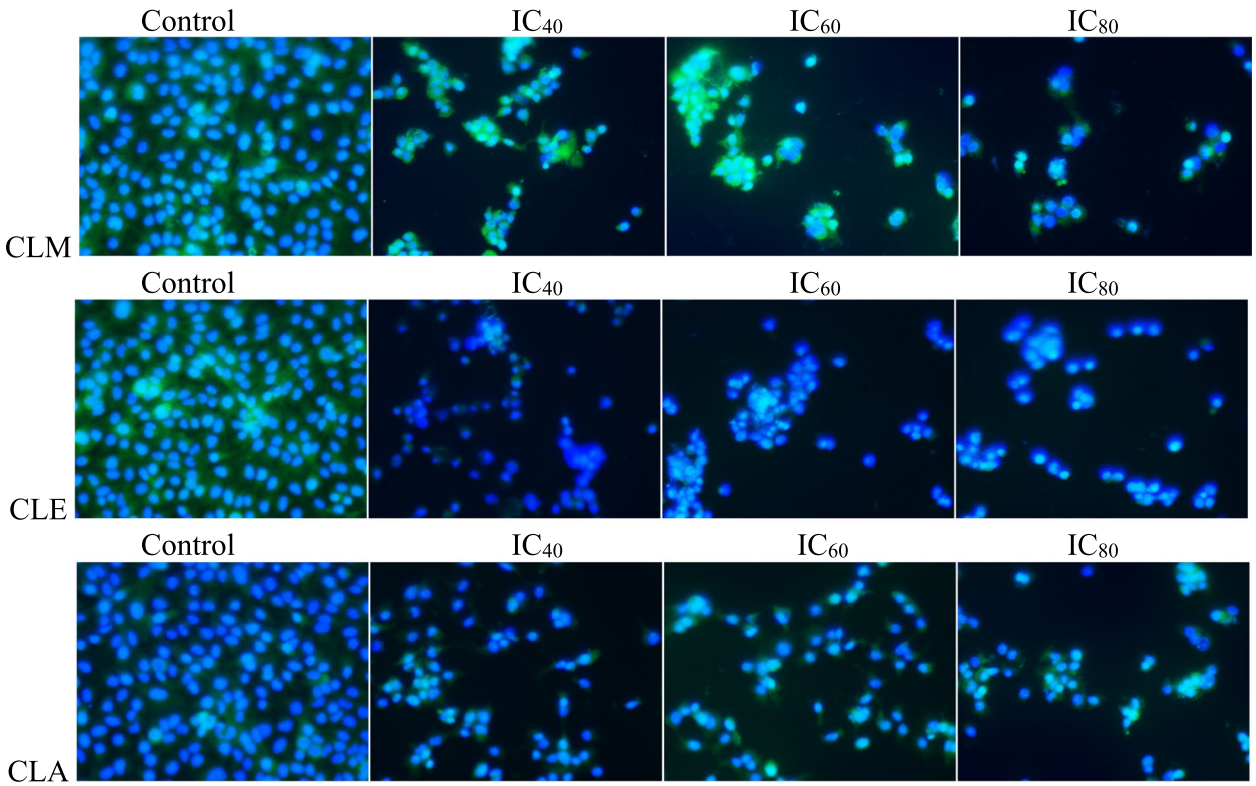


Fig. 4 Morphological alterations in KON cells after 24 h of exposure to IC_{40}, IC_{60}, and IC_{80} doses of CLM, CLE, and CLA, as detected by DAPI and FDA double staining

adhering to the surface of the 6-well culture plate. The KON cells exhibited typical epithelial cell behavior, indicating normal growth. Treatment with the IC_{40}, IC_{60}, and IC_{80} concentrations of CLM, CLE, and CLA altered the morphology and cell structure, and morphological alterations were observed. At the low concentration of IC_{40}, the cells appeared polygonal or spindle-shaped, with well-defined boundaries, similar to the untreated cells. However, as extract concentration was increased to IC_{60} and IC_{80}, the cells shrank, developed small nuclei, and became irregular, with condensed cell components and cell structure, as evidenced by an alteration in cell shape from epithelial-like cells to rounded and small nuclei. The cytotoxic effects of the extract manifested in repressing the growth and proliferation of KON cells, inducing loss of membrane integrity, shrinkage, membrane blebbing, and apoptosis. The proportion of cell death and apoptosis increased in a concentration-dependent manner. Based on the study's findings, *C. lentillifera* extract has strong anti-cancer properties and may soon be developed into a useful oral cancer treatment.

Effects of the extracts of *C. lentillifera* inducing cell death and cell apoptosis in KON cells

PI labeling and flow cytometry were utilized to induce apoptotic cell death in KON cells treated with extracts of *C. lentillifera*, as shown in Fig. 6. The abnormal modification of the nucleus by binding DNA inside the KON cells was examined using PI staining. The results demonstrated that the CLM, CLE, and CLA induced cell death in a dose-dependent manner after treatment with IC_{40}, IC_{60}, and IC_{80} concentrations for 24 h. The proportion of PI-positive cells in the KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLM was 39.68 ± 2.702, 31.76 ± 1.348, and $45.93 \pm 0.339\%$; the proportion of PI-positive cells in KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLE was 4.10 ± 0.223, 36.10 ± 0.407, and $52.16 \pm 0.192\%$; and the proportion of PI-positive cells in KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLA was 3.70 ± 0.165, 38.76 ± 1.202, and $61.89 \pm 2.653\%$, respectively. The results thus revealed that these extracts have the ability to induce cell death.

Effects of the extracts of *C. lentillifera* on intracellular reactive oxygen species (ROS) levels

ROS signaling molecules perform important functions in signal transduction and have been shown to be vital for maintaining cellular homeostasis. ROS can be an effective therapeutic strategy in anticancer therapy because it causes oxidative damage when it accumulates. To determine whether extracts of *C. lentillifera* have a similar role

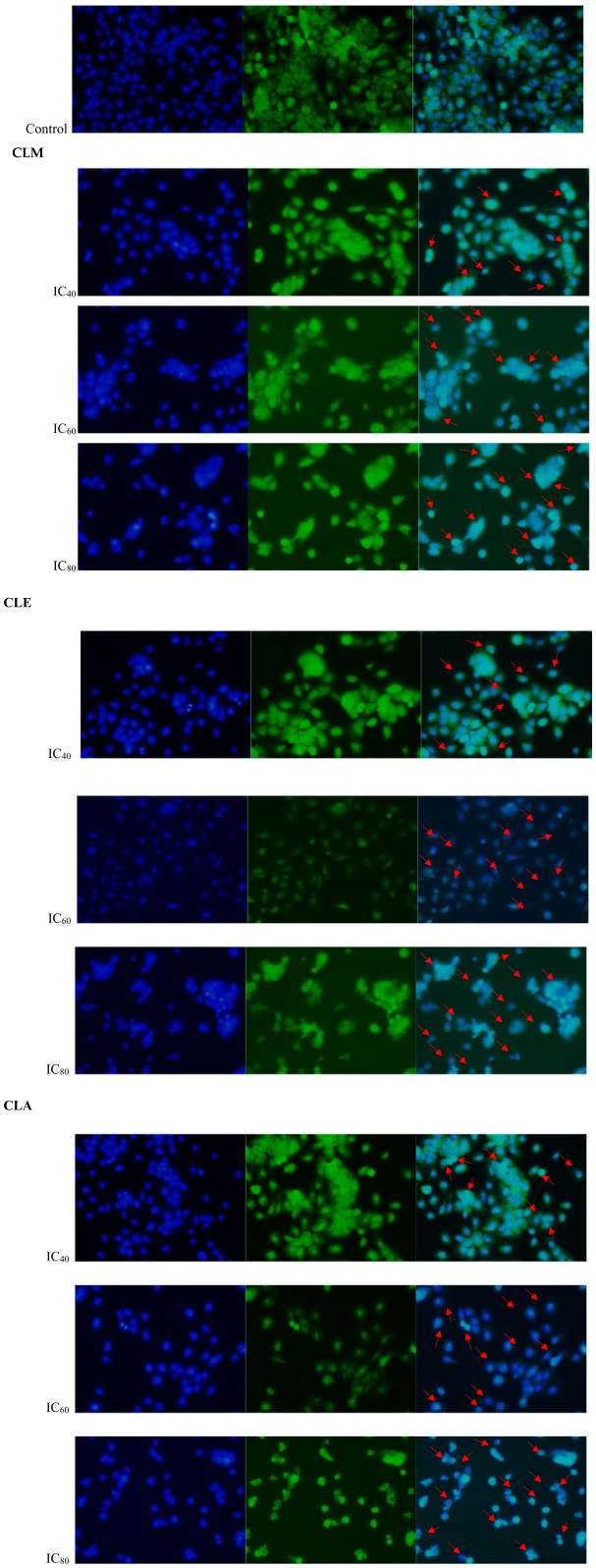


Fig. 5 Morphology of KON cells after 24 h with IC_{40}, IC_{60}, and IC_{80} concentrations of CLM, CLE, and CLA. The red arrows denote cell death and apoptotic nuclei

in KON cells, we examined the ROS levels in the KON cells. Increasing ROS levels were identified using the cell-permeable fluorogenic probe DCF-DA and validated with inverted fluorescence microscopy and flow cytometry. The KON cells were treated with extracts of *C. lentillifera* for 24 h before being labeled with DCF-DA to evaluate the intracellular ROS accumulation, which was then studied using an inverted fluorescence microscope and flow cytometry. As demonstrated in Fig. 7, inverted fluorescence microscopy of extracts of *C. lentillifera*-treated cells revealed a considerable increase in ROS accumulation compared to the untreated cells (Fig. 7a). The flow cytometry data showed that extracts of *C. lentillifera* caused a significant accumulation of ROS, and the extracts of *C. lentillifera*-treated cells accumulated much more ROS than the untreated cells (Fig. 7b). The percentages of ROS generation in the KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLM were 28.24 ± 4.566, 34.18 ± 2.636, and 9.427 ± 0.802%; the percentages of ROS generation in the KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLE of *C. lentillifera* were 11.41 ± 0.293, 17.87 ± 3.971, and 16.14 ± 0.575%; and the percentages of ROS generation in KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLA were 8.34 ± 1.226, 14.00 ± 0.9735, and 8.46 ± 2.271%, respectively, compared to the control group, which was 7.127 ± 2.488%. These findings suggest that the extracts of *C. lentillifera* can produce intracellular ROS in KON cells.

The effects of the extracts of *C. lentillifera* on the loss of mitochondrial membrane potential

To determine the role of mitochondria in triggering apoptosis in KON cells, the changes in mitochondrial membrane potential following *C. lentillifera* extract administration was examined. Rho123 dye was used to assess the mitochondrial membrane potential. Rho123 enters mitochondria only when the membrane potential is intact and remains there. When the membrane potential is lost, the dye is leached out of the mitochondria, resulting in a drop in fluorescence, which is proportional to the mitochondrial membrane potential. As shown in Fig. 8, the findings indicate that the extracts of *C. lentillifera* reduced the mitochondrial membrane potential of KON cells after 24 h of incubation according to the average % reduction of mitochondrial membrane potential in KON cells treated with IC_{40}, IC_{60}, and IC_{80} concentrations of CLM, CLE, and CLA. As shown in Fig. 8a and b, when treatment dosages were increased from IC_{40} to IC_{80} concentrations, the loss of mitochondrial membrane potential was greater than that of the control. The percentages of Rho123 uptake in the KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLM were 39.31 ± 1.970, 56.55 ± 1.467, and 87.26 ± 0.2853%; the percentages of Rho123 uptake in KON cells treated with IC_{40},

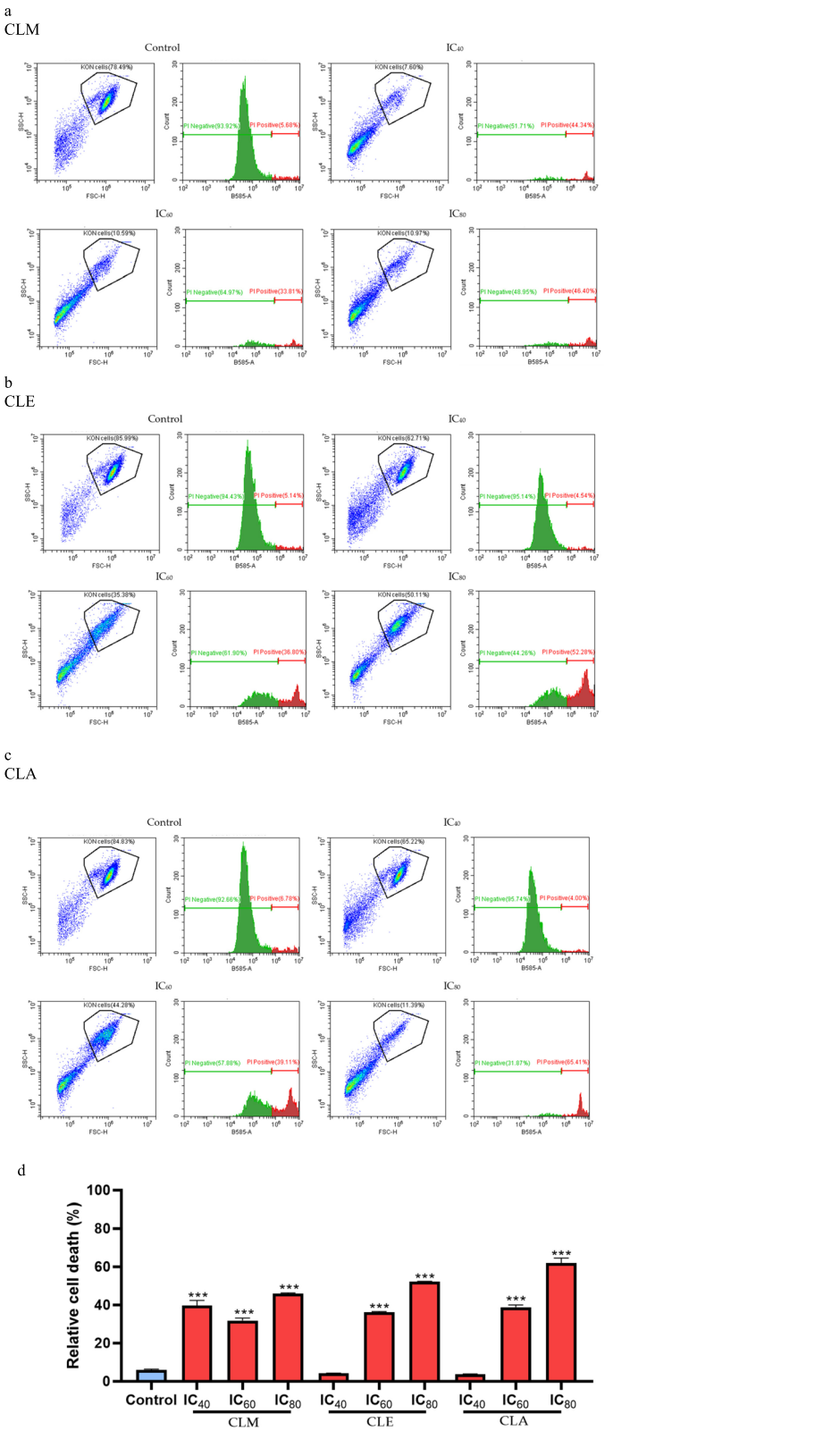


Fig. 6 Calculation of the cell death rate in the KON cells treated with IC_{40}, IC_{60}, and IC_{80} of CLM (**a**), CLE (**b**), and CLA (**c**) by employing flow cytometer analysis and PI staining. The data showed the relative cell death in the KON cells treated with CLM, CLE, and CLA at IC_{40}, IC_{60}, and IC_{80} after treatment for 24 h (**d**). The data are presented as the mean ± SEM ($n = 3$). ***$p < 0.001$ significantly different from the control

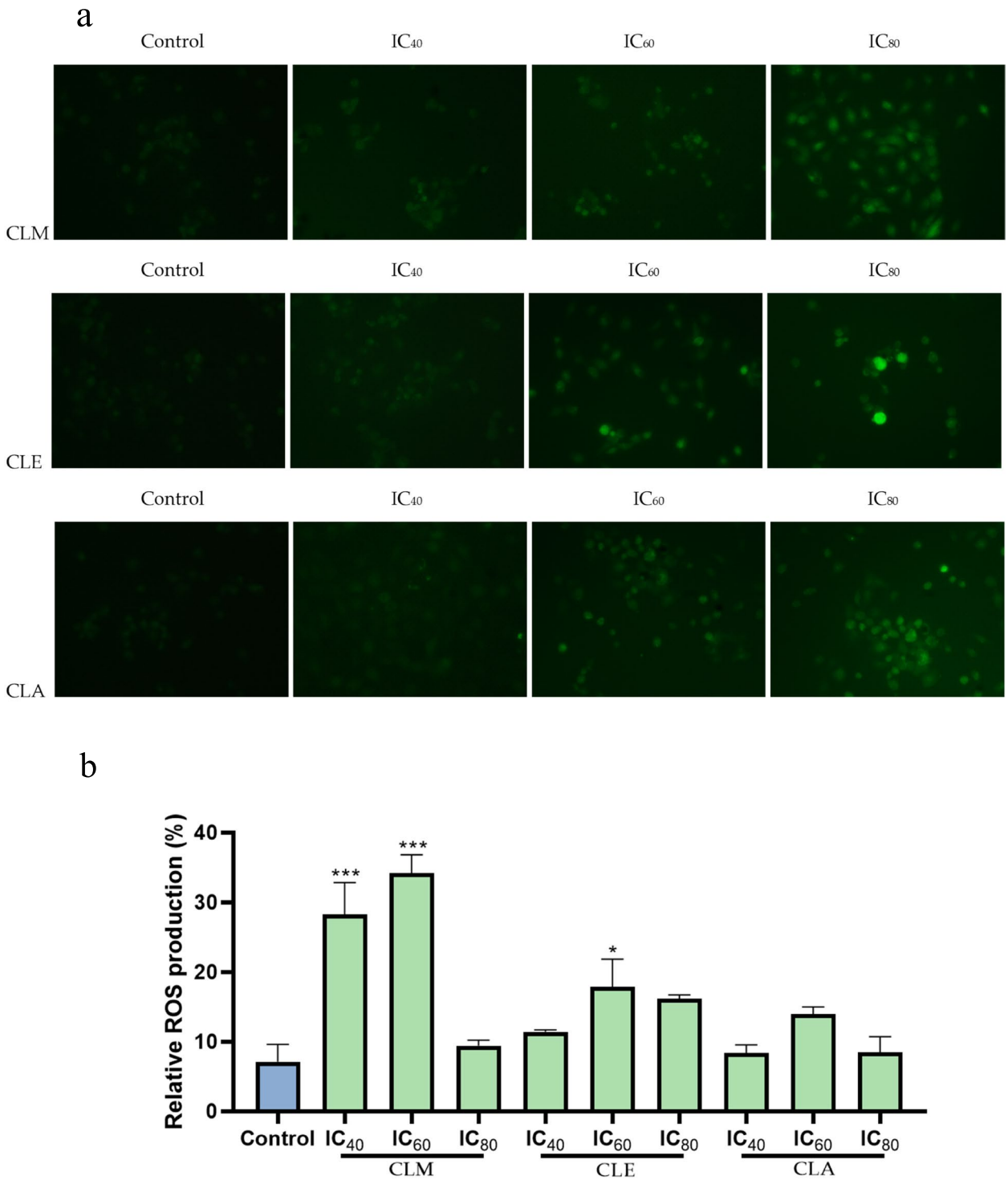


Fig. 7 The extracts of *C. lentillifera* caused intracellular reactive oxygen species buildup in KON cells. Inverted fluorescence microscopy images of ROS generation in the control and *C. lentillifera* extracts after 24 h of treatment (**a**). Flow cytometry study of reactive oxygen species (ROS) production in control cells and the *C. lentillifera* extract-treated KON cells throughout 24 h (**b**). A representative histogram of DCF-DA staining in treated KON cells with the columns presenting the average ± SEM of three experiments. * $p < 0.05$ compared to control; *** $p < 0.001$ compared to the control

IC_{60}, and IC_{80} of the CLE were 26.75 ± 3.362, 47.55 ± 2.919, and 48.25 ± 3.335%; and the percentages of Rho123 uptake in KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLA were 30.31 ± 2.084, 83.58 ± 1.037, and 85.13 ± 1.609%, respectively, compared to the control group, which was 14.92 ± 3.962%. All findings were statistically significant when compared to the untreated control group ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

Effects of the extracts of *C. lentillifera* on cell cycle progression in KON cells

The cell cycle distribution of the KON cells was substantially modified following a 24 h treatment with CLM (IC_{40}, IC_{60}, and IC_{80}). As seen in Fig. 9 and Table 4, the KON cell population treated with IC_{40}, IC_{60}, and IC_{80} concentrations in the S and G2-M phases decreased after treatment for 24 h compared to the untreated control cells. The KON cells were accumulated in the sub-G and G0-G1 phases, indicating that the CLM caused cell cycle arrest at these phases. After 24 h of treatment with CLE (IC_{40}, IC_{60}, and IC_{80}), the KON cells' cell cycle distribution changed noticeably. Also shown in Fig. 9 and Table 4, the KON cells displayed an increase in the percentage of cells in the G0-G1 phase after treatment. Correspondingly, the reduction in KON cells during the S-G2-M phase of the cell cycle indicates cell cycle arrest at this phase. While the IC_{60} and IC_{80} concentrations had a lower population in the G0-G1, S, and G2-M phases, the KON cells were accumulated in the sub-G phase after treatment for 24 h because the CLE induces cell death and cell apoptosis. Moreover, after being exposed to the CLA for 24 h, the KON cells' cell cycle distribution was significantly altered (IC_{40}, IC_{60}, and IC_{80}). As demonstrated in Fig. 9 and Table 4, in comparison to the untreated control cells, the numbers of KON cells in the S and G2-M phases of the cell cycle decreased following treatment. Additionally, KON cells accumulated in the sub-G and G0-G1 phases of the cell cycle, indicating that the CLA encouraged cell cycle advancement during these phases. These results imply that CLM, CLE, and CLA may cause cell cycle arrest and apoptosis in KON cells, and that the effect is concentration-dependent.

Effects of the extracts of *C. lentillifera* on colony formation in KON cells

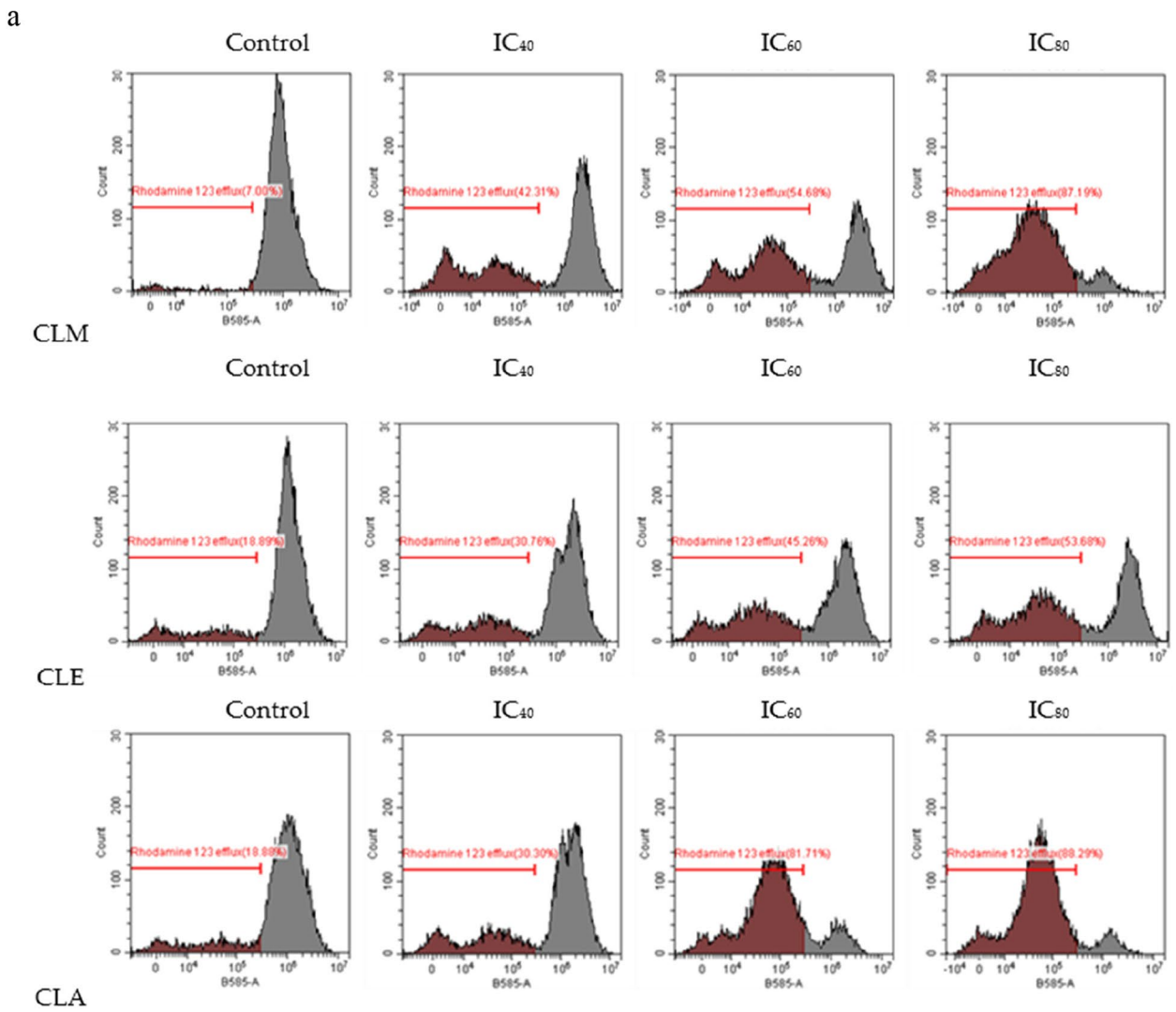
The KON cells' anchorage-dependent growth was assessed using colony formation assays. In general, cancer cells grow in colonies in contact with adjoining cells; losing contact with the adjacent cells causes the cancer cells to die. The colony formation experiment demonstrates that dosing the cells with the *C. lentillifera* extracts greatly decreased the KON cells' colony formation when compared to the control group. As demonstrated in Fig. 10, the percentages of colony formation in the KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLM were 33.33 ± 3.33, 0.00 ± 0.00, 0.00 ± 0.00%; the percentages of colony formation in the KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLE were 80.00 ± 5.77, 26.67 ± 6.66, 0.00 ± 0.00%; and the percentages of colony formation in the KON cells treated with IC_{40}, IC_{60}, and IC_{80} of the CLA were 53.33 ± 3.333, 3.33 ± 3.333, 0.00 ± 0.000%, respectively, compared to the control group, which was 100.0 ± 0.000%. The density quantification revealed that the *C. lentillifera* extracts at IC_{40} concentrations reduced colony growth by more than 20–70% compared to the control group ($p < 0.01$, $p < 0.001$), whereas treatment with the extracts at IC_{60} and IC_{80} concentrations reduced colony formation by more than 73–100% ($p < 0.001$). This experiment confirmed the anti-cancer properties of the *C. lentillifera* extracts.

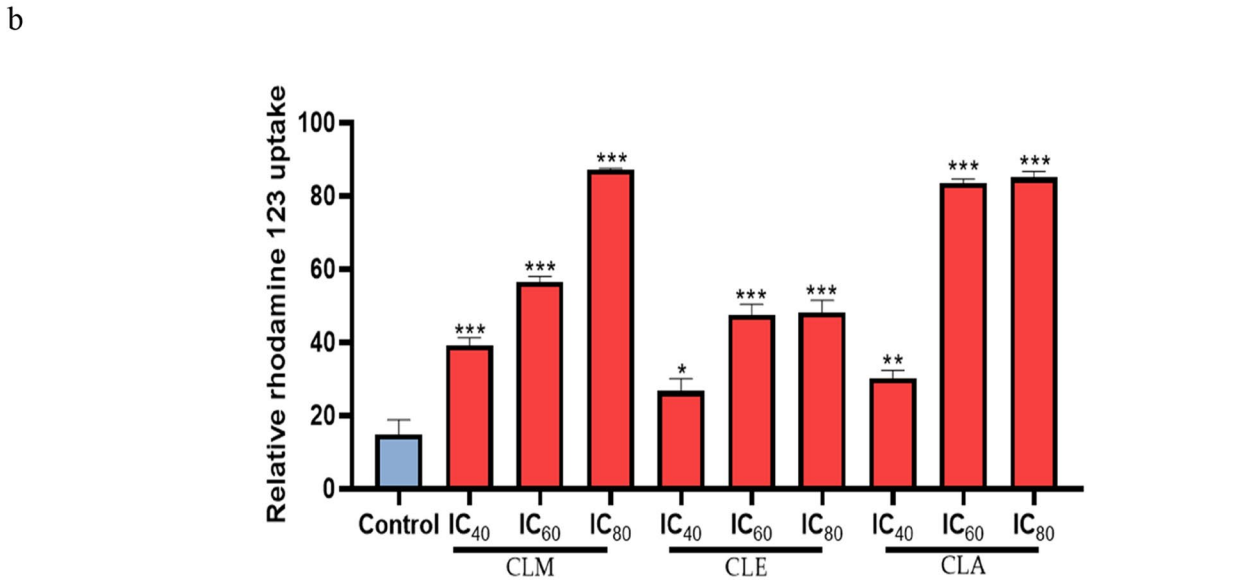
Effects of the extracts of *C. lentillifera* on cell migration in KON cells

Subcytotoxic doses of the compounds were chosen with the IC_{40} concentration that did not significantly affect cell viability in order to study the effect of the compounds on the migration of the cells. As demonstrated in Fig. 11, CLM, CLE, and CLA were found to be effective in suppressing KON cell migration to the wound area, as demonstrated by the outcomes of the wound scratch experiment. The percentage of migrating cells that reached the cell-free region serves as an indicator of the inhibition of migration. After 24–48 h of treatment, the reduction of KON cell migration occurred at IC_{40}. Reduced migration capacity, a concomitant drop in cell density in the cell-occupied area, and cell shrinkage coincided with the decline in the number of cells migrating to the cell-free area. At time 24 h, the percentages of cell migration inhibition in the CLM, CLE, and CLA were 8.217 ± 1.963 ($p < 0.05$), 3.99 ± 1.542, and 15.11 ± 2.271, ($p < 0.001$), respectively, compared to the control group, which was 0.00 ± 0.000. At 48 h, the percentages of cell migration inhibition in the CLM, CLE, and CLA were 12.02 ± 2.057 ($p < 0.001$), 4.718 ± 1.116, and 16.08 ± 0.2604,

(See figure on next page.)

Fig. 8 The decrease in mitochondrial membrane potential was quantified using rhodamine 123 labeling. KON cells were treated with CLM, CLE, and CLA at IC_{40}, IC_{60}, and IC_{80} for 24 h. Following incubation, the KON cells were stained with rhodamine 123 and flow cytometrically examined (**a**). These are the representative results (**b**). Data are from the evaluation of fluorescence intensity using three measurements. *$p < 0.05$, significantly different from the control; **$p < 0.01$, significantly different from the control; ***$p < 0.001$, significantly different from the control





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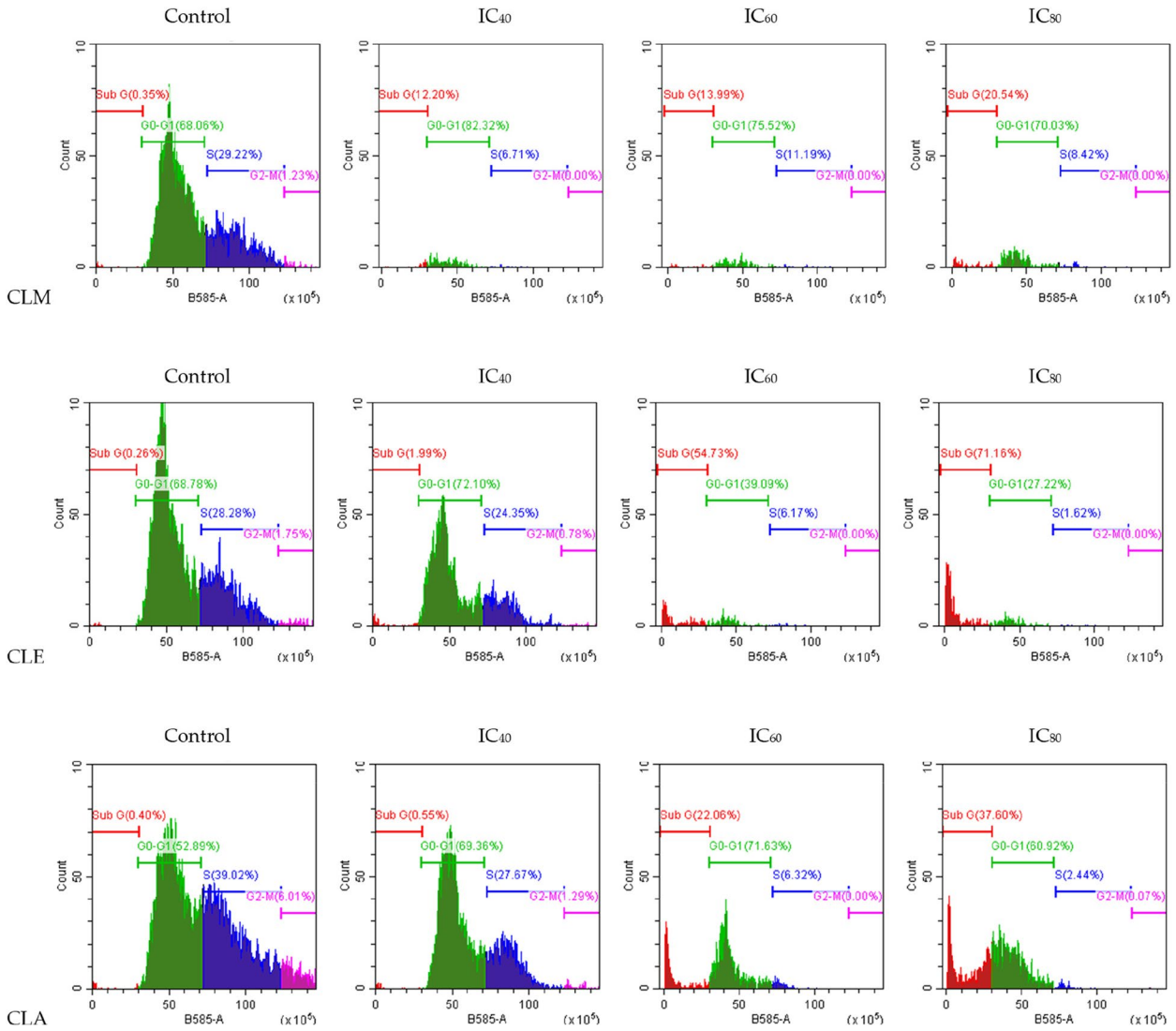

Fig. 9 Cell cycle arrest investigation using flow cytometry in KON cells treated with CLM, CLE, CLA for 24 h. The representative flow cytometry histograms show the cell cycle phases (% in the sub-G, G0-G1, S, and G2-M phases)

($p < 0.001$), respectively, compared to the control group, which was 0.00 ± 0.000. These findings show that *C. lentillifera* extracts may alter KON cells' capacity to migrate.

Effects of the extracts of *C. lentillifera* on cell migration and invasion in KON cells

As demonstrated in Fig. 12, the effects of *C. lentillifera* extract on cell migration and invasion were investigated using the transwell migration assay and the matrigel invasion test, respectively. The extracts were utilized in the experiment at an IC_{40} concentration that had negligible to no impact on cell viability. The ability of KON cells to migrate and invade was diminished by the *C. lentillifera* extracts, and CLA significantly reduced the KON cells' ability to migrate and invade after incubation for 48 h. The percentages of cell migration inhibition at IC_{40} concentration of the CLM, CLE, and CLA were 29.01 ± 12.60 ($p < 0.05$), 96.59 ± 0.760 ($p < 0.001$), and $100.0 \pm 0.000\%$ ($p < 0.001$); the percentages of cell invasion inhibition at IC_{40} concentrations of the CLM, CLE, and CLA were 47.28 ± 12.33 ($p < 0.01$), 58.38 ± 7.245 ($p < 0.001$), and $94.74 \pm 0.4459\%$ ($p < 0.001$), respectively; and the CLA produced the highest anti-migration as well as anti-invasion properties on the metastatic behavior of KON cells. Taken together, these findings suggest that the extracts of *C. lentillifera* are able to inhibit the migratory and invasive potential of KON cells.

Table 4 The percentages of cell cycle distribution in the KON cells treated with *C. lentillifera* extracts at IC_{40}, IC_{60}, and IC_{80} after 24 h

C. lentillifera extract	Cell cycle distribution (%)			
	sub-G	G0-G1	S	G2-M
Control	0.44 ± 0.073	61.64 ± 4.536	33.10 ± 3.006	3.36 ± 1.404
CLM IC_{40}	11.24 ± 1.734	79.50 ± 3.403	9.52 ± 2.626	0.00 ± 0.000
CLM IC_{60}	19.94 ± 3.896	70.04 ± 3.461	10.32 ± 0.450	0.00 ± 0.000
CLM IC_{80}	23.32 ± 1.800	70.47 ± 0.361	5.65 ± 1.574	0.31 ± 0.157
CLE IC_{40}	3.84 ± 1.853	71.07 ± 0.809	23.03 ± 1.179	1.17 ± 0.195
CLE IC_{60}	52.30 ± 1.973	44.25 ± 2.831	3.92 ± 1.153	0.00 ± 0.000
CLE IC_{80}	64.87 ± 3.162	32.70 ± 2.812	2.53 ± 0.497	0.00 ± 0.000
CLA IC_{40}	0.57 ± 0.119	68.29 ± 0.883	28.81 ± 0.798	1.32 ± 0.090
CLA IC_{60}	24.49 ± 1.217	70.12 ± 0.781	5.46 ± 0.452	0.00 ± 0.000
CLA IC_{80}	34.73 ± 2.070	61.64 ± 0.777	4.13 ± 1.140	0.09 ± 0.058

The values are expressed as mean ± SEM ($n = 3$)

Impact of the extracts of *C. lentillifera* on variations in the transepithelial electrical resistance (TEER) values in KON cells

As presented in Fig. 13, following treatment with the CLM, CLE, and CLA at IC_{40}, IC_{60}, and IC_{80} concentrations, the TEER values in the CLM treatment were 375.7 ± 1.764, 303.6 ± 1.225 ($p < 0.001$), and 279.1 ± 0.1202 millivolts ($p < 0.001$); TEER values in the CLE treatment were 319.0 ± 0.6692 ($p < 0.001$), 291.4 ± 0.6360 ($p < 0.01$), and 279.0 ± 0.7371 ($p < 0.001$) millivolts; and the TEER values in the CLA treatment were 380.9 ± 3.528 ($p < 0.001$), 286.2 ± 0.06667 ($p < 0.001$), and 275.7 ± 4.222 ($p < 0.001$) millivolts, respectively, in contrast to the control, which had a TEER value of 303.6 ± 0.5175 millivolts. The application of the CLM, CLE, and CLA resulted in a substantial increase in TEER values when compared to the control group at IC_{40} concentrations. These findings suggest that *C. lentillifera* extracts can modify the integrity of the cellular barrier and influence cell permeability, allowing extracts to migrate into KON cells. However, the IC_{60} and IC_{80} concentrations of *C. lentillifera* extracts significantly decreased the TEER values. The reduction of TEER values is an indicator of a compromised barrier or the loosening of the tight junctions of KON cells resulting from these extracts attacking the cell membrane and cell structure, as seen in the apoptotic staining experiment.

The cytotoxicity of the extracts of *C. lentillifera* on viable MRC-5 fibroblast cells

As demonstrated in Fig. 14, the cytotoxic effects of the extracts were evaluated against MRC-5 fibroblast cells to investigate their hazardous effects on normal cells. When compared to cancer cell lines, all extracts showed less cytotoxicity than the normal cell line, indicating that its effects are limited to cancer cells. The KON cells showed more growth inhibition and increased receptiveness to the extracts' treatment. The *C. lentillifera* extracts had a dose- and time-dependent cytotoxic effect on the cells. High levels of growth inhibition were seen at higher doses. The IC_{50} values of CLM, CLE, CLA were 729.8 ± 153.0, 664.2 ± 120.9, and 583.9 ± 44.42 µg/mL, respectively.

Discussion

The powerful actions of marine carotenoids against numerous cancer cell lines have been linked to transcription factors, cell proliferation, metastasis, and angiogenesis. Seaweed has valuable bioactive chemicals with anti-inflammatory, antioxidant, antidiabetic, anti-obesity, and anti-cancer properties. Marine green algae produce a variety of active components with different functional structures and biological capabilities, including polyphenols, peptides, and polysaccharides [29, 30]. Interestingly, natural populations of *C. lentillifera* have varying nutritional and biochemical properties due to environmental factors such as predation, sedimentation, salinity, temperature, pollution, and nutrients; thus, different geographical growing fields can contribute to varying levels of nutrients and secondary metabolites [21]. The yield of bioactive compounds, the kind of compounds extracted, and the degree of biological activity are largely determined by the type of extraction solvents used. In this study, the extracts were obtained by the maceration process. This traditional extraction approach was chosen for its simplicity, cost-effectiveness, and low equipment requirements. The extraction at room temperature while protected from light helped to reduce thermal and light degradation substances [31]. Furthermore, maceration was commonly employed in previous studies of *C. lentillifera* and related algae, allowing for a direct comparison [21, 32]. Three solvents with different polarity including methanol, ethanol, and acetone were used to create the *C. lentillifera* extracts. Stronger polarity was correlated with the highest concentration of the extract in the methanol solvent, and it is thought that methanol may dissolve both hydrophilic and lipophilic components in plants, thereby increasing yield [33, 34].

TPC, TFC, chlorophyll a, and chlorophyll b were found to be greater in the CLA. TPC values in the previously described investigation differed considerably depending on the extraction solvent utilized [35]. In accordance with earlier research, phenolic groups exhibit varying solvent affinities, ranging from very polar to nonpolar. It has been demonstrated that acetone is the most efficient extraction medium for polyphenols found in a variety of seaweeds [36]. Consequently, the strong antioxidant activity of the *C. lentillifera* extracts may be explained by the

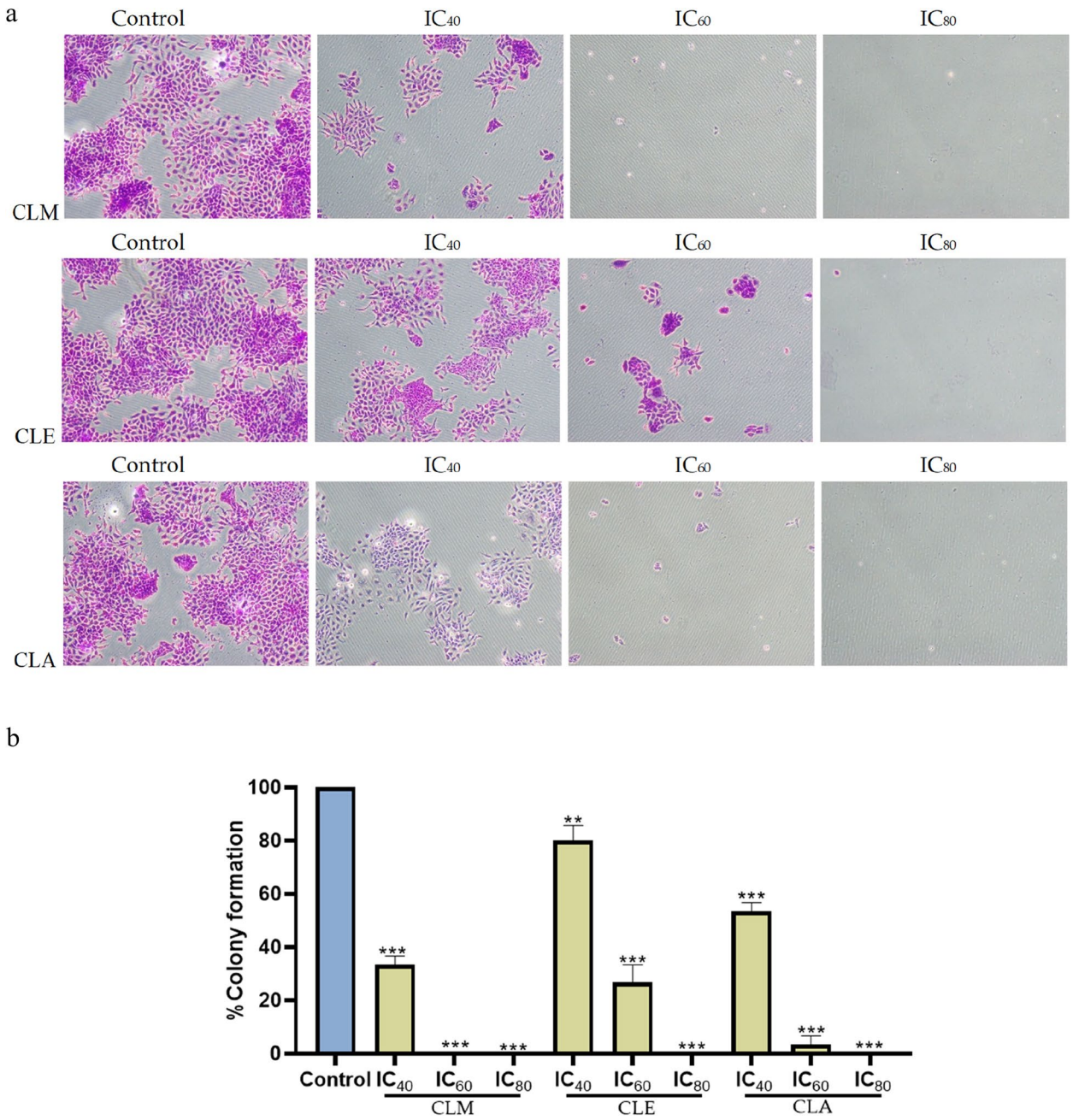

Fig. 10 Effects of the extracts of *C. lentillifera* on colony formation in KON cells (**a**). KON cells were grown with IC_{40}, IC_{60}, and IC_{80} amounts of CLM, CLE, and CLA for 24 h. Cells were washed with 1X PBS and further cultured in a 6-well plate for 7 days. Each bar shows the mean ± SEM of the three separate experiments (**b**). $^{**}p < 0.01$, significantly different from the control, $^{***}p < 0.001$ significantly different from the control

presence of TPC, TFC, chlorophyll a, and chlorophyll b in them. The highest total polyphenol concentration was found in brown seaweed species, and this amount was connected with the highest potential for antioxidants. Through LC-QTOF MS/MS analysis of the CLA, its phytoconstituents were characterized, and the most abundant compounds were 13-sophorosyloxydocosanoic acid (11.994%), jubanine C (7.938%), cysteinyl-tyrosine (4.860%), 11,12-dihydroxybenzo[a]pyrene (4.860%), D-Vacciniin (4.857%), 2-amino-4-oxo-6-(1,2-dioxoprolyl)−7,8-dihydroxypteridine (4.856%), momordicoside K (3.390%), polyoxyethylene (600) mono-ricinoleate (3.165%), na-p-hydroxycoumaroyltryptophan (2.640%), and oryzarol (2.30%). Eight seaweeds were found to have a total of 54

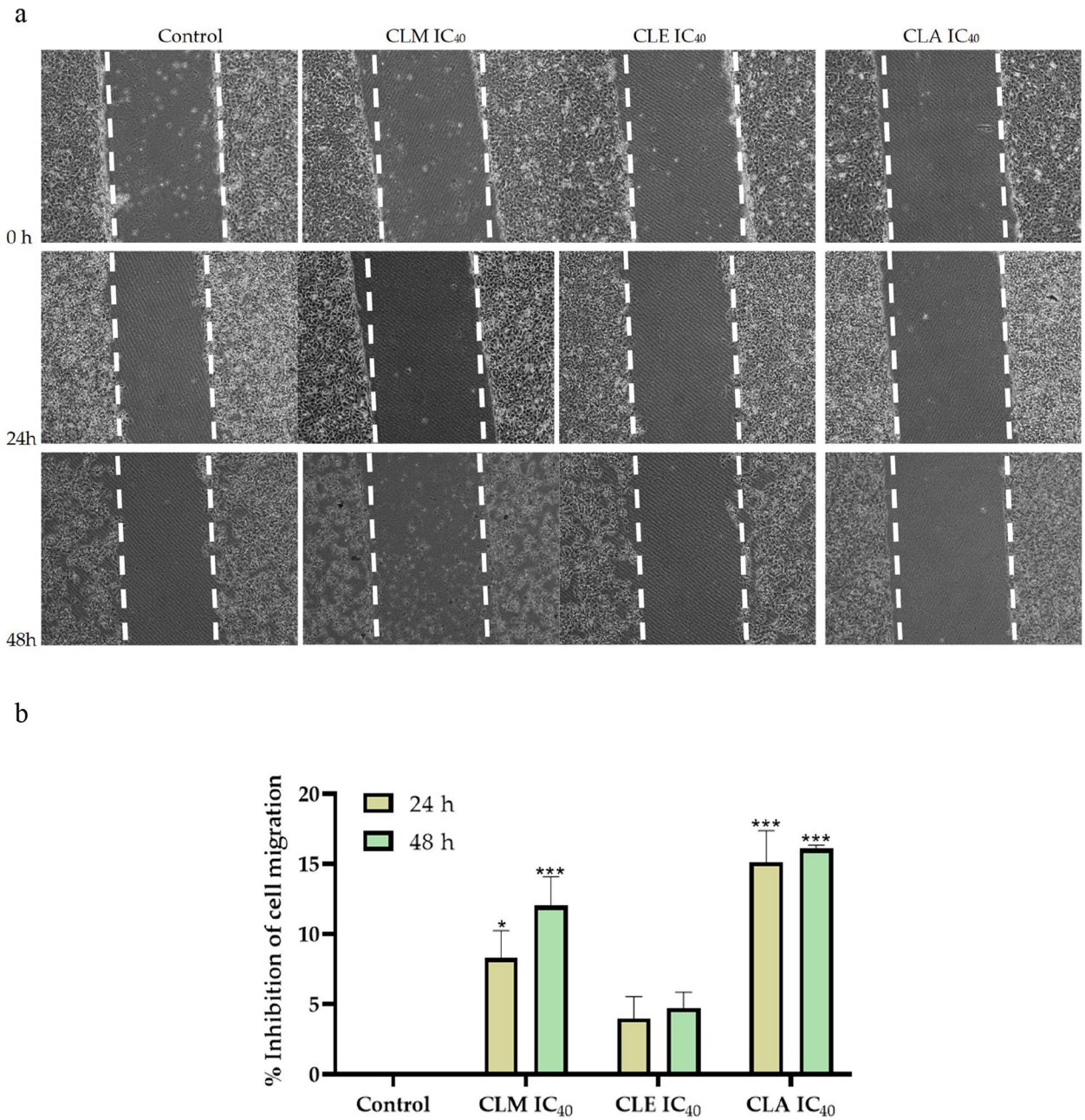

Fig. 11 Representative images (10x) of the open wound area of KON cells after incubation with CLM, CLE, and CLA at 0, 24, and 48 h (**a**). The inhibition of cell migration (%) of the KON cells in each treatment is displayed in the bar graph. CLM, CLE, and CLA at IC_{40} were added to the KON cells and incubated for 24 and 48 h (**b**). At every stage, the extent of the wounds was measured and reported as a percentage of the reduced area when compared to the initial 0 h of incubation. Each bar shows the mean ± SEM of three separate experiments. *$p < 0.05$, significantly different from the control, ***$p < 0.001$ significantly different from the control

phenolic compounds, according to the LC-ESI-QTOF-MS/MS analysis. *Centroceras* sp. had the highest concentration of phenolic chemicals, with *Ecklonia* sp. and *Caulerpa* sp. following closely, and is a good source of antioxidant-rich polyphenols that are compatible with seaweed's potential health benefits in culinary, medicinal, and nutraceutical applications [37]. However, the different solvent extracts might contain different chemical components, thus the full LC–MS profile of CLM and CLE should be further investigated. The yield of CLE was consistently higher than that of the CLA and CLM. Nevertheless, all three extracts showed signs of antioxidative activity, with the CLE displaying the

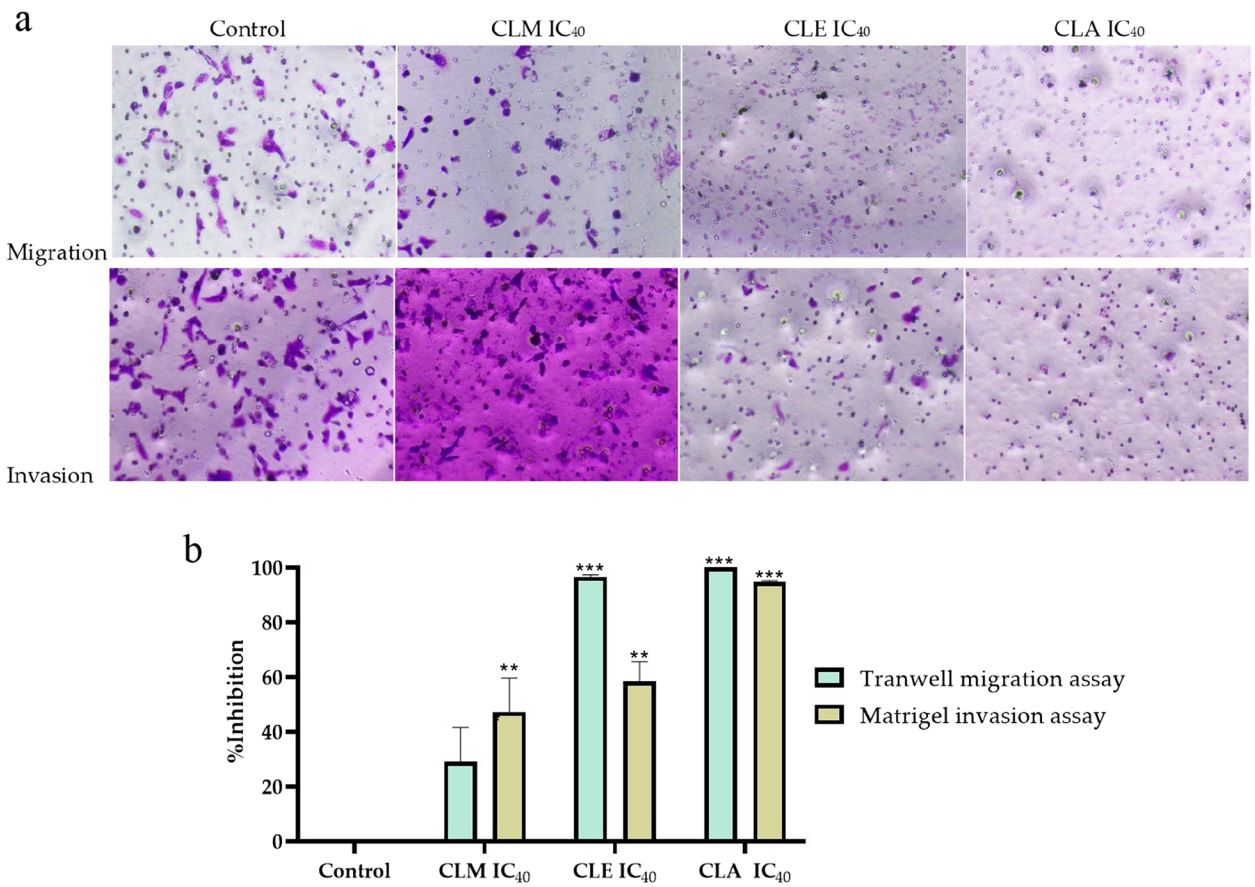

Fig. 12 For the migratory and invasive assays, the KON cells were reconstituted in a medium devoid of serum. The cells were then seeded with CLM, CLE, and CLA at IC_{40} concentration in the upper chamber of the apparatus for 48 h. In order to serve as a chemoattractant, 10% FBS was added to the DMEM medium in the lower system. The migrating and invaded cells were preserved with ice-cold methanol and then colored with crystal violet. The KON cells were photographed using an inverted microscope with 10 × magnification (**a**). The data are presented as a percentage of suppression of cell migration and invasion (**b**). $^{**}p < 0.01$ significantly different from the control, $^{***}p < 0.001$ significantly different from the control

highest levels of these qualities in comparison to the methanol and acetone extracts. The variations in the quantity and type of antioxidant chemicals, such as carotenoids, phenol, and ascorbic acid, that are present in these extracts may be the cause of the variations in antioxidant content [38]. Previous studies have demonstrated the total antioxidant activity of organic extracts from 37 algal samples, which included 30 species of Hawaiian algae from 27 taxa, was measured. The extract of *Turbinaria ornata* was discovered to be the most active. Bioassay-guided fractionation of this extract resulted in the separation of several distinct carotenoids as active principles. Fucoxanthin, a carotenoid, was discovered to be the most powerful bioactive antioxidant. The Hawaiian algae have high antioxidant activity, which could lead to their usage in several valuable healthcare or related goods, as well as in the chemoprevention of a variety of diseases, including cancer [39]. Another study found a link between antioxidant and anti-cancer activities. The effects of Italicize Spirulina (Spirulina), and two cell lines of A549 and HFF were treated with the IC_{50} concentration for 24 h. MTT tests revealed that the largest loss in cell viability occurred at a dose of 500 µg/mL. The results demonstrated that the extract affected the A549 cell cycle. A. platensis extract had a notable effect on the lung cancer cell cycle, arresting it in phase G2, preventing cancer cells from entering phase M and proliferating. Furthermore, the algal extract demonstrated concentration-dependent antioxidant activity [40].

The present study showed that the CLM, CLE, CLA may be potential novel anti-cancer agents that originate from marine environments and have specificity for cancer cells. The MTT assay revealed that *C. lentillifera* extracts effectively inhibited the number of viable KON cells compared with the control group. The viability of the KON cells was most efficiently decreased by the CLA compared to the CLE and CLM. As previously studied,

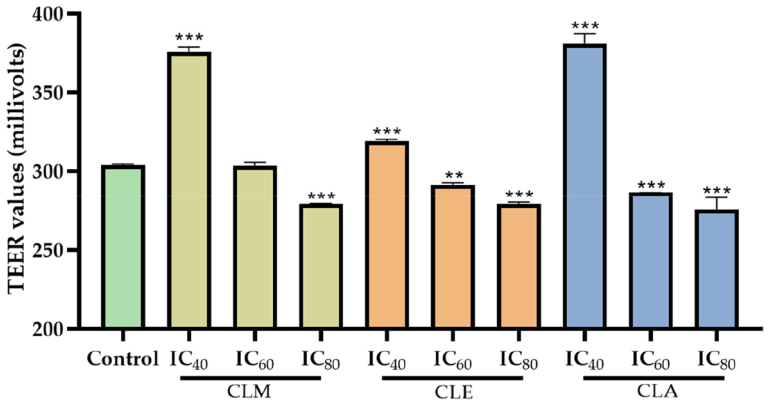

Fig. 13 To measure the transport of *C. lentillifera* extracts to KON cells and assess the TEER values as a measure of the cell barrier's integrity, KON cells were planted in a 6-well plate and incubated with *C. lentillifera* extracts for a duration of 24 h. A Millicell® ERS-2 Voltohmmeter was used on the cell monolayer to quantify the TEER readings. The data are presented as TEER values (millivolts). mean ± SEM ($n = 3$) was used to express the data. $^{**}p < 0.01$, significantly different from the control, $^{***}p < 0.001$, significantly different from the control

24 h after incubation, A172 human glioblastoma (GBM) cell viability was significantly reduced by increasing the concentrations of *C. lentillifera* hexane solvent extracts (CLHE) from 200 to 1,000 µg/mL in comparison to the untreated and 0.25% DMSO-treated groups. The CLHE extracts' 50% inhibitory concentration (IC_{50}) against A172 cells was 224.7 ± 5.205 µg/mL [23]. Moreover, *C. lentillifera* showed anti-cancer properties in ME (Maceration—Ethanol), MH (Maceration—n-Hexane), and SE (Soxhletation—Ethanol) as potential bioresource agents for anti-cancer medications, particularly for leukemia, breast, colorectal, and hepatoma malignancies in humans [21]. The correlations between the variables: cytotoxicity, antioxidant activity, TPC, TFC, chlorophyll a, and chlorophyll b constituents were analyzed using Pearson's correlations. The results indicated that cytotoxicity exhibited a strong negative correlation with TPC ($|r| = 0.95$, $p = 0.205$), TFC($|r| = 0.98$, $p = 0.114$), chlorophyll a ($|r| = 0.83$, $p = 0.379$), and b ($|r| = 0.83$, $p = 0.373$) whereas antioxidant showed a strong correlation with TFC ($|r| = 0.83$, $p = 0.374$), moderate correlation to TPC ($|r| = 0.46$, $p = 0.693$), and weak correlation to chlorophylls a ($|r| = 0.21$, $p = 0.868$), and b ($|r| = 0.22$, $p = 0.861$). None of these correlations were statistically significant ($p > 0.05$). Regarding relationships among chemical constituents, strong positive correlation was observed between TPC and TFC ($|r| = 0.88$, $p = 0.319$), TPC and chlorophyll a ($|r| = 0.96$, $p = 0.174$), TPC and chlorophyll b ($|r| = 0.97$, $p = 0.168$), TFC and chlorophyll a ($|r| = 0.71$, $p = 0.494$), TFC and chlorophyll b ($|r| = 0.72$, $p = 0.487$). However, these correlations were not statistically significant. The only statistically significant correlation was observed between chlorophyll a and chlorophyll b ($|r| = 0.99$, $p = 0.006$).

DAPI and FDA double staining revealed that *C. lentillifera* extracts affect cell structure and morphology, impacting the ability to attach the coverslip to the culturing plate, resulting in programmed cell death. Moreover, the apoptotic characteristics were established by Hoechst 33,258 and AO double staining, which demonstrated that *C. lentillifera* extracts cause cell death and cells undergoing apoptosis at IC_{40}, IC_{60}, and IC_{80} concentrations. Apoptosis is characterized by many morphological and biochemical changes, including nuclear condensation, DNA fragmentation, apoptotic body formation, cell shrinkage, and blebbing [41]. KON cells' apoptotic-like characteristics were observed with an inverted fluorescent microscope. The KON cells treated with *C. lentillifera* extracts had the following characteristics: cell rounding, shrinkage, membrane blebs, loose contact with neighboring cells, production of apoptotic bodies, and cell vacuolization. For many anti-cancer treatments, one of the most crucial requirements is the ability to inhibit the growth of cancer cells and/or induce apoptosis [42,

(See figure on next page.)

Fig. 14 The shape of MRC-5 cells after treatment with various doses of *C. lentillifera* extracts (**a**). The cytotoxic effects of CLM (**b**), CLE (**c**), and CLA (**d**) on MRC-5 cells. The three extracts were added to MRC-5 cells at concentrations of 7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL for an entire day. Following treatment for 24 h, graphs were created to show the percentage of cytotoxicity (**e**). Cell cytotoxicity was assessed using the MTT colorimetric assay. mean ± SEM ($n = 3$) was used to express the data. $^{*}p < 0.05$, significantly different from the control, $^{**}p < 0.01$ significantly different from the control, $^{***}p < 0.001$ significantly different from the control

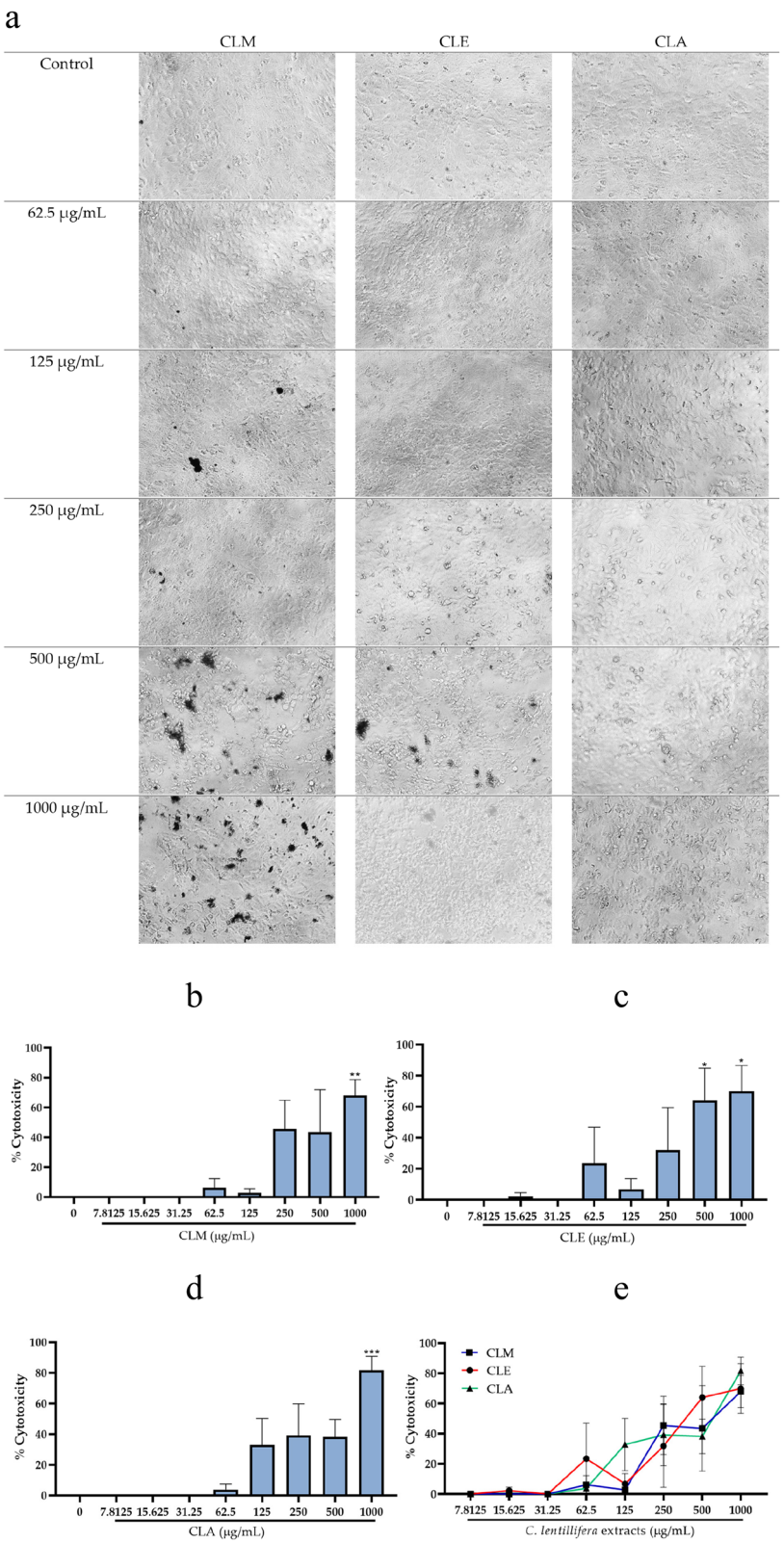

Fig. 14 (See legend on previous page.)

43]. KON cells treated with *C. lentillifera* extracts showed significant increases in apoptotic cell populations, suggesting potential proapoptotic characteristics. Furthermore, increasing the concentration of *C. lentillifera* extracts resulted in the formation of late apoptotic or secondary necrotic cells, which demonstrated a loss of membrane function as seen when using propidium iodide with a flow cytometer. The study's flow cytometric analysis demonstrated that KON cells experienced a high incidence of PI positive labeling and cell death when exposed to *C. lentillifera* extracts. One component of seaweed known as polyphenols can be employed as an anti-cancer drug by promoting oxidative stress, causing apoptosis, and inhibiting proliferation [44]. Previous research on HeLa cells, which are cervical cancer cells, demonstrated the antiproliferative activity of *Caulerpa racemosa* (*C. racemose*), which was characterized by a decrease in cell viability and an increase in apoptotic activity. *C. racemosa* extracts at 50 µg/mL, 100 µg/mL, 200 µg/mL were administered, and 0 µg/mL was used for the control. The extract from *C. racemosa* markedly boosted the production of the proapoptotic protein BAX, cleaved caspase-3, and overall apoptosis, while reducing the viability of HeLa cells [45]. Cancer cells are more susceptible to sudden increases in ROS levels than normal cells, and ROS generated during various conventional treatments can mediate proapoptotic effects in cancer cells [46]. As a result, some chemicals that increase the intracellular ROS in cancer cells to a hazardous level can cause mitochondrial damage and cell death [47]. In the current work, higher ROS levels in *C. lentillifera* extract-treated cells indicated that the extract induces ROS-mediated death in KON oral cancer cells. In accordance with previous research, laminarin-induced apoptosis and mitochondrial impairment were evidenced by depolarized mitochondrial membranes, calcium disruption, and suppression of migratory cells caused by reactive oxygen species production and related intracellular signaling pathways [48]. The depolarization of the mitochondrial membrane and subsequent rise in permeability of the outer membrane following pore formation are critical and reflective stages in the intrinsic apoptotic process. This is followed by the release of proapoptotic proteins and cytochrome c. Rho123, a fluorescent dye, is a particular probe for detecting ΔΨm variations in live cells [49, 50]. The current investigation also used flow cytometry to assess the impacts of the extracts on mitochondrial membrane disruption in KON oral cancer cells. The outcomes showed that the cells provided a strong reduction in ΔΨm following treatment with the extracts, which confirmed that the mitochondrial membrane played a significant role in *C. lentillifera* extract induced cell death in KON oral cancer cells. Mitochondrial malfunction has been found to play a role in the production of apoptosis and has even been proposed as a major component of the apoptotic cascade. Opening the mitochondrial permeability transition pore has been shown to depolarize the transmembrane potential (ΔΨm), release apoptogenic substances, and reduce oxidative phosphorylation. In some apoptotic systems, loss of ΔΨm may occur early in the process [51]. In the current study, an increased ROS level along with reduced mitochondrial function in *C. lentillifera* extract-treated cells indicate that *C. lentillifera* extracts induce ROS-mediated apoptosis in KON oral cancer cells. The production of ROS disrupted mitochondrial function, leading to both internal and extrinsic pathways of apoptosis. Certain anti-cancer and DNA damaging agents are known to function by stopping the cell cycle at certain points and causing cancer cells to undergo apoptosis. The sequence of actions that take place in a cell and cause it to divide and duplicate is known as the cell cycle. The four distinct phases of the cell cycle are sub-G, G0-G1, S, and G2-M, which are characterized by sets of macromolecular assemblies. Cyclin-dependent kinases (CDKs) interact with certain partner proteins, called cyclins, to regulate the cell cycle and protect cell division at multiple checkpoints. Any stage of dysfunction triggers an unchecked cell cycle that eventually results in cancer formation, which is mostly linked to changes in CDK activity [52, 53]. Notably, the results from the flow cytometer analysis presented the proportion of cell death and PI-positive KON cells. Further analysis demonstrated that *C. lentillifera* extracts blocked the cell cycle of KON cells in the sub-G and G0-G1 phases, thereby decreasing the progression of KON cells to the S and G2-M phases of the cell cycle. At the IC_{80} concentration, high doses of *C. lentillifera* extracts bring KON cells together in the sub-G phase and cause cell death and debris cells. The alteration pattern of cell division in the KON cells treated with *C. lentillifera* extracts was observed, which further confirms the antiproliferative potential of these extracts. Depending on the cell line, extract concentration, and treatment duration, the extract's reaction and decreases in cell growth can occur [54]. Additional experiments will be performed in the future to further investigate the anti-cancer mechanisms of *C. lentillifera* extracts toward the inhibition of KON cells and to confirm their potency. Since the primary cause of death for cancer patients is connected to metastatic progression, the study of cell migration in cancer research is quite interesting and beneficial. The extracellular matrix (ECM), blood circulation, and distant foci are the pathways via which cancer cells move and infiltrate in order to propagate and disseminate throughout the body [55, 56]. Clonogenic cell survival and colony formation assays verified the *C. lentillifera* extracts' antiproliferative efficacy. The clonogenic assay

is a versatile and widely used instrument for quantifying reproductive cell survival in vitro, and it is based on a single cell's ability to develop into a colony. This assay assesses cancer cells' capacity to multiply into colonies unhindered by growth inhibitors [57, 58]. The present investigation found that the colony-forming potential of KON cells was greatly suppressed by *C. lentillifera* extracts, thus confirming its efficiency against metastatic colonization in KON cells. Similarly, the non-polar chemicals found in the hexane fraction of *Chnoospora minima* (*C. minima*) decreased cell growth and triggered apoptosis-mediated cell death in RMS and MCF-7 cells. Additionally, the hexane fractions of *C. minima* greatly hindered RMS and MCF-7 cells' capacity to form colonies [59].

One of the key characteristics of cancer is metastasis, which continues to be the primary cause of mortality resulting from cancer. Through local invasion, cancer cells separate from the main tumor, enter the circulatory system, and then flee from the blood vessels to colonize other locations. Once the metastatic cells have split off from the original tumors, they start producing matrix metalloproteinases that break down the basal membrane and invade the extracellular matrix [60–63]. To determine the anti-metastatic effects of the extracts, the non-toxic concentrations at IC_{40} concentration and allowing over 60% cell survival were selected. To further verify the effects of *C. lentillifera* extracts on cell motility, wound healing, trans-well migration, and invasion, experiments were carried out. In the current work, the anti-metastatic qualities of *C. lentillifera* extracts were ascertained using the in vitro wound scratch assay. According to our findings, KON cells migration was considerably reduced in *C. lentillifera* extract-treated groups at IC_{40} dose as compared to the untreated control samples. This finding may point to the extract's potential anti-metastatic qualities. The findings also showed that *C. lentillifera* extracts could drastically decrease KON cell movement and invasion compared with the untreated cells. While the exact mechanism underlying the anti-migration and anti-invasion actions is yet unknown, the results showed that the *C. lentillifera* extract could inhibit oral cancer cell lines' ability to metastasize. Our data show that *C. lentillifera* extracts can effectively suppress cell migration and invasion, in addition to its antiproliferative and proapoptotic actions. Our study is the first to show how *C. lentillifera* extracts affect the cell cycle, apoptosis, and motility of oral cancer cells, adding to our understanding of how it works. To create a novel chemotherapeutic drug in the future, more research must concentrate on the mechanisms involving anti-metastasis and the main active ingredients both in vitro and in vivo. A commonly used quantitative method for assessing the integrity of tight junction dynamics in cell culture models of endothelial and epithelial monolayers is known as transepithelial/transendothelial electrical resistance, or TEER. Cell barrier integrity is a crucial factor in assessing the passage of medications, materials, or chemicals of interest across an epithelial barrier, and TEER values are a necessary step towards achieving this goal [64, 65]. The TEER results additionally demonstrated that the treatment with IC_{40} concentrations of *C. lentillifera* extracts raised the TEER of the KON cell monolayer. These findings verify that the KON cell monolayer's permeability was dramatically reduced following treatment with *C. lentillifera* extracts [66]. The TEER results consistently show that the carnosine treatment raised the TEER of the EA.hy926 endothelial cell monolayer. These findings demonstrate that the carnosine therapy considerably reduced the permeability of the EA.hy926 colorectal cancer cells, which in turn affected the regulation of cell adhesion and extravasation during metastasis [67]. However, the higher concentrations of *C. lentillifera* decreased the TEER values, which means that the cell structure and cell integrity were disrupted. The cytotoxic effects of the *C. lentillifera* extracts were evaluated against the MRC-5 fibroblast cell line in order to investigate the extracts' harmful effects on these cells. When compared to the KON oral cancer cell lines, the extracts showed less cytotoxicity towards the normal cell line, indicating that their actions are limited to cancer cells. Normal cells are less sensitive to drugs or chemicals than cancer cells; nonetheless, genetic mutations or other changes make cancer cells more vulnerable to specific therapies. The inability of existing treatments to target cancer cells while sparing the normal cells that surround the cancer cells is one of their primary problems. The results of this investigation, however, showed that the *C. lentillifera* extracts exhibited mild toxicity against normal cells. Previous research found 1446 samples of 306 species of marine algae along Japan's beaches. The algal extracts were produced in two steps: first with phosphate-buffered saline (PBS), then with methanol, and then examined for in vitro selective anti-cancer activity against murine lymphoid leukemia L1210 cells and minimal cytotoxic activity against NIH-3T3 mouse fibroblast cells. 47 species of algae exhibited strong cytotoxic activity against L1210 cells, as well as equivalent cytotoxicity against mouse NIH-3T3 normal cells. However, four species of green algae had high action against L1210 cells while causing no harm in normal cells [68]. This study was the first to show how *C. lentillifera* extracts affect oral cancer cells, with particular attention paid to how they reduce metastatic behavior, induce apoptosis, mitochondrial dysfunction, and ROS-mediated cell death, and exhibit cell cycle arrest properties at the sub-G/G0-G1 phase. The study investigated the toxicity of *C. lentillifera*

extracts on MRC-5 fibroblast cells to verify their selective effects on cancer cells as opposed to normal cells. These findings are promising because the development of innovative anti-cancer medicines depends on their specificity for cancer cells. Based on the data reported above, the *C. lentillifera* extracts can be considered prospective new therapeutic candidates with a promising new mode of action and antiproliferative capabilities, which could be used to design novel anti-cancer medicines. However, the underlying mechanisms of action and how specific compounds contribute to observed anti-cancer effects could be further elaborated.

Conclusions

The present investigation discovered that CLM, CLE, and CLA can inhibit KON oral cancer cells. Ten major chemicals contained in acetone extract were able to be isolated and identified through the use of LC-QTOF MS/MS. The most abundant compound is sophorosyloxydocosanoic acid. The three extracts exhibited antioxidative activity, corresponding to the quantities of components of TPC, TFC, chlorophyll a, and chlorophyll b in the extracts. The CLA selectively inhibited cell proliferation and promoted apoptosis. All three *C. lentillifera* preparations produced ROS-mediated cell death and mitochondrial dysfunction and also stopped KON cells in the sub-G/G0-G1 cell cycle. Additionally, all extracts inhibited KON cells' ability to form colonies, migrate, and invade. The integrity of the cell membrane was disrupted. Furthermore, the three *C. lentillifera* extracts exhibited lower toxicity to lung fibrocyte cells than to KON carcinoma cells. According to these results, *C. lentillifera* extracts may be utilized to treat oral cancer and offer information about possible therapy approaches; further in-depth investigations, including in vivo studies and clinical trials, are essential to validate their therapeutic potential for oral cancer treatment.

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Clinical trial number
Cultured cells were used for this investigation. There are no human trials or clinical studies to report, hence there is no clinical trial number.

Authors' contributions
S.M. contributed to the conceptualization, investigation, methodology, data curation and statistical analysis, validation, visualization, supervision, resources and also wrote the manuscript and review & editing. T.S., S.T. and A.P. contributed to the methodology and investigation. S.M., T.S., S.T., A.P. and T.C. contributed revised the manuscript. All authors have read and approved the final manuscript.

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Data availability
Data is contained within the article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests

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