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Ethyl acetate extract from red coralline macroalgae *Mastophora rosea*, rich in polyphenolic catechins, induces apoptosis and inhibits metastasis in melanoma cell line

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Abstract

Background The rise in global temperatures and subsequent increase in extreme heatwaves is implicated to have a significant contribution to the growing incidence of various skin diseases, particularly melanoma skin cancer. The existing therapeutic approaches for skin cancer such as chemotherapy induce significant adverse effects. This highlights the need for further development of novel medicinal substances from natural sources which has less side effects implications. Macroalgae, often known as seaweeds, are recognized for their diverse array of bioactive compounds, which have numerous potential applications. The red coralline macroalgae or seaweed *Mastophora rosea* is abundant in the tropical Indo-Pacific region, including Indonesia. However, little is known regarding the phytochemical profile and potent bioactivity of this unique seaweed. The objective of this study is to assess the phytochemical profile, antioxidant and cytotoxic activity of *M. rosea* in melanoma cell line.

Methodology The dried macroalgae *M. rosea* sample was extracted using various solvents with differing polarities: ethanol, ethyl acetate (EA), and n-hexane. Subsequent biochemical evaluations included total phenolic content and antioxidant capability. Additionally, cytotoxic experiments were conducted using the mammalian melanoma cell line SK-MEL-2.

Results The ANOVA results revealed that the EA solvent was the most efficient in producing extracts with high total phenolic content (368.81 ± 3.16 mg GAE/g). This high total phenolic content is accompanied by potent antioxidant capacity ($IC_{50} = 99.52 \pm 19.58$ μ g/mL). In addition, *M. rosea* EA extract is attributed with the presence of various polyphenolic catechins, such as gallic acid, gallocatechin, epigallocatechin (EGC), catechin, epicatechin, epigallocatechingallate (EGCG), and epicatechingallate (ECG). Furthermore, *M. rosea* EA shows strong cytotoxic activity against the melanoma cell line SK-MEL-2 ($IC_{50} = 37.10 \pm 0.85$ μ g/mL). In addition to the melanoma cell line, the *M. rosea* EA extract shows similar cytotoxicity in epithelial HeLa cells ($IC_{50} = 34.32 \pm 1.19$ μ g/mL) and low cytotoxicity in normal human dermal fibroblast cells ($IC_{50} > 200$ μ g/mL).

Conclusion The current results show that *M. rosea* could induce apoptosis and also suppress metastasis activity in SK-MEL-2 cells. Further characterization and isolation of the possible bioactive compounds are necessary to better comprehend its mechanism of action against melanoma. Nevertheless, this study indicates that more study needed

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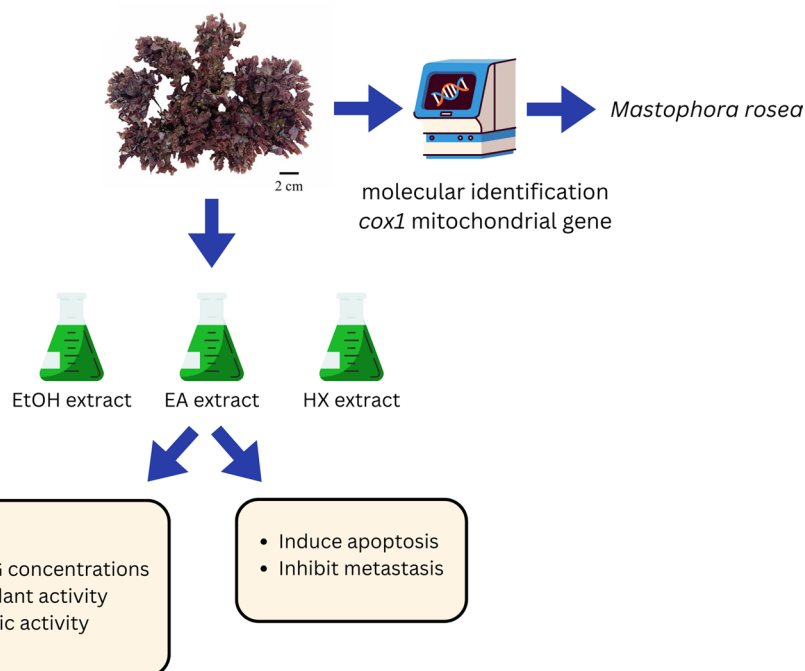


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to be done on various macroalgae regarding their phenolic components that may be beneficial for the development of novel therapeutic agents for cancer treatment.

Keywords Antioxidant, Cancer, Coralline, Cytotoxicity, Macroalgae, Melanoma, Seaweed

Graphical abstract



1 Introduction

Cancer remains the second leading cause of death worldwide, after heart disease [1]. It is characterized as a disease caused by the uncontrolled growth of abnormal cells. Melanoma has been demonstrated to have the most severe clinical symptoms and the greatest mortality rate among various types of skin cancer [2]. In addition, the annual incidence of melanoma has increased by up to 6% over the past decade [3]. This may be attributed to the substantial increase in ultraviolet (UV) radiation that reaches the Earth's surface. Currently, clinically approved treatments for melanoma are usually radiation and chemotherapy. However, these conventional treatments continue to produce severe side effects [4, 5]. Therefore, the development of alternative treatments with minimal side effects is urgently needed to enhance the clinical outcomes of melanoma treatment.

Over the past few decades, the attention and interest in the discovery of novel antitumour agents have turned to natural products originating from marine organisms, including macroalgae or seaweeds [6]. This is mainly

due to the vast secondary metabolites produced by macroalgae as a result of a defence mechanism to survive in extreme environmental conditions [7, 8]. Macroalgae synthesize a broad range of bioactive compounds, which also vary within species, geographic origin, and environmental conditions [9]. The general profile of macroalgae bioactive compounds includes polysaccharides, polyphenols, phycobiliproteins, carotenoids, pigments, terpenes, and phlorotannins [10]. Among these compounds, polyphenols are one of the most biologically relevant due to their anticancer activity [11].

The red coralline macroalgae *Mastophora rosea* has a distinctive physicochemical feature and demonstrates various bioactivities, including anticoagulant, antimicrobial, anti-inflammatory, and antifungal [12–14]. However, there remains no report of the bioactivity and physiochemical properties of *M. rosea* collected from Indonesia despite its abundance. Hence, this study investigated the phytochemical composition, cytotoxic activity, and potential of *M. rosea* as a novel source of bioactive compounds for melanoma therapy. In addition,

the *M. rosea* specimen used in this study was determined with molecular tools using the mitochondrial biomarker cytochrome oxidase subunit 1 (*cox1*).

2 Materials and methods

2.1 Materials

The reagents 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu's phenol reagent, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Fluorescence dye Hoechst33342 was purchased from Dojindo (Kumamoto, Japan). The mammalian cell culture medium, Dulbecco's Modified Eagle's Medium (DMEM), was acquired from Gibco (Thermo Fisher Scientific, NY, USA). All extract solvents used, such as ethanol (EtOH), n-hexane (Hx), and ethyl acetate (EA), were HPLC grade. The standard polyphenols gallic acid (GA), gallic acid (GC), epigallocatechin (EGC), catechin (C), epicatechin (EC), epigallocatechingallate (EGCG), gallic acid-gallate (GCG), and epicatechingallate (ECG) were purchased from Wuhan ChemFaces Biochemical Co., Ltd (Wuhan, Hubei, China) with purity > 98%.

2.2 Macroalgae sample preparation and extraction

The macroalgae samples were collected from Lendang luar coast, North Lombok, Indonesia (8°27' 23.8"S 116°02'10.2"E). The collected *Mastophora rosea* samples were thoroughly rinsed with distilled water to remove sand and epiphytic organisms. The biomass was dried at room temperature (24 °C) in an air-conditioned room for four days until it reached a constant weight. During the drying process, the samples were sprayed with distilled water (dH₂O) containing 1% fungicide (Virkon, PA, the USA) to prevent fungal contamination. Once the dried samples reached constant weight, the samples were ground with a food-grade grain miller. Approximately 50 g of dried algal powder was submerged with the extraction solvent, which was either ethanol, n-hexane, or ethyl acetate (1:5 w/v). The dried algal sample was extracted by three cycles of maceration and incubated at room temperature for 3×24 h with continuous agitation. After 24 h, the mixture was filtered using a vacuum pump (VP-15L, Joanlab, Huzhou, China) and Whatman no. 1 filter paper. All the filtrates were collected, and the solvents were evaporated using a rotary evaporator at 50 °C and 50 rpm. The extracts were collected in sterilized 60 mL glass screw-cap vials with Teflon inserts and stored at −20 °C until required for use.

2.2.1 Determination of *M. rosea* macroalgae extract total phenolic content (TPC)

The Folin–Ciocalteu method was employed to ascertain the total phenolic contents (TPC) of macroalgae *M. rosea*

extracts [15]. The reaction was neutralized by the addition of 10% sodium carbonate after oxidation with the Folin–Ciocalteu reagent. The UV–Vis microplate reader (Multiskan-GO, Thermo Fisher Scientific) was used to measure the absorbance at 725 nm after the sample was incubated at ambient temperature (24 °C) for 1 h. Gallic acid powder (Sigma-Aldrich, MO, the USA) of 10 mg was prepared in 10 mL ethanol and diluted to logarithmic concentrations (1, 3, 10, 30, 100, 300, and 1000 µg/mL) to generate the standard curve. The results were reported as milligrams of gallic acid equivalent (GAE) per gram of dried extract weight (mg GAE/g).

2.2.2 Antioxidant radical scavenging assay based on DPPH

The antioxidant activity of the macroalgae *M. rosea* extracts was measured by DPPH [16]. A sample of 100 µL DPPH reagent (200 µM) was mixed with 100 µL *M. rosea* extract with various concentrations (1–1000 µg/mL) in a 96-well plate. The DPPH reagent was also mixed with the corresponding solvents of each extract for the blank measurement. The plate was maintained at ambient temperature in the dark for 20 min. The UV–VIS multiplate reader (Multiskan GO, Thermo Scientific, MA, the USA) was used to measure the absorbance at 520 nm after the reaction was complete.

2.2.3 HPLC analyses of polyphenol compounds in macroalgae extracts

The polyphenolic constituents of the selected macroalgae extracts were analysed using high-performance chromatography (HPLC) (HP 1200 series, Agilent Technologies, CA, the USA) equipped with an LC column (3.0 mm×150 mm, 5 µm particle size) and a specific C18 guard column. The column was injected with 20 µL of the samples, and the samples were analysed using UV detection at 280 nm [17]. For quantitative determination of polyphenolic compound amounts, a calibration curve from known five standard compounds was used: gallic acid (GA), gallic acid (GC), epigallocatechin (EGC), catechin (C), epicatechin (EC), epigallocatechingallate (EGCG), gallic acid-gallate (GCG), and epicatechingallate (ECG).

2.3 Macroalgae *Mastophora rosea* molecular determination

The identification of red macroalgae *M. rosea* was confirmed by molecular determination using mitochondrial marker cytochrome oxidase subunit 1 (*cox1*). The *cox1* marker is often used to determine red macroalgae, and it can be very successful in the identification of a potentially new species [18]. Ten small fragments (2–3 cm) from the tips of the thallus were dissected and

preserved in a Ziplock bag containing silica gel. DNA extraction was performed on approximately 10 mg of the dried seaweed fragments. The DNA of the macroalgae sample was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) by following the manufacturer's protocol. The dried macroalgae fragments were submerged in lysis buffer with the addition of RNase and proteinase K. The PCR mix Ampliqon Taq DNA polymerase 2×Master Mix RED (Germany, Odense, Denmark) was used to amplify the extracted DNA. The primer used was *cox1* COX143F (5'-TCA ACAATCATAAAGATATTGGWACT-3') and C880R (5'-CCTGTNTTAGCAGGWGCTATTACAATGC-3'). The PCR product was subjected to Sanger sequencing. The molecular taxonomical status was determined using the Basic Local Alignment Search Tool (BLAST) in NCBI.

2.4 Macroalgae *M. rosea* cytotoxic activity against melanoma cell line

The SK-MEL-2 melanoma cell line (ATCC HTB-68), HeLa cell line, and NHDF normal cell line were acquired from the European Collection of Authenticated Cell Cultures (ECACC). The SK-MEL-2, HeLa, and normal cell lines were regularly grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) with the addition of 10% foetal bovine serum (FBS) and 1% penicillin [19]. The cells were kept at 37 °C in a 5% CO₂ humidified incubator (Forma Steri-Cycle, Thermo Fisher Scientific). The culture medium was changed regularly (at least every two days for all experiments).

The cytotoxicity of the macroalgae extracts was assessed using the MTT test [20]. The cancer (SK-MEL-2 and HeLa) and normal cell lines (NHDF) were seeded in a 96-well plate at a density of 5×10^3 cells/well. After 24 h of incubation, the cells were exposed to different concentrations of the macroalgae extracts (1–1000 µg/mL). The cells were then incubated for an additional 48 h. After treatment, the medium was removed, and a fresh culture medium (50 µL/well) containing MTT reagent (50 µL/well) was added. The reaction was conducted at 37 °C with humidified conditions (5% CO₂). After a 3-h incubation, the mixture was aspirated and replaced with 150 µL MTT solvent (DMSO). The reaction was conducted in the dark at room temperature (24 °C). The absorbance of purple colour from the MTT reaction was measured with a UV–VIS microplate reader (Multiskan-GO, Thermo Fisher Scientific) at 590 nm. Doxorubicin was used as a positive control. Respective IC₅₀ of the macroalgae extracts was calculated with GraphPad Prism software using nonlinear regression curve fit [21].

2.5 Hoechst33342 nuclear staining for early detection of apoptosis

Further evaluation was conducted to confirm the potential anticancer activity of the *M. rosea* extracts. The blue fluorescence dye Hoechst33342 was utilized to identify apoptotic processes in cells [22, 23]. The SK-MEL-2 cells were seeded in 35-mm cell culture dishes at a seeding density of 3×10^5 cells/mL. After 24 h, the media was discarded and replaced with medium containing IC₅₀ concentration of selected macroalgae extract and doxorubicin. After a period of 48 h, the original treatment solution was substituted with a new culture medium containing a concentration of 40 µM of Hoechst33342. Subsequently, the cells were cultured for 20 min at 37 °C in an environment supplemented with 5% carbon dioxide (CO₂). The cells were washed twice with PBS before observation under an inverted-fluorescence microscope at 20× magnification. The quantification of apoptotic events was performed by measuring the condensed nuclear DNA using the corrected total cell fluorescence (CTCF) equation. The formula for CTCF is calculated by subtracting the fluorescence intensity of the area of the selected cell and the background readings from the integrated density [24].

2.6 Scratch assay for anti-metastatic activity

The scratch assay could be used to determine the anti-metastatic activity of a certain therapeutic agent [25]. Briefly, 2×10^5 cells/mL were seeded in 24-well plates and were left to grow overnight at 37 °C with 5% CO₂. Subsequently, the cells were rinsed with PBS and a wound was created using a sterile 200 µL pipette tip. The detached cells were removed by washing twice with PBS. The cells were then treated with the selected macroalgae extracts with low non-toxic concentration (20 µg/mL). The morphological changes of the cells were observed and documented with an inverted microscope (Primo Vert, Zeiss). The experiments were performed in triplicate ($n=3$). The width of the gap closure at different time intervals (0, 24, 48, and 72 h) was quantified using ImageJ software. The wound healing inhibition activity is expressed as a percentage from the formula below [26].

Inhibition (%) = $[(\text{wound width day 0} - \text{wound width day 3}) / \text{wound width day 0}] \times 100$.

2.7 Statistical analysis

All assays were carried out in triplicate. The data presented are mean ± SD. Significant relationships between treatments were determined based on statistical analysis of variance (ANOVA) with Dunn–Sidak

multiple comparison. The IC_{50} values and statistical calculations were determined using GraphPad Prism software (v9.1.10).

3 Results

3.1 Identification of the macroalgae *Mastophora rosea* sample

The macroalgae *Mastophora rosea* sample was collected from the western coastal area of Lombok Island, Indonesia. The morphological features of all the collected samples were identified based on an electronic database [27]. Figure 1A shows the gross morphology documentation of the red macroalgae *M. rosea* used in this study. The thallus is thick and has a dark red colour with a linear, ribbon-like structure. The branches often appear as dichotomies, occurring quite close together, giving the morphological look of a fan-based appearance. The upper thallus often appears to be much more pigmented compared to the lower surfaces. The thallus dimensions range from 3 to 10 cm [28]. The determination of the

macroalgae *M. rosea* was confirmed with molecular tools using the mitochondrial DNA marker *cox1* (Cytochrome oxidase subunit I). The 590 bp DNA sequence was analysed with the Basic Local Alignment Search Tool (BLAST) in NCBI. The BLAST findings indicate a similarity range of 95.83% to 99.83% when compared to other *M. rosea* voucher sequences in the NCBI GenBank. This suggests that the macroalgae specimen used in this study is *M. rosea*. In addition, the sequence was submitted to NCBI GenBank (Accession no. PQ212778).

3.2 Macroalgae *M. rosea* extract biochemical properties

For initial screening of *M. rosea* biochemical properties, the macroalgae specimen was extracted using solvents with different polarity: ethanol (EtOH), ethyl acetate (EA), and n-hexane (Hx). After extraction, the biochemical properties, including extraction yield (%), total phenolic content (TPC), and antioxidant capacity, were analysed (Fig. 1B–D). The ethanol solvent produced a larger quantity of macroalgae extract than the n-hexane

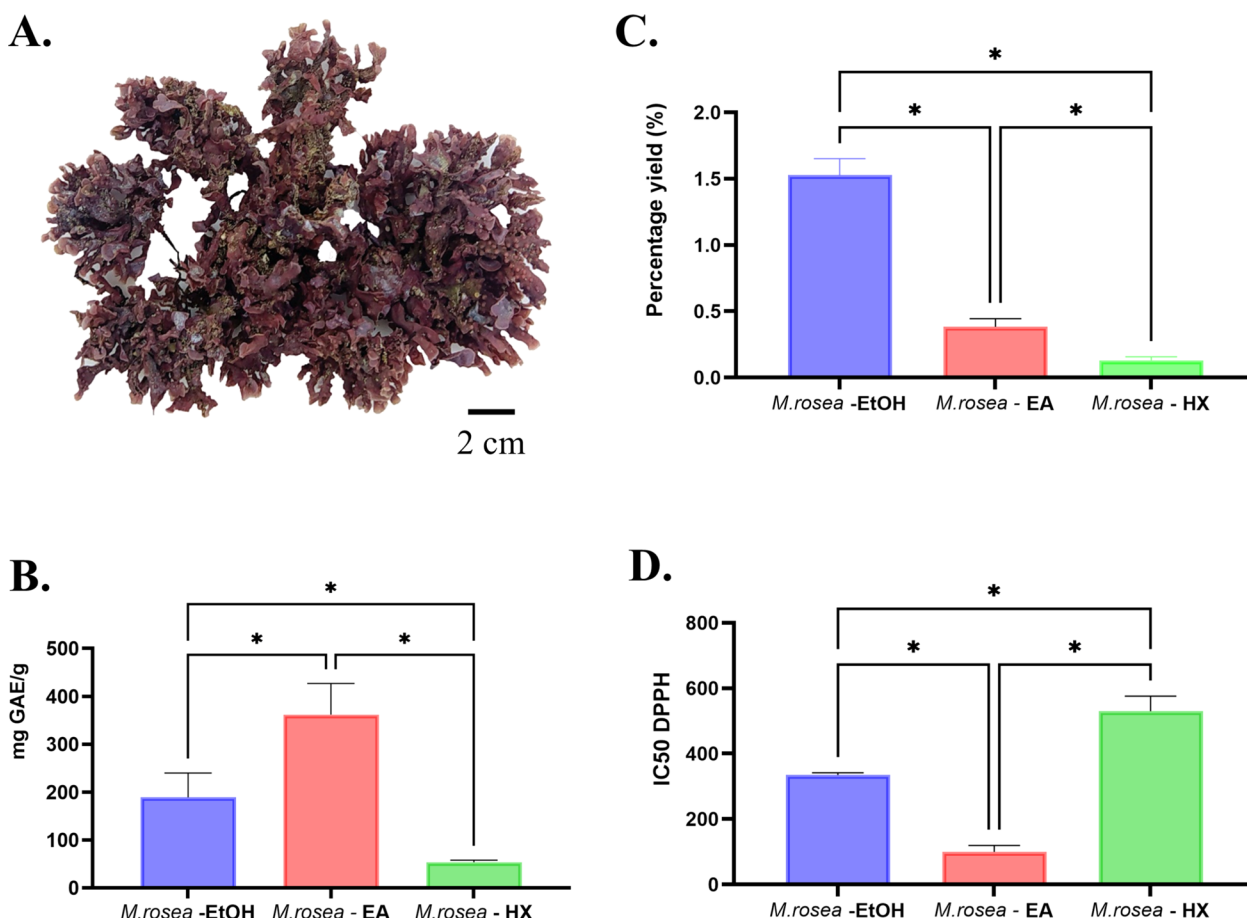


Fig. 1 Morphological documentation of the **A** Red macroalgae *M. rosea* used in this study and biochemical properties, including **B** extraction yield, **C** total phenolic content (TPC), and **D** antioxidant activity. Results are expressed as mean $\pm$ SD of triplicate from three independent experiments. Asterisks (*) indicate a significant difference compared to positive control doxorubicin ($p < 0.05$)

and ethyl acetate solvents. Extracts obtained from n-hexane solvent demonstrated reduced yields (<0.1%). The TPC values of *M. rosea* varied across different extraction solvents. The ethyl acetate fraction of *M. rosea* exhibited the highest total phenolic content (TPC) value at 368.81 ± 3.16 mg GAE/g, followed by the ethanol extract at 189.16 ± 50.73 mg GAE/g, and the n-hexane extract at 53.36 ± 4.42 mg GAE/g. Generally, elevated TPC values were predominantly achieved by the EA extract in comparison with other solvents.

The extracts of the macroalgae *M. rosea* were also analysed for DPPH antioxidant activity. The ANOVA statistical analysis indicates that the ethyl acetate extract exhibited the highest antioxidant activity ($IC_{50} = 99.52 \pm 19.58$ μ g/mL), followed by the ethanol extract ($IC_{50} = 334.97 \pm 6.79$ μ g/mL), and finally the n-hexane extract ($IC_{50} = 530.13 \pm 45.76$ μ g/mL). The antioxidant activity of *M. rosea* extracts exhibits trends analogous to the total phenolic content (TPC).

3.3 HPLC analyses of polyphenolic compounds in *Mastophora rosea* extracts

The HPLC analyses were performed to further evaluate the polyphenolic catechin compounds distributed in the macroalgae *M. rosea* extracts (Table 1). The concentrations of polyphenolic compounds were determined based on five phenolic standards: gallic acid (GA), galocatechin (GC), epigallocatechin (EGC), catechin (C), epicatechin (EC), epigallocatechingallate (EGCG), and epicatechingallate (ECG; Fig. 2A–D). The ethyl acetate extract showed a higher concentration of phenolic compounds compared to ethanol and n-hexane extract, which is expected due to their high TPC. The hydroxybenzoic derivative gallic acid (GA) is primarily found in all the macroalgae extracts, whereas the flavan-3-ol catechin hydrate (C) is only present in some of the extracts.

Our results also show that the red coralline algae *M. rosea* EA extract showed significantly high concentrations of polyphenolic catechins, including GA (8.01 ± 0.11 mg/g), EGC (8.70 ± 1.23 mg/g), C (5.93 ± 0.55 mg/g), and ECG (9.88 ± 1.04 mg/g). The ethanol extract of *M. rosea* contained seven identified polyphenolic catechins. The catechin ECG was not present in the *M. rosea* ethanol extract. However, the catechin EGCG (7.73 ± 0.39 mg/g) was significantly higher compared to the other extracts. Meanwhile, the *M. rosea* HX extract showed trace amounts of polyphenolic catechins. Only four catechins were present: GA, EGC, C, and ECG. The current study underscores the important role of solvent extraction in efficiently recovering polyphenolic compounds, as evidenced by the HPLC analyses of macroalgae extracts, which revealed significant variations in compound yield and composition depending on the solvent system used.

3.4 Cytotoxicity activity of macroalgae extracts against SK-Mel-2

The SK-Mel-2 is an established cell line that can be used as a melanoma cell model [29]. The cytotoxic effects of the macroalgae extracts were also evaluated in the epithelial cancer cell line (HeLa) and normal human dermal fibroblast cell line (NHDF; Fig. 3A–C). The cytotoxicity of the macroalgae extracts was assessed using the MTT assay over a 48-h period, with doses ranging from 1 to 1000 μ g/mL. The chemodrug doxorubicin was used as a positive control. The IC_{50} of macroalgae *M. rosea* EA extract was below 200 μ g/mL in both SK-MEL-2 cells ($IC_{50} = 37.10 \pm 0.85$ μ g/mL) and HeLa cells ($IC_{50} = 34.32 \pm 1.19$ μ g/mL). Crude extracts are considered to have a potential cytotoxic compound if the IC_{50} value is below 200 μ g/mL [30]. The EtOH extract of *M. rosea* showed slightly lower cytotoxicity in SK-MEL-2 cells ($IC_{50} = 311.00 \pm 0.95$ μ g/mL) and

Table 1 Polyphenolic contents (mg/g sample) of macroalgae extracts according to eight polyphenolic catechin standards: gallic acid (GA), galocatechin (GC), epigallocatechin (EGC), catechin (C), epicatechin (EC), epigallocatechingallate (EGCG), galocatechingallate (GCG), and epicatechingallate (ECG)

Phenolic compound	<i>M. rosea</i> EtOH extract	<i>M. rosea</i> EA extract	<i>M. rosea</i> HX extract
GA	7.68 ± 2.21^a	8.01 ± 0.11^a	2.49 ± 0.12^b
GC	1.05 ± 0.14^b	1.06 ± 0.28^b	–
EGC	4.62 ± 1.17^b	8.70 ± 1.23^a	1.98 ± 0.33^b
C	4.96 ± 0.72^b	5.93 ± 0.55^a	3.03 ± 0.59^b
EC	1.59 ± 0.39^b	3.54 ± 1.15^b	–
EGCG	7.73 ± 0.39^a	5.15 ± 1.31^b	–
GCG	5.60 ± 1.43^b	3.91 ± 0.25^b	–
ECG	–	9.88 ± 1.04^a	1.84 ± 0.36^b

Results are expressed as mean $\pm$ SD of triplicate from three independent experiments. Values with the same superscript letters in different groups of the same column imply statistical non-significance. Same superscript letters imply no statistical difference

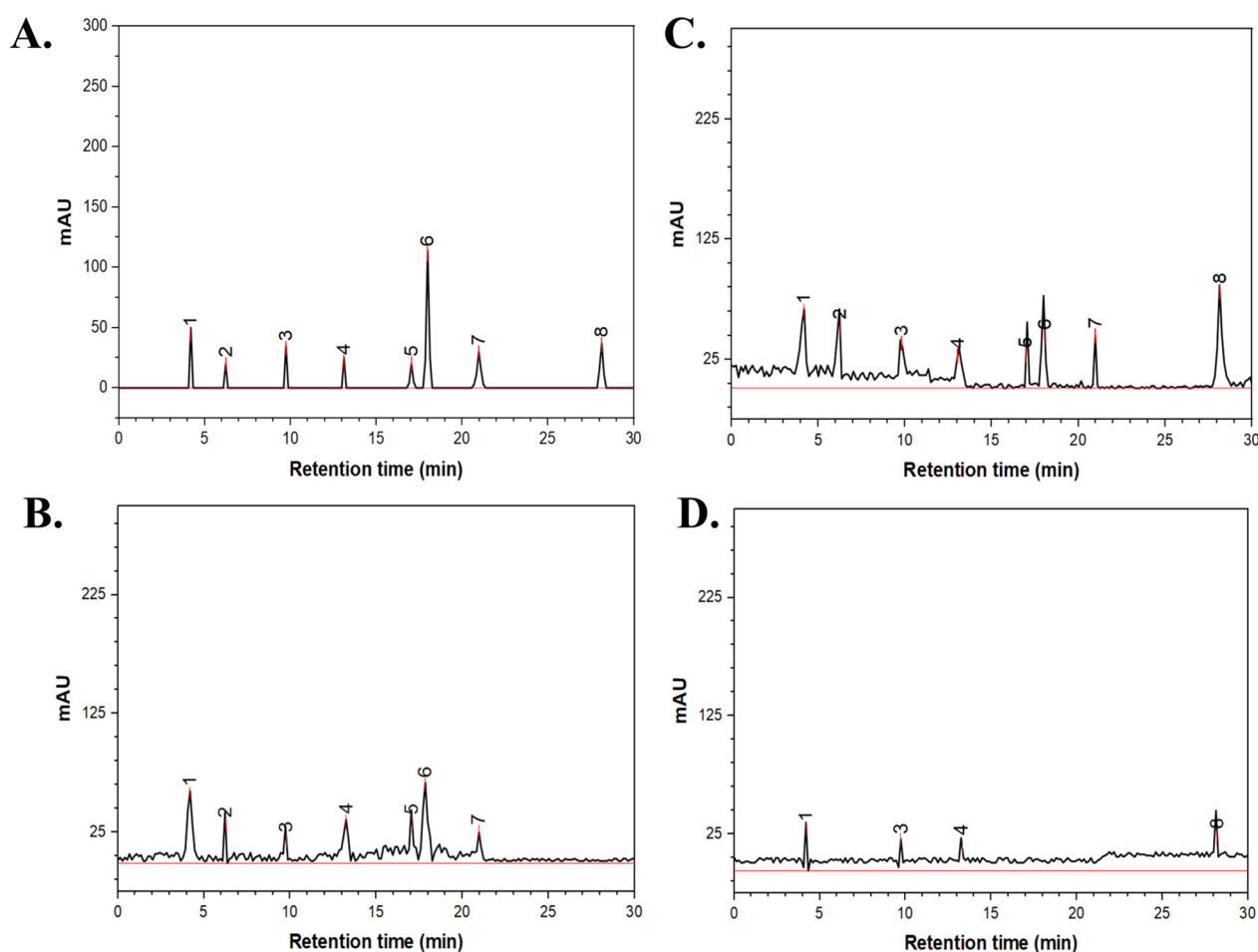


Fig. 2 HPLC chromatograms of polyphenolic catechins, including **A** the standard polyphenols: 1. gallic acid (GA), 2. gallic acid (GA), 3. epigallocatechin (EGC), 4. catechin (C), 5. epicatechin (EC), 6. epigallocatechingallate (EGCG), 7. gallic acid (GA), 8. epicatechingallate (ECG); **B** *M. rosea* EtOH extract; **C** *M. rosea* EA extract; and **D** *M. rosea* HX extract

HeLa cells ($IC_{50}=254.84 \pm 0.73 \mu\text{g/mL}$). Meanwhile, the *M. rosea* HX extract demonstrated the lowest IC_{50} cytotoxicity effects in both SK-MEL-2 and HeLa cells, $IC_{50}=655.37 \pm 1.34 \mu\text{g/mL}$ and $IC_{50}=599.82 \pm 1.57 \mu\text{g/mL}$. Notably, all macroalgae *M. rosea* extracts demonstrate less cytotoxic effects against normal fibroblast cells ($IC_{50} > 200 \mu\text{g/mL}$).

The extracts of macroalgae exhibit notable cytotoxic effects on cancer cells while demonstrating less cytotoxicity in normal cells. However, the exact mechanism of action remains unknown. Further chemical characterization and compound isolation should be conducted to understand more about the potential mechanism of action.

In terms of extract solvents, the ethyl acetate extract showed more cytotoxic effects compared to ethanol and n-hexane. Among all the macroalgae *M. rosea* extracts, the ethyl acetate extract of *M. rosea* exhibited promising cytotoxic and anti-proliferative effects in the tested

cell lines (Fig. 3D). Currently, there remains no information regarding the cytotoxic effects of red coralline macroalgae *Mastophora rosea* in human cancer cells. The strong cytotoxic effect of *M. rosea* EA extract is potentially attributed to the high concentration of polyphenolic compounds, notably EGC and ECG, compared to other extracts.

3.5 Apoptotic inducing activity of *M. rosea* EA extract

Apoptosis is considered a target therapy for novel candidates of anticancer therapeutic agents [31]. Apoptosis is widely known for its prominent characteristic of chromatin condensation [32]. The apoptotic-inducing effects of macroalgae *M. rosea* extracts were evaluated in the SK-MEL-2 melanoma cell line (Fig. 4). For this purpose, doxorubicin which is a widely used positive control for observing DNA damage-inducing anticancer drug was used. The nuclear Hoechst33342 staining could be used to detect condensed chromatin which is one of

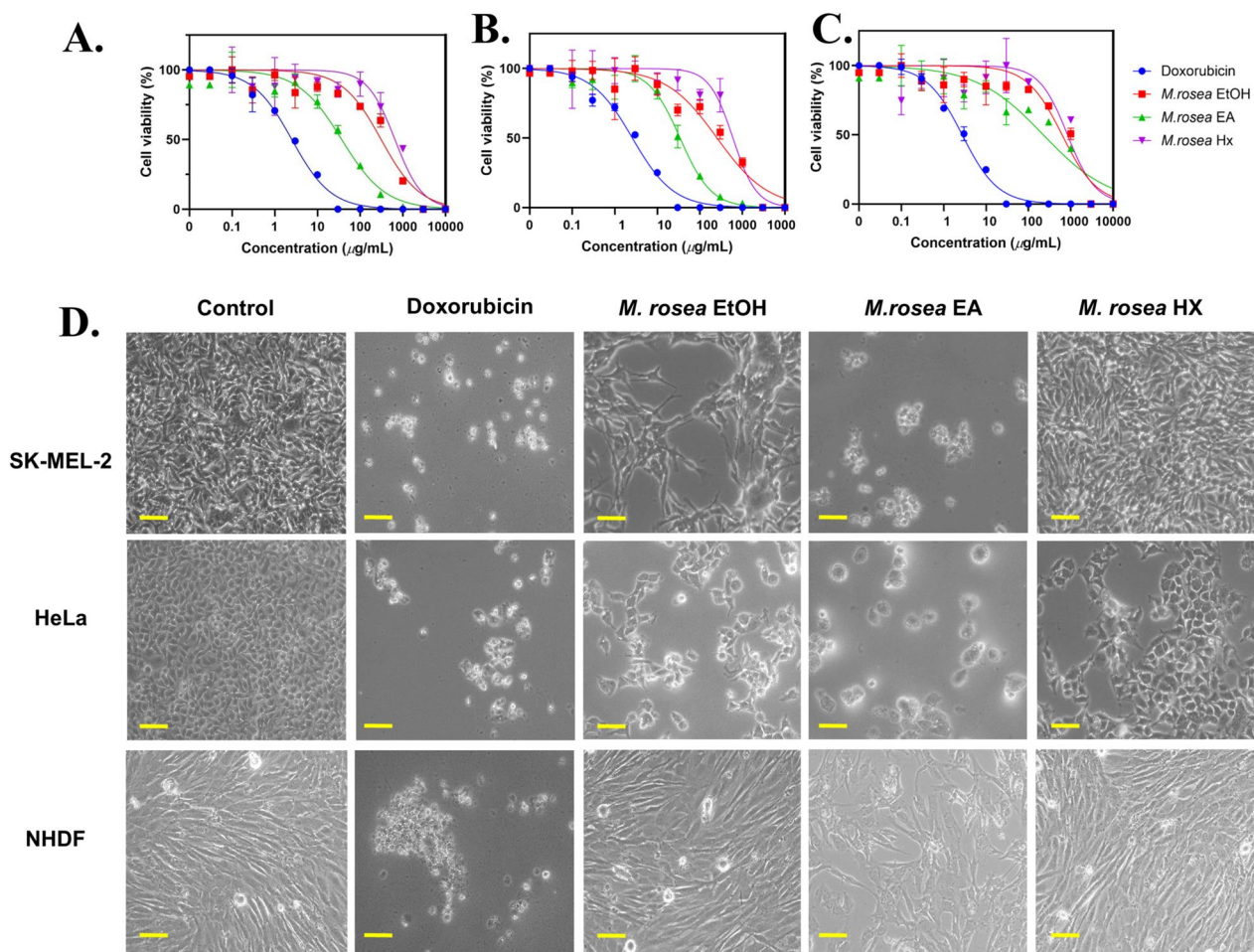


Fig. 3 Dose–response cytotoxic activity of *M. rosea* **A** EtOH, **B** EA, and **C** HX extracts. **D** The Microscopic documentation of *M. rosea* EA in 100 µg/mL concentration. Scale bar = 100 µm

the primary features of cells undergoing apoptosis [22]. SK-MEL-2 cells treated with IC₅₀ concentrations of *M. rosea* EA extract and doxorubicin showed increased blue fluorescence exposure of cells. This indicates condensation of chromatin, which indicates the cell undergoing apoptotic cell death [33]. Meanwhile, the SK-MEL-2 cells treated with *M. rosea* EtOH and HX extracts showed similar blue fluorescence exposure to the normal cells.

In addition, the apoptotic nuclei of the apoptotic cells could be evaluated based on the fluorescence intensity. The apoptotic nuclei percentage was significantly higher in *M. rosea* EA extract and doxorubicin compared to control and other extract treatments. Furthermore, based on corrected total cell fluorescence (CTCF) values, the treated cells with ethyl acetate extract of *M. rosea* and doxorubicin show no significant difference. This strongly implies that the cells in these treatments with *M. rosea* ethyl acetate extract and doxorubicin are undergoing apoptosis. The corrected total cell fluorescence (CTCF)

read out provides additional justification to estimate the levels of nuclei condensation in the treated cells [24]. The CTCF values also correlate with the number of apoptotic nuclei of treated cells, which could be distinguished based on the emitted blue fluorescence. However, apoptosis characteristics could be assessed in other ways, such as DNA fragmentation and caspase activity [34]. Further studies related to apoptosis activity would be needed to conclude the significance of apoptotic-inducing effects attributed to these macroalgae *M. rosea* extracts.

3.6 Macroalgae *M. rosea* extracts effect on melanoma cell migration

Melanoma is considered a highly malignant metastatic tumour, which is commonly incurable with an extremely poor prognosis [35]. Hence, the selected extracts were also evaluated for their potential anti-metastatic activity in SK-MEL-2 cells using the scratch assay [36]. The extracts were tested at low non-toxic concentration 20 µg/

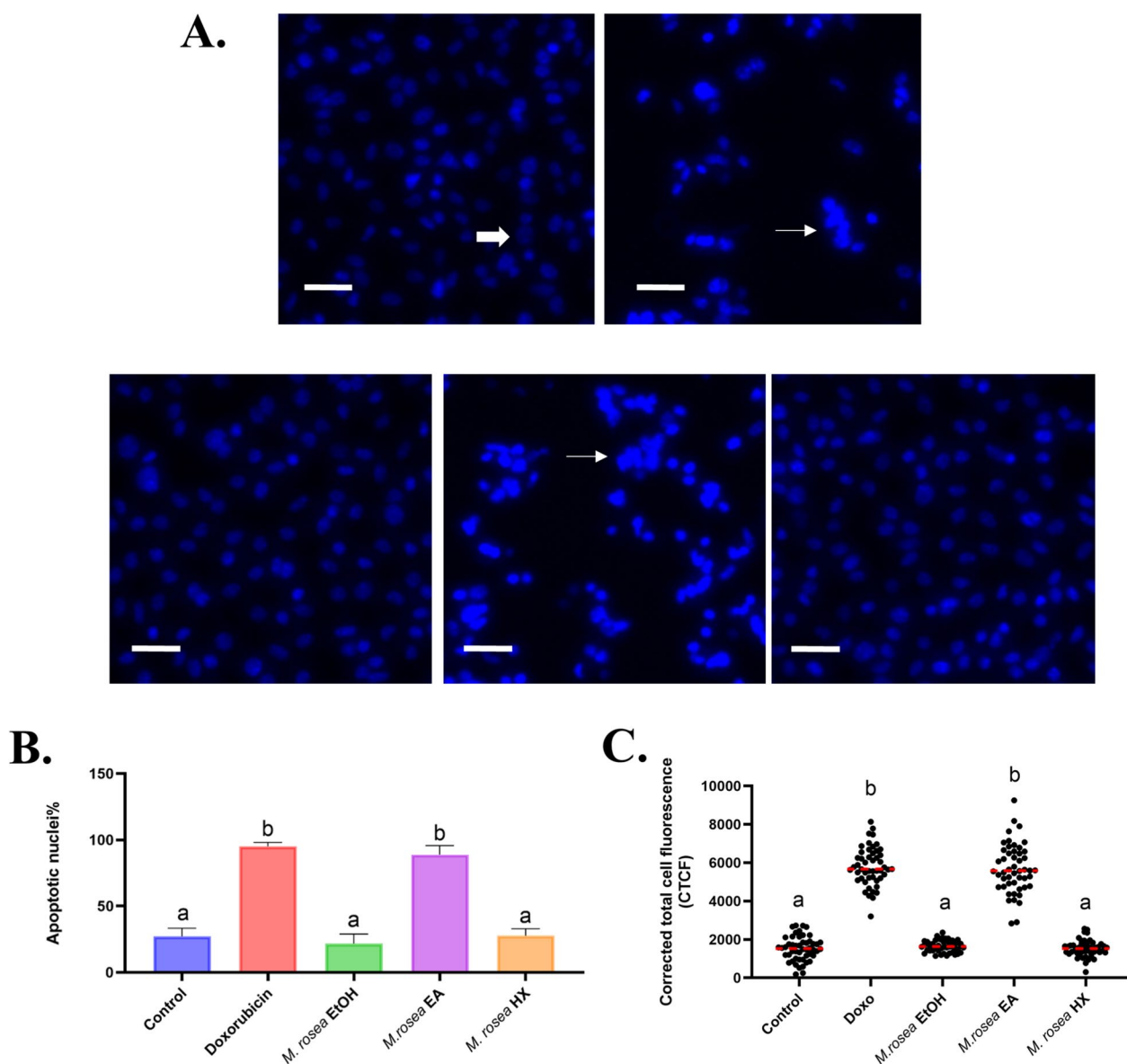


Fig. 4 Apoptosis-inducing effects of selected macroalgae extracts based on chromatin condensation. **A** Cells undergoing chromatin condensation emit blue fluorescence, **B** Apoptotic nuclei percentage, **C** Corrected total cell fluorescence (CTCF) values of treated cells with doxorubicin and macroalgae extracts at IC_{50} concentration. The thick arrow indicates healthy cells, and the thin arrow indicates chromatin condensation in cells. Results are expressed as mean $\pm$ SD of triplicate from three independent experiments. Different alphabetic notations indicate significant differences between treatments ($p < 0.05$). The superscript letters imply no statistical difference. The thick white arrow shows a normal cell. The thin white arrow shows condensed chromatin. Scale bar = 25 μ m

mL, as determined by the previous antiproliferative assay. The treated cells were then documented at 0, 24, 48, and 72 h after incubation to determine the anti-metastatic activity based on wound closure (Fig. 5). Significant differences ($p < 0.05$) between treatments were evident at 48 and 72 h. At the end of the assay (72 h), the ethyl acetate extract ($62.00 \pm 13.08\%$) and EtOH extract ($60.67 \pm 6.66\%$) of *M. rosea* showed significant inhibition of wound closure

when compared to the control ($27.00 \pm 2.65\%$). Only the HEX extract of *M. rosea* showed significantly weaker cell migration closure activity at $9.85 \pm 7.37\%$. Higher inhibition percentage indicates fewer cells are migrating. This wound healing assay is a simple, non-expensive, and highly reproducible method to study the effects of certain substances on cancer cell migration in vitro. Hence, the anti-metastatic activity of *M. rosea* EA extract is expected. However,

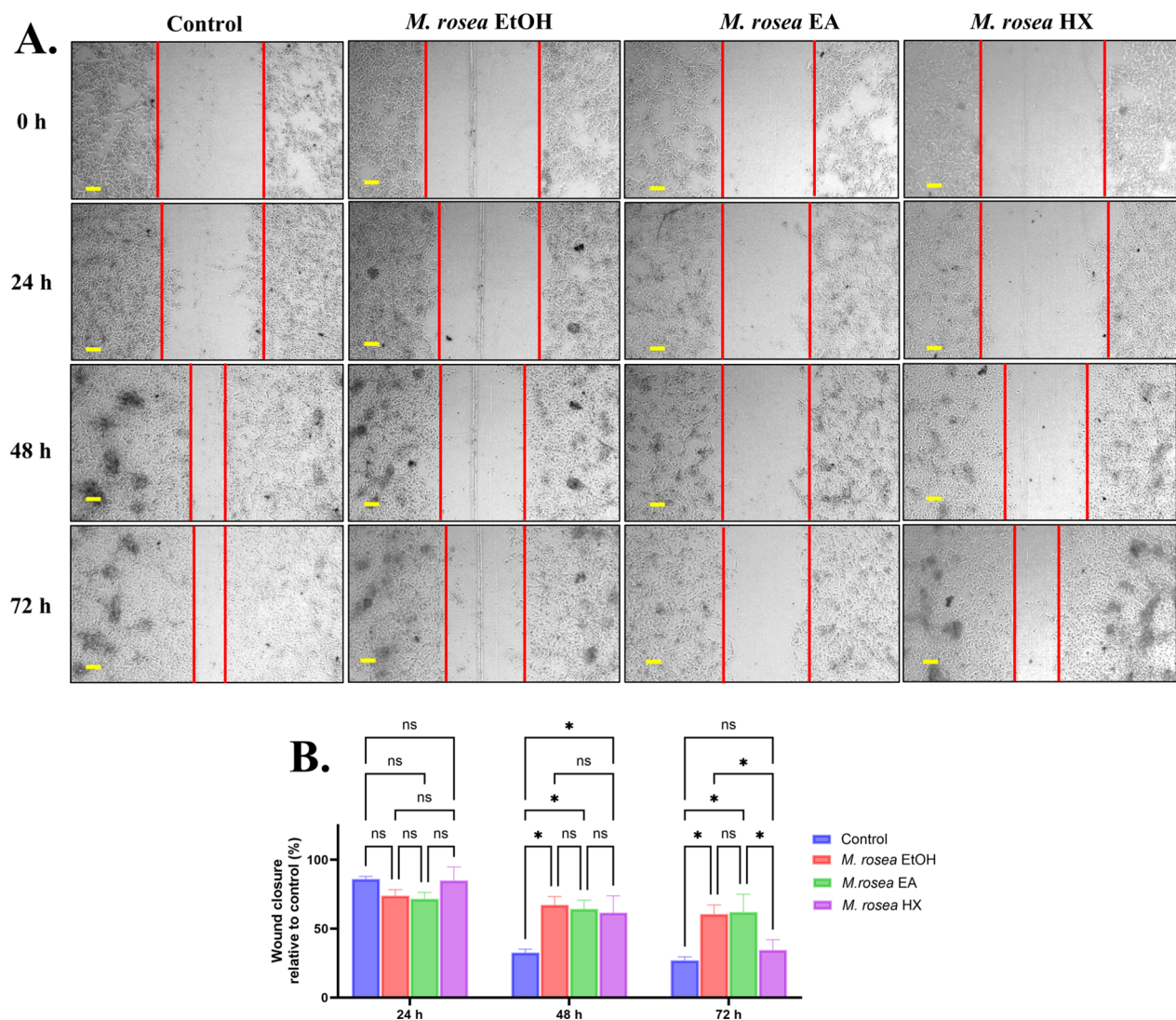


Fig. 5 Scratch assay of selected macroalgae extracts (20 µg/mL) against a melanoma cell line. **A** Documentation of wound coverage in 0–72 h and **B** wound coverage percentage. Results are expressed as mean $\pm$ SD of triplicate from three independent experiments. Different letters in groups indicate a significant difference between treatments ($p < 0.05$). The same letters in groups indicate no significant difference between treatments. Asterisks (*) notations indicate significant differences between treatments ($p < 0.05$). ns notations indicate no significant differences between treatments. Scale bar = 100 µm

surprisingly the EtOH extract of *M. rosea* also showed inhibitory effects towards the SK-MEL-2 cell migration. Hence, further advanced molecular investigation is needed to understand this mechanism.

Hence, further chemical fractionation and characterization are needed to disclose the mechanism of action behind the biological activities of *M. rosea* extracts.

4 Discussion

Currently, cancer continues to be a prominent cause of mortality and a significant health concern worldwide. Among all the different types of cancer, breast

and lung cancer continue to be the primary causes of death resulting from cancer [37]. Nevertheless, the occurrence rates of skin cancer have increased significantly on a global scale. This is presumed to be a result of the many impacts of global climate change, such as increased global temperature and ultraviolet radiation intensity [38]. Conventional cancer therapies, such as radiotherapy and chemotherapy, can cause a range of side effects that reduce the chances of survival, raise the risk of death, and even lead to the return of cancer [39]. Therefore, there is an urgent need to develop alternative bioactive compounds that might complement

conventional cancer treatments and potentially minimize the associated adverse effects.

Macroalgae or seaweed have been well studied as producers of bioactive compounds with various biological activities, including antioxidant, anti-inflammatory, and anticancer properties [23, 40–42]. Nevertheless, the primary obstacle in utilizing these significant marine natural resources is the accessibility of biomass. Furthermore, Indonesia, known for its vast array of seaweed species, still lacks comprehensive research on a significant amount of its biodiversity [43]. Further efforts should be made to investigate the pharmacological possibilities of the seaweed found in Indonesian coastal regions, especially the abundant species.

The red macroalgae, *M. rosea*, possesses a distinctive thallus feature where the cell walls are calcified with calcite crystals. Therefore, it is classified as a member of the coralline algae group, also known as corallines. This red macroalgae exhibits a worldwide distribution and can be found in the intertidal zone, extending to the furthest boundary of the photic zone (>250 m). The ability of this macroalgae to adapt to a wide range of habitats indicates that it produces a diverse array of secondary metabolites, which enhance its potential for adaptation and survival.

The correct identity of the red macroalgae *M. rosea* was confirmed with the mitochondrial gene cytochrome oxidase subunit 1 (*cox1*). This report is the initial confirmation of the molecular identification of *M. rosea* in Indonesian waters. In addition to the report on the distribution of the red macroalgae *M. rosea* in Indonesia, this study also presents the phytochemical profile, specifically polyphenols. Polyphenols, a category of bioactive compounds, show promise as phytochemicals in the prevention of skin cancer generated by UV radiation. These substances are regarded as photoprotective agents because of their antioxidant, anti-inflammatory, anti-proliferative, and anti-carcinogenic characteristics [44]. The polyphenolic catechins epigallocatechin (EGC), epigallocatechingallate (EGCG), and epicatechingallate (ECG) have demonstrated promising anticancer activity in various studies [45–47]. The ethyl acetate extract of *M. rosea* exhibited very high quantities of EGC and ECG, which likely contribute to its potent cytotoxic activity against melanoma cell lines. Furthermore, ECG specifically triggers apoptosis, programmed cell death, in a melanoma cell line by activating reactive oxygen species (ROS) [48]. Although polyphenolic catechins have pharmacological importance, their presence in macroalgae or seaweeds has only been reported in a small number of studies [16, 49, 50]. The notable abundance of these catechins in seaweeds may also be linked to their great antioxidant capacity [51].

The red macroalgae *M. rosea* underwent extraction using solvents of various polarity. Previous investigations indicate that the extract obtained with ethanol solvent yields the largest quantity. This is demonstrated in numerous experiments indicating that solvents with greater polarity produce higher extract yield. The principle "like dissolves like" indicates that polar chemicals are more soluble in polar solvents, while nonpolar compounds are best dissolved in nonpolar solvents. Nevertheless, certain research indicates that polar solvents can extract a wider array of molecules, including those possessing antioxidant characteristics. However, the total phenolic content was not the highest in ethanol solvent extract but ethyl acetate solvent extract. While polyphenols are typically regarded as polar substances owing to the presence of many hydroxyl (-OH) groups on their aromatic rings, certain polyphenols may display a degree of nonpolar characteristics. These nonpolar polyphenols can contribute to the stabilization of lipid membranes and provide protection against oxidation in lipid-dense settings. An example of this is certain tocopherols (vitamin E) which are nonpolar owing to their structural composition. They may possess a greater or lesser number of hydrophobic (nonpolar) groups, resulting in increased solubility in lipids and decreased solubility in water.

Other reports also show that EA extract exhibited higher TPC compared to other extract solvents [52, 53]. Prior investigations have indicated that ethyl acetate extracts from the macroalgae *Gracilaria arcuata* and *Ulva lactuca* yield significant TPC and exhibit robust antioxidant action [54]. Similar results were also observed in brown macroalgae, such as *Padina tetrastrum*, which produced higher TPC values in the ethyl acetate fraction (587.79 ± 7.14 mg GAE/g) compared to methanol (220.04 ± 5.12 mg GAE/g) and n-hexane (70.81 ± 0.44 mg GAE/g) fractions [55]. These studies suggest ethyl acetate may be the most effective solvent to extract natural antioxidants from macroalgae.

The high total phenolic content of the ethyl acetate extract correlates with its strong antioxidant activity compared to the other extracts. It is suggested that a positive correlation typically exists between total phenolic content and antioxidant activity in diverse natural products [56]. Samples with elevated amounts of phenolic chemicals typically demonstrate enhanced antioxidant capabilities. Phenolic chemicals function as antioxidants by contributing hydrogen atoms or electrons to free radicals, thereby stabilizing them [57]. Although brown macroalgae are mostly common as the primary source for polyphenolic compounds, some red macroalgae may exhibit high polyphenolic contents. As reported by Yoshie et al., the red macroalgae *Chondrococcus hornemannii* contains high amounts of catechin

(2170 ± 95 $\mu\text{g/mL}$) and EGCG (16.000 ± 760 $\mu\text{g/mL}$) [49]. Other studies also report the presence of various phenolic compounds, such as catechins and flavonols, in red macroalgae [58]. The higher extraction of polyphenolic compounds in polar solvents can be attributed to the fact that free phenolic compounds are inherently polar, leading to their increased solubility in the solvent [59]. Besides the extraction solvent, the concentration of flavonols also depends on the sample's geographical location and the seasonal time of collection. This difference is proven by the fact that the flavonol content in *S. polycystum* and *Paustralis* from the Seribu Island, Indonesia, only contained trace amounts of catechins [60]. Meanwhile, other reports have shown that brown macroalgae, including *Sargassum* and *Padina*, generally contain high amounts of polyphenolic compounds [61]. This is in accordance with other findings, which suggest a similar conclusion [62]. The ethyl acetate extract of *M. rosea* shows strong cytotoxic activity in melanoma cell line SK-MEL-2 cell, while exhibiting minimal cytotoxic activity in normal cells. Certain seaweed extracts demonstrate specific cytotoxicity, effectively eliminating cancer cells while preserving normal cells [63]. This selective activity arises from the existence of particular bioactive chemicals in seaweed that specifically target the distinct properties of cancer cells [64].

Seaweed polyphenols have demonstrated selective cytotoxicity, targeting cancer cells while demonstrating minimal to no harm to normal cells [65]. In a previous study, a comprehensive screening was carried out on 47 species of macroalgae. The results revealed a significant cytotoxic effect of these macroalgae on L1210 cells, while demonstrating minimal cytotoxicity on normal cells [66, 67]. The collective findings, in conjunction with this investigation, suggest that the bioactive compounds of macroalgae may interact with specific cancer-associated receptors or cancer cell-specific molecules, thereby initiating pathways that induce cancer cell apoptosis. This distinctive characteristic renders them intriguing candidates for cancer therapy. Research demonstrates that seaweed polyphenols can trigger apoptosis in cancer cells and suppress their proliferation.

Among the catechins, EGCG, EGC, GC, and ECG are known to have strong cancer preventive activity compared to other catechins, while others, such as EC and C, are usually inactive [68, 69]. A previous study demonstrated the strong anticancer activity of the Japanese brown macroalgae *Hiziki fusiformis*, which is possibly due to its high EGC content (3770 ± 1200 $\mu\text{g/g}$) [60, 70].

Currently, there remains no information regarding the cytotoxic effects of red coralline macroalgae *Mastophora rosea* in human cancer cells. The strong cytotoxic effect of *M. rosea* EA extract is potentially attributed to the

high concentration of polyphenolic compounds, notably EGC and ECG, compared to other extracts. Both EG and ECG, major catechins found in green tea, exhibit strong anticancer activity. EGCG, in particular, is known for its prominent anticancer effects, even at low concentrations. ECG also shows significant anticancer activity, sometimes even surpassing EGCG in certain cancer cell types.

In addition strong anti-proliferative effects of extracts which are commonly accompanied by anti-metastatic effects [71–73]. Certain compounds and extracts have shown cancer cell migration activity inhibition and were not cytotoxic to that cell line [74]. In several studies, the desired outcome is to inhibit the metastasis of cancer cells without simultaneously causing cell death [75]. In addition, the low-dose applications of certain substances are preferable to inhibit cell migration, which is a crucial event of cancer development, leading to poor prognosis and clinical outcomes [76]. In addition, low doses of anticancer drugs decrease the chances of drug resistance [77].

Different seaweed extracts may induce different effects towards cell migration, as demonstrated in another study where the treatment extract of *Sargassum ilicifolium* and *Ulva Lactuca* showed increased cell migration and proliferation in L929 cells [78]. Similar bioactivity was demonstrated by another study, where *Sargassum ilicifolium* effectively increased cell migration of keratinocytes (HaCaT) at a very low concentration (4 $\mu\text{g/mL}$). Seaweeds can generate a wide range of secondary metabolites that exhibit a diverse array of biological functions. Multiple studies have demonstrated that extracts derived from various species of macroalgae exhibit distinct and potent antioxidant activity through diverse mechanisms. These mechanisms include scavenging free radicals and reactive oxygen species (ROS), preventing lipid oxidation, and chelating metal ions [79]. This may also occur for anticancer activities which involve a much more complex molecular mechanism, including induction of apoptosis, anti-metastatic effects, anti-angiogenesis effects, inhibition of cell cycle, and cytotoxic effects [80].

5 Conclusion

The red macroalgae, scientifically known as Rhodophyta, comprises the largest category of seaweeds. However, a significant portion of the species members of this group remains insufficiently studied. This study reports, for the first time, the presence of *M. rosea* in the waters of Lombok, Indonesia. This is supported by the fact that there is limited information for *M. rosea* DNA sequence originating from Indonesia available in the NCBI GenBank. Most of the studies are done based on morphological observations, which is difficult to rely

on when it comes to seaweed identification. In addition to the report of *M. rosea* macroalgae distribution and molecular identification, this study also evaluates the phytochemical profile and potent anticancer effects against melanoma skin cancer cells. Furthermore, it was evident that ethyl acetate solvent was the most efficient in producing extract with the highest total phenol content, antioxidant activity, and cytotoxic activity. This information is important for further phytochemical profiling and fractionation of various studies for targeting macroalgae phenols that would be promising for the discovery of novel anticancer compounds.

Abbreviations

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BLAST	Basil Local Alignment Search Tool
C	Catechin
Cox1	Cytochrome oxidase subunit 1
CO ₂	Carbon dioxide
CTCF	Corrected total cell fluorescence
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EA	Ethyl acetate
EC	Epicatechin
ECACC	European Collection of Authenticated Cell Cultures
ECG	Epicatechingallate
EGC	Epigallocatechin
EGCG	Epigallocatechingallate
FBS	Foetal bovine serum
EtOH	Ethanol
GA	Gallic acid
GAE	Gallic acid equivalent
GC	Gallocatechin
HaCaT	Keratinocytes
HeLa	Immortalized cell line
Hoechst33342	Cell-permeant blue-emitting fluorescent dye
HPLC	High-performance liquid chromatography
HX	N-hexane
IC ₅₀	Half-maximal inhibitory concentration
<i>M. rosea</i>	<i>Mastophora rosea</i>
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
NHDF	Normal human dermal fibroblast cell lines
PCR	Polymerase chain reaction
SK-MEL-2	A type of human melanoma cell line
TPC	Total phenolic content
UV	Ultraviolet

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Author contributions

Conceptualization was done by ESP, NWRM, and AF. Data curation was done by NWRM and PBAS. Formal analyses were done by ESP and AF. Investigation was done by NWRM and PBAS. Methodology was done by ESP and AF. Project administration was done by NWRM. Resources was done by ESP. Software was done by ESP and AF. Supervision was done by ESP and AF. Validation was done by AF, SW, and MK. Writing original draft was done by ESP. Writing review was done by AF, SW, and MK.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

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