

# The Suppression of Signal Transducer and Activator of Transcription-3 in A549 human Lung Carcinoma Cells Induced by Marine Sponge *Callyspongia aerizusa*

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**Introduction:** Lung cancer is recognized as a highly lethal disease, demanding swift and accurate solutions. Previous analysis showed the cytotoxic impact of *Callyspongia aerizusa* (*C. aerizusa*) extract containing ergost-22-en-3-one and ergost-7-en-3-ol against A549 lung cancer cells, with an IC<sub>50</sub> value of 9.38 µg/mL. However, the extract did not have cytotoxicity towards Het-1A esophagus epithelial cells. Several reviews also validated the upregulation of pro-apoptotic molecules and the inhibition of anti-apoptotic molecules linked to the caspase-dependent signaling pathway.

**Purpose:** The objective of this research was to extend the understanding of the effects of *C. aerizusa* extract on A549 lung carcinoma, examining its influence on various signaling pathways, malignancy, migration, and invasion.

**Materials and Methods:** PCR was used to measure mRNA expression, targeting *PTEN*, *Akt*, *mTOR*, *STAT-3*, *IL-6*, *VEGF*, and *HIF1α*. Additionally, Western Blot analysis was adopted to assess PTEN, p-Akt, Akt, p-mTOR, and p-STAT-3 protein expressions. Wound healing and invasion assays were performed to measure the migration and invasion capabilities of A549 cells post-treatment with *C. aerizusa* extract.

**Results:** The mRNA expression analysis showed an increase in *Akt* and *mTOR* but a decrease in *PTEN* and *STAT-3* after 24 hours of treatment with *C. aerizusa* extract. At the protein level, there was a downregulation of p-Akt, Akt, p-mTOR, and p-STAT-3, while PTEN increased during 24-hour treatment. Wound healing and invasion assay results showed a weakened ability of A549 cells after a 24-hour treatment with *C. aerizusa* extract. Moreover, *IL-6* and *HIF-1α* mRNA expression levels decreased during 24 hours, while *VEGF* mRNA had a slight decrease compared to untreated cells.

**Conclusion:** In conclusion, the ergosteroids present in marine sponge *C. aerizusa* extract signified a remarkable reduction in malignancy, migration, and invasion capabilities in A549 lung carcinoma cells. These results suggested their promising candidacy for future anti-angiogenesis in anticancer therapy.

**Keywords:** *Callyspongia aerizusa*, A549 cells, PI3K/Akt signaling, STAT-3, wound healing, invasion

## Introduction

Lung cancer is responsible for 1.8 million deaths out of 2.2 million new cases in 2020, making it the leading cause of cancer mortality.<sup>1</sup> The incidence is expected to rise, particularly in countries where the epidemic is in its earlier stage.<sup>1</sup>

Countries such as China, Indonesia, and several African countries are experiencing whether recent peaks in smoking rates or ongoing increases.<sup>1</sup> Without interventions to hasten smoking cessation or reduce initiation, this trend is likely to persist for at least the next few decades.<sup>1</sup>

Bio-natural products derived from marine creatures are regarded to be the potential solution to drug resistance, particularly in cancer therapy.<sup>2</sup> This has led to the development of various drugs currently used in clinical settings.<sup>2</sup> Among the invertebrate marine creatures, sponge plays a prominent role in producing bio-natural products that are increasingly considered for therapeutic purposes.<sup>2</sup> Examples of such products, approved by the FDA and used globally, include Cytarabine (Ara-c) sourced from *Cryptotethia crypta* and Eribulin mesylate (Halaven) derived from *Halichondria okadai*.<sup>2</sup> Ongoing research continues to explore the potential of sponge in the context of anticancer applications.<sup>2</sup>

*Callyspongia aerizusa* (*C. aerizusa*) is a significant sponge due to its widely acknowledged anti-cancer potential and varying levels of cytotoxicity against multiple cancer cells.<sup>3</sup> Reports on *C. aerizusa* show Callyaerins E and H as contributors to its cytotoxic activities.<sup>3</sup> Previous reviews examined the cytotoxic effect of *C. aerizusa* extract containing ergost-22-en-3-one and ergost-7-en-3-ol on A549 cancer cells, signifying its ability to induce programmed cells death or apoptosis.<sup>4</sup> The extract upregulated caspase-9, caspase-3, and poly(ADP-ribose) polymerase-1 (PARP-1,) while down regulating  $\beta$ -lymphoma cell 2 (BCL-2) at both mRNA and protein expression levels.<sup>4</sup> As concluded in the previous research, a commitment was made to characterize additional properties of the crude methanol extract, conducting various in vitro tests and investigating malignant pathways associated with *C. aerizusa* extract.<sup>4</sup>

High levels of signal transducer and activator of transcription 3 (STAT-3) and p-STAT-3 expression are associated with poor prognosis of patients with NSCLC.<sup>5</sup> STAT-3 regulates several genes that play a role in apoptosis, metastasis, proliferation and angiogenesis such as BCL-2 and vascular endothelial growth factor (VEGF).<sup>6</sup> STAT-3 can be activated by cytokines-receptor binding, such as interleukin-6 (IL-6), or janus kinases (JAK) phosphorylation.<sup>7</sup>

This research aims to investigate the Jak-Stat Signaling pathways used in the processes of cells division and tumor formation in A549 cells treated with *C. aerizusa* extract through the AKT pathway. Additionally, wound healing and invasion assays will be conducted to assess the ability of A549 cells after the treatment.

## Materials and Methods

### *C. aerizusa* Extraction Preparation

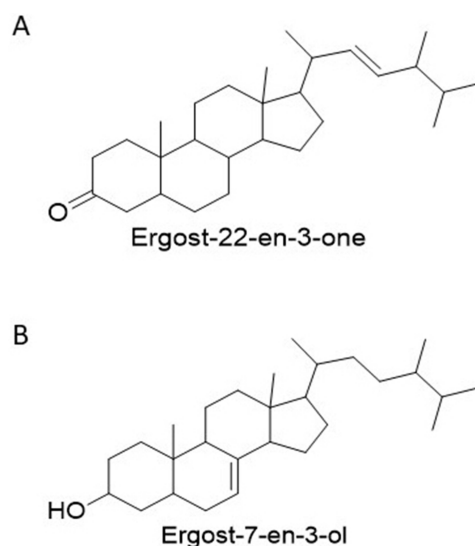
The Sponges *C. aerizusa* obtained by scuba diving 20–30 m depth from Polewali Mandar Sea, West Sulawesi, Indonesia and identified as *Callyspongia aerizusa* Desqueyroux-Faúndez by Oceanographic Research Center, Indonesian Institute of Sciences, Jakarta, Indonesia.<sup>4</sup> To extract *C. aerizusa*, 200 g of the cleaned wet sponge as subjected to chopping, grinding, and three rounds of maceration with total 2 L of methanol and changed every day (500 mL x 4×24 hours).<sup>4</sup> The total macerate was concentrated using a rotary evaporator and subsequently lyophilized, leading to 15.05 g (7.525%) of *C. aerizusa* sponge extract dry powder and stored at –20 °C. The dry powder was dissolved in 0.9% NaCl saline for biological activity testing.<sup>4</sup> *C. aerizusa* sponge extract dry powder contained ergost-22-en-3-one and ergost-7-en-3-ol (Figure 1) detected using Gas Chromatography-Mass Spectrometry (GC-MS) Analysis.<sup>4</sup>

### Cells Line Culture

The human lung carcinoma cells A549, obtained from the American Type Culture Collection (ATCC), were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Darmstadt, Germany). The medium was supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were incubated in an incubator with CO<sub>2</sub> 5% and temperature 37°C. Cells observation was conducted using an Inverted Microscope Axio Vert.A1 (Zeiss, Baden-Württemberg, Germany).

### RNA Isolation and Reverse Transcription-Polymerase Chain Reaction Analysis (RT-PCR)

For gene expression, three A549 cell culture groups were used in triplicate. Two groups were treated with 10 µg/mL *C. aerizusa* extract and incubated for 12 and 24 hours, while the untreated cells culture served as the control group. RNA



**Figure 1** Chemical structure of (A) Ergost-22-en-3-one and (B) Ergost-7-en-3-ol. Ergosteroids are predicted to induce a cytotoxic effect by triggering apoptotic programmed death in A549 cells.<sup>4</sup>

from harvested cells was isolated using InnuPrep DNA/RNA Mini Kit (Analytik Jena, Jena, Germany) following the kit protocol. The purity of the RNA was assessed through the A260/A280 ratio by the Infinite M200 PRO microplate reader (Tecan, Männedorf, Switzerland). The RNA isolate represented a template for RT-PCR, with primers designed using BLAST on the NCBI homepage (Table 1).<sup>4</sup> The *mRNA* expression of genes associated with malignancy markers,

**Table 1** Primer That Designed and Used in Present study

Primer	Sequence	GC%	Tm (°C)
<i>PTEN_R</i>	5'-CCCCCACTTTAGTGCACAGT-3'	55	62.6
<i>PTEN_F</i>	5'-CATAACGATGGCTGTGGTTG-3'	50	58.2
<i>Akt_R</i>	5'-CTTAATGTGCCCGTCCTTGT-3'	50	59.8
<i>Akt_F</i>	5'-TCTATGGCGCTGAGATTGTG-3'	50	58.6
<i>mTOR_R</i>	5'-GCTGCCACTCTCCAAGTTTC-3'	55	61.2
<i>mTOR_F</i>	5'-CCAACAGTTCACCCTCAGGT-3'	55	63.3
<i>STAT3_R</i>	5'-AAGGCACCCACAGAAACAAC-3'	50	60.7
<i>STAT3_F</i>	5'-AGTGAGTAAGGCTGGGCAGA-3'	55	63.1
<i>HIF-1α_R</i>	5'-TGACTTGATGTTTCATCGTCCTC-3'	52	61
<i>HIF-1α_F</i>	5'-AGCTTCTGTTATGAGGCTCACC-3'	50	60.9
<i>IL-6_R</i>	5'-TCACCAGGCAAGTCTCCTCA-3'	55	64.1
<i>IL-6_F</i>	5'-CCACCGGAACGAAAGAGAA-3'	55	63.7
<i>VEGF_R</i>	5'-TCATCTCTCCTATGTGCTGGC-3'	52	61
<i>VEGF_F</i>	5'-ATGAACCTTCTGCTCTCTGG-3'	45	56.5
<i>GAPDH_R</i>	5'-CTTGATTTTGGAGGGATCTCG-3'	52	57.3
<i>GAPDH_F</i>	5'-AAGGTGAAGGTCGGAGTCAAC-3'	52	57.3

including *phosphatase and tensin homolog (PTEN)*, *protein kinase B (Akt)*, *mammalian target of rapamycin kinase (mTOR)*, *STAT-3*, *hypoxia-inducible factor 1-alpha (HIF-1α)*, *IL-6*, *VEGF*, and *GAPDH* as an internal expression control (reference gene), was determined using MyTaq™ One-Step RT-PCR kit (Bioline, London, UK).

The RT-PCR, performed in a thermal cycler (Biometra, Göttingen, Germany) followed the instructions of the manufacturer. All primer sequences, detailed in Table 1, were procured from Macrogen Inc. (Singapore) for 40 cycles.

For detection, 20 µL of each amplified cDNA solution was mixed with 5 µL Fermentas® LD 6x (Thermo Fisher Scientific, MA, USA) and subjected to gel electrophoresis. The gel matrix consisted of 2% agarose in Tris-Acetate-EDTA buffer (Thermo Fisher Scientific) and was stained with SYBR Safe Gel Stain (Invitrogen MA, USA). Subsequently, ImageJ ver. 1.53e (NIH, MD, USA) was adopted for quantifying the *mRNA* expression level of various genes. The total experiments were performed nine times.

## Western Blot Analysis

For protein expression, three A549 cell culture groups were used in triplicate. Two groups were treated with 10 µg/mL *C. aerizusa* extract and incubated for 12 and 24 hours, while the untreated cells culture served as the control group. Proteins were extracted from cells using PRO-PREP solution (iNtRON Biotechnology, MA, USA). The extracted protein samples (10 µL/lane) were separated by 10% SDS-PAGE in the electrophoresis tank (Invitrogen) and were subsequently transferred to a nitrocellulose membrane by adopting a blot transfer device (Invitrogen). The membrane was washed with milli-Q water, blocked with 1% skim milk in phosphate-buffered saline tween-20 (PBST), washed with 0.1% PBST, and incubated with primary antibodies at 4°C overnight. After incubation, the membrane was subjected to a 0.1% PBST wash before probing with secondary antibodies, followed by incubation at room temperature for 1 hour and a final 0.1% PBST wash.

Visualization occurred through electrochemiluminescence methods using an enhanced chemiluminescence (ECL) solution (GF Healthcare, IL, USA). Scanning was done with LI-COR (NE, USA), and quantification was performed by adopting ImageJ ver. 1.53e (NIH). The total experiments were performed nine times.

## Wound Healing Assay

To assess A549 cells ability in wound healing, a scratch-wound assay was performed on cells treated with 10 µg/mL *C. aerizusa* extract.<sup>8</sup> Cells were seeded in 6-well plates and incubated until 80% confluence. Wound was created through the monolayer using a sterile 200 µL pipette tip, and cells debris was removed by washing with PBS three times.<sup>8,9</sup> Wound was observed under a microscope and measured to calculate the migration rate using the formula, including percentage wound healing for 24 and 48 hours = [(wound length at 0 hour) - (wound length at 24 or 48 hours)]/(wound length at 0 hour) x 100. The experiments were performed nine times.

## Cells Invasion Assay

Cells invasion was examined using the BD BioCoat Matrigel invasion chamber (8.0 µm, BD Bioscience, CA, USA) based on the instructions of the manufacturer. About 500 A549 cells treated with 10 µg/mL *C. aerizusa* extract were added to each invasion chamber (500 µL). After 12 hours of incubation, cells were stained with Giemsa stain (Sigma-Aldrich), observed and counted under the microscope. The parental groups were used for normalization, and all samples were tested six times.

## Statistical Analysis

The quantitative data for protein and *mRNA* expression were calculated using ImageJ 1.53v (NIH) with triplicate measurements. Data normality was assessed by adopting a stem-and-leaf plot, followed by the statistical significance calculation using one-way ANOVA and Tukey's post hoc test for groups with homogenous distribution and the Mann-Whitney test for the other group. Statistical significance was considered when the *P*-value was <0.05. The analysis was performed using IBM SPSS statistic 26 (IB Corp., NY, USA).

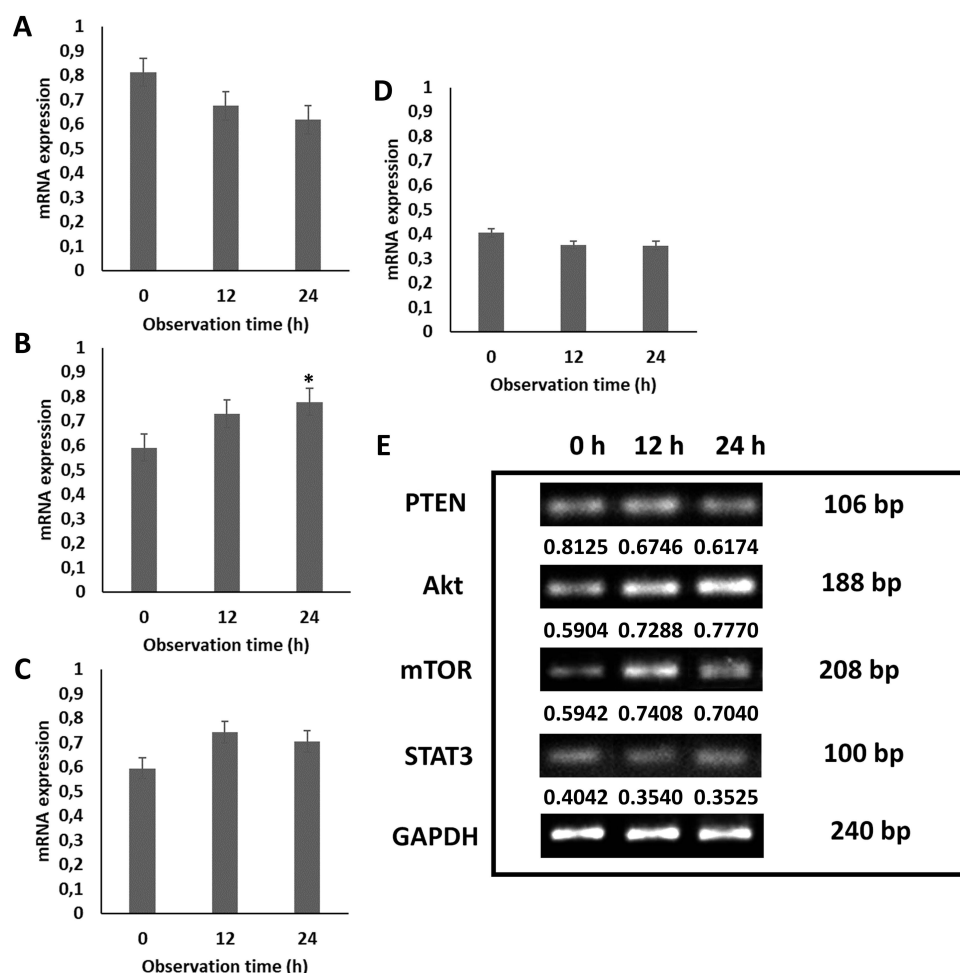
## Results

### mRNA Expression Levels in A549 Cells

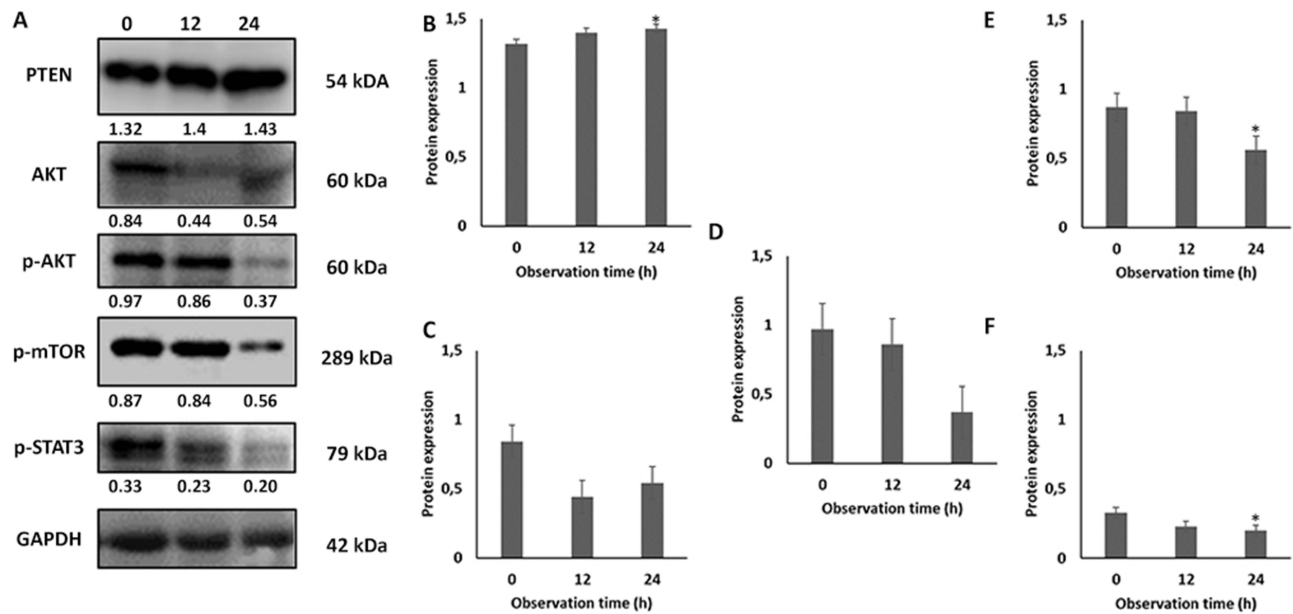
The *mRNA* expression of various genes in A549 cells after the *C. aerizusa* extract treatment was observed through RT-PCR, electrophoresis gel visualization, and quantification using ImageJ ver. 1.53e (NIH) (Figure 2). After a 24-hour treatment with the extract, *PTEN* expression (106 bp) decreased compared to the 0-hour expression (Figure 2A and E). A similar gene expression alteration was observed in *STAT-3* (100bp) (Figure 2D and E). The *mRNA* expression level of *Akt* (188 bp) increased at 12 hours then increased slightly at 24 hours (Figure 2B and E), while *mRNA* expression level of *mTOR* (208 bp) increased at 12 hours and then decreased slightly at 24 hours (Figure 2C and E). However, on both *Akt* and *mTOR mRNA* expression, compared to the expression at 0 hours, the expression at 24 hours remained higher. These results suggested that *C. aerizusa* extract suppressed *STAT-3* at the *mRNA* level.

### Protein Expression Levels in A549 Cells Extract

To validate the synthesis and activation of responsible proteins, a Western blot analysis was conducted (Figure 3A). *PTEN* expression (54 kDa) increased at 12 and 24 hours compared to 0 hours (Figure 3A and B), while *AKT* activity (60 kDa) (Figure 3A and C), had a pattern characterized by a decrease at 12 hours and an increase at 24 hours. This signified the role of *PTEN* as a regulator of *Akt* activation through  $PI(3,4,5)P_3$  inhibition, thereby affecting *Akt* phosphorylation. The expression of p-*AKT* (60 kDa) (Figure 3A and D) and p-*mTOR* (289 kDa) (Figure 3A and E) decreased at 12 and



**Figure 2** PI3K/Akt Signaling *mRNA* Expression (*PTEN*, *Akt*, *mTOR*, and *STAT-3*) in A549 cells treated with *C. aerizusa*. (A) *PTEN*, (B) *Akt*, (C) *mTOR*, (D) *STAT-3* *mRNA* expression was visualized using electrophoresis gel. Quantitative data of band intensity was measured with ImageJ ver. 1.53e (NIH). (E) Bar graph showing *mRNA* expression of *PTEN*, *Akt*, *mTOR*, and *STAT-3* obtained by RT-PCR in A549 cells treated with *C. aerizusa* extract. One-way ANOVA followed by Tukey's post hoc test was performed. \* $p < 0.05$ .

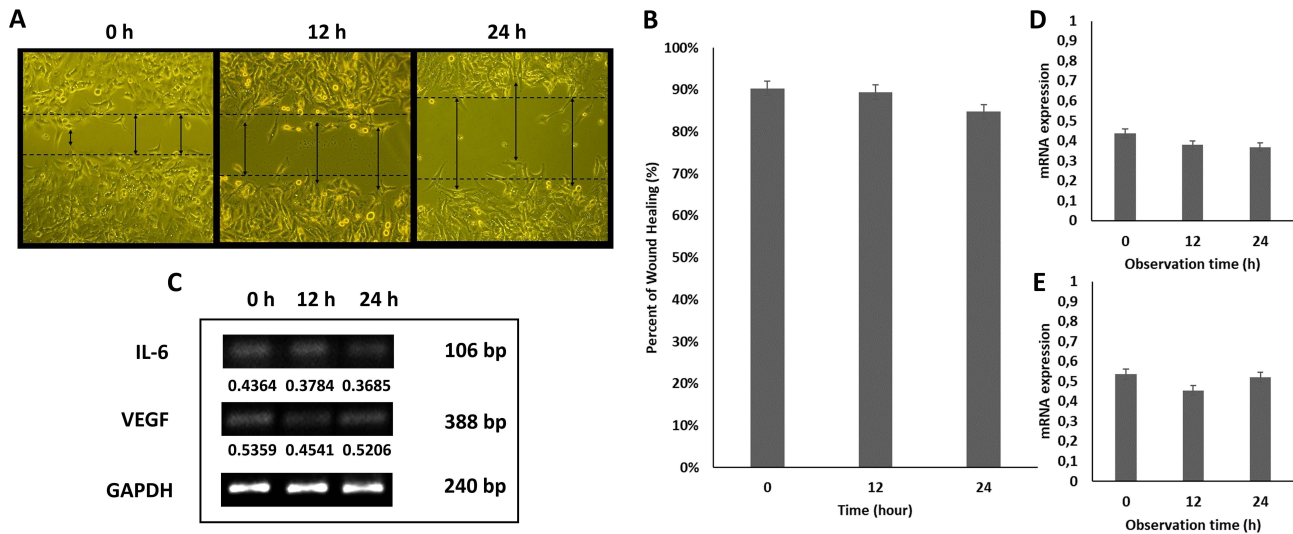


**Figure 3** Jak/Stat Signaling Protein expression (PTEN, AKT, p-AKT, p-mTOR, and STAT-3) in A549 cells treated with *C. aerizusa*. (A) Visualization of protein expression was normalized with  $\beta$ -actin. Quantitative data of band intensity was measured with ImageJ ver. 1.53e (NIH). Bar graph showing protein expression of (B) PTEN, (C) AKT, (D) p-AKT, (E) p-mTOR, and (F) STAT-3 obtained through Western blotting in A549 cells treated with *C. aerizusa* extract. One-way ANOVA followed by Tukey's post hoc test was performed. \* $p < 0.05$ .

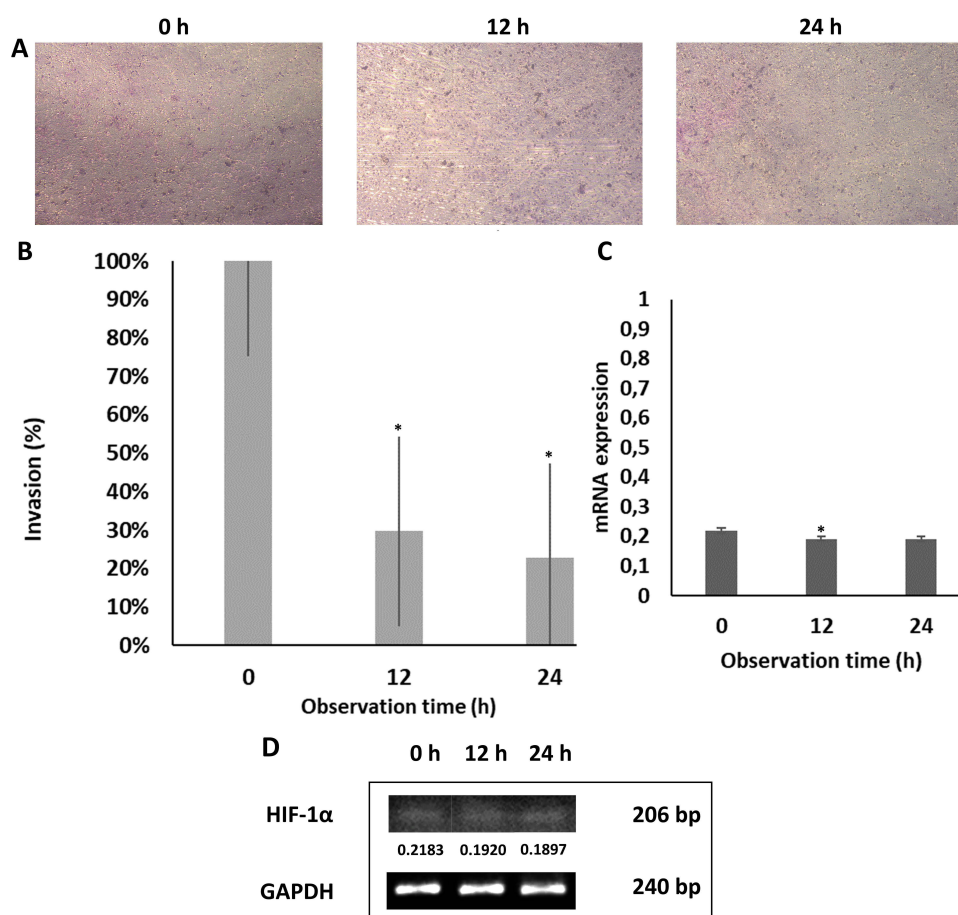
24 hours, similar to p-STAT-3 (79 kDa) (Figure 3A and F). The results showed that *C. aerizusa* extracts could suppress STAT-3 at both the protein and *mRNA* levels.

## Wound Healing Assay and mRNA Expression of IL-6 and VEGF

The ability of A549 cells to cover scratches after treatment with *C. aerizusa* extract for 24 hours decreased over time (Figure 4). The untreated A549 cells covered 90.34% of the scratch after 24 hours, while cells treated with the extract for 12 hours covered 89.41%, and for 24 hours, the coverage reduced to 84.74% (Figure 4A and B).



**Figure 4** Wound healing assay and IL-6/VEGF *mRNA* expression in A549 cells treated with *C. aerizusa*. (A) Photograph of A549 cells after 24 hours of scratching, 0 hours for untreated cells, 12 hours, and 24 hours after treatment with the extract 10  $\mu$ g/mL. (B) Percentage of scratch coverage in wound healing assay (%). (C) Visualization of *mRNA* expression of IL-6 and VEGF was performed using electrophoresis gel. Quantitative data of band intensity for (D) IL-6 and (E) VEGF obtained by RT-PCR in A549 cells treated with *C. aerizusa* extract. One-way ANOVA followed by Tukey's post hoc test was performed.



**Figure 5** Invasion assays validated with *HIF-1α* mRNA expression in A549 cells treated with *C. aerizusa* extract. **(A)** Invasive ability of A549 cells without *C. aerizusa* extract treatment (0 h) and with the extract treatment. **(B)** Significant reduction in the invasive ability of A549 cells without *C. aerizusa* extract treatment (0 h) compared to A549 cells with treatment (12 and 24 hours). Invasion in the treated cells (%) was normalized to untreated cells. \* $p < 0.05$ . **(C)** Visualization of mRNA expression of *HIF-1α* was performed using electrophoresis gel. **(D)** Quantitative data of band intensity for *HIF-1α* obtained by RT-PCR in A549 cells treated with *C. aerizusa* extract. One-way ANOVA followed by Tukey's post hoc test was performed. \* $p < 0.05$ .

Wound healing results, along with Inflammation markers such as *IL-6* and *VEGF* mRNA expression were used to strengthen and validate the observed phenomenon. *IL-6* decreased over 24 hours (Figure 4C and D), while *VEGF* slightly decreased at 12 hours and increased again at 24 hours in A549 cells treated with *C. aerizusa* extract (Figure 4C and E).

## Cells Invasion Assay and mRNA Expression of HIF-1α

The invasive ability of A549 cells treated with *C. aerizusa* extract was weakened compared to untreated cells (Figure 5A and B). After 12 and 24 hours of treatment, cells reaching the bottom of the chamber decreased to 29.75% and 22.80% respectively, while untreated cells reached 100%.

To validate the decreased invasion ability of A549 cells by *C. aerizusa* extract, the mRNA expression detection to *HIF-1α* was conducted (Figure 5C and D). This showed the downregulation of *HIF-1α* mRNA expression occurred within 12 and 24 hours after treatment with the extract.

## Discussion

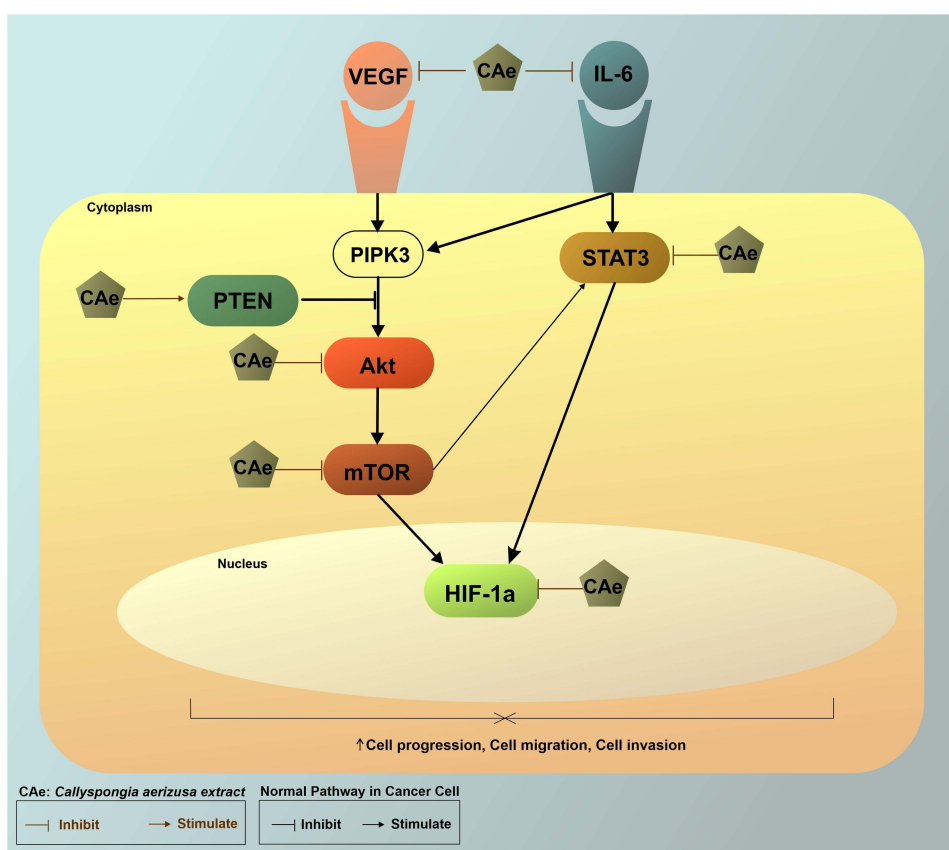
*C. aerizusa* had established strong cytotoxicity against L5178Y mouse lymphoma cells lines, attributed to compounds such as Callyaerins E and H.<sup>3</sup> Previous investigation showed that the *C. aerizusa* extract containing ergost-22-en-3-one and ergost-7-en-3-ol (Figure 1) had cytotoxic effects against A549 lung cancer cells, with an  $IC_{50}$  value of 9.38  $\mu\text{g/mL}$ .<sup>4</sup> However, it did not showcase cytotoxicity to Het-1A esophagus epithelial cells.<sup>4</sup> The extract also inhibited A549 cells colony formation and modulated pro-apoptotic and anti-apoptotic molecules, implicating caspase-dependent signaling

pathways.<sup>4</sup> This research further explored the effect of the *C. aerizusa* extract on A549 lung carcinoma through apoptosis-related signaling, specifically focusing on the activation of Casp-9 and inhibition of Bcl-2, often associated with p-Akt and STAT-3.<sup>5–7</sup> Consequently, phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling-related protein analysis was crucial to further explore the molecular mechanisms. The *mRNA* expression showed an increase in *Akt*, while *PTEN* remained unchanged (Figure 2A, B and E). The expression of *mTOR* increased at 12 hours and slightly decreased at 24 hours, while *STAT-3* expression decreased at 12 hours and continued to decrease slightly at 24 hours (Figure 2C, D and E). At the protein level, there was downregulation of p-Akt (Figure 3A and D), p-mTOR (Figure 3A and E), and p-STAT-3 (Figure 3A and F), coupled with increased PTEN (Figure 3A and B). Akt expression decreased at 12 hours but had a slight increase at 24 hours (Figure 3A and C). The observed downregulation of malignancy in A549 lung cancer cells was supported by weakened wound healing and invasion abilities (Figures 4 and 5). Wound healing assay showed a reduction from 90% to 80% in cells capacity to cover the stretch (Figure 4A and B). Concurrently, the invasion assay had a significant decrease in the number of A549 cells capable of traversing the matrigel membrane, reaching 22.80% (Figure 5A and B). The results were in line with the suppression of *IL-6* and *HIF-1 $\alpha$*  *mRNA* expression levels during the 24-hour treatment period (Figures 4C, D and 5C, D). *VEGF* *mRNA* level showed a decrease at 12 hours, followed by a slight increase at 24 hours (Figure 4C and E).

The expression patterns of PTEN, Akt, and mTOR at both *mRNA* and protein levels showed non-linearity. While *PTEN mRNA* expression decreased in the graphical representation (Figure 2A), the band visualization (Figure 2E) suggested marginal differences. PTEN protein expression also seemed to increase in the graph (Figure 3B), but the band expression (Figure 3A) lacked significance. In contrast, *Akt* and *mTOR* *mRNA* expression increased, but their protein expression decreased (Figures 2B, C, E and 3A, C and E). This discrepancy could be attributed to potential *mRNA* decay after treatment with *C. aerizusa* extract, resulting in inhibition during transcription and translation, thereby impeding the activation of Akt and mTOR proteins at 12 and 24 hours, respectively (Figures 2E and 3A).<sup>10,11</sup> The most cancer malignancy biomarker, STAT-3, had a decrease at 12 hours and nearly disappeared at 24 hours in protein level, despite a slight continued decrease in *mRNA* at 24 hours (Figures 2D, E and 3A, F).

The expression of PTEN, Akt, p-Akt, p-mTOR, and STAT-3 proteins held significance as they influenced the phenomena that occurred in A549 cells (Figure 3A). A previous report on apoptotic cells death in A549 cells treated with *C. aerizusa* extract was in line with the downregulation of anti-apoptotic protein Bcl-2, attributed to the suppression of cell survival signaling pathways. PTEN protein expression in A549 cells treated with *C. aerizusa* extract increased at 12 hours, coinciding with Akt suppression (Figure 3A–C). Moreover, the suppression of phosphorylated Akt was observed in A549 cells at 24 hours after treatment (Figure 3D). This was in line with previous research on the anticancer activity of a dipeptide Cyclo(-Pro-Tyr) from marine sponge *C. fistularis* in the liver cancer HepG2 cells lines, which inhibited Akt phosphorylation and increased PTEN.<sup>12</sup> The inhibition of Akt/mTOR signaling pathway induced apoptotic cells death in various cancers, including A549 cells.<sup>13</sup> As shown in Figure 3A, the suppression of phosphorylated Akt within 12 hours also led to the downregulation of phosphorylated mTOR expression. Compared to previous reviews, such as the Microalga *Chlorella sp.* extract (300 and 400  $\mu$ g/mL), marine sponge *C. aerizusa* extract required a much smaller dose (10  $\mu$ g/mL) to achieve a similar effect.<sup>13</sup> The IL-6/STAT-3 pathway, known for its role in regulating malignancy, experienced abnormal activation related to tumor angiogenesis and migration.<sup>14,15</sup> The decrease in phosphorylated Akt subsequently led to decreased phosphorylated mTOR and STAT-3 protein level expression at 12 hours and nearly disappeared at 24 hours (Figure 3A, E and F). A similar phenomenon was experienced in the Akt/mTOR/STAT-3 signaling pathway of TE-8 cells treated with *Curcuma zedoaria*.<sup>16</sup>

As the downregulation of STAT-3, a malignancy marker associated with inflammation similar to IL-6, was detected in A549 cells treated with *C. aerizusa* extract, it was anticipated that the wound healing assay ability of the lung cancer cells would be impaired. Wound healing assay showed a significant weakening of cells ability to cover the scratch up to 20% (Figure 4A and B). Although *mRNA* expression of *IL-6* and *VEGF* in cells treated with *C. aerizusa* extract showed downregulation at both 12 hours and 24 hours, the analysis contradicted previous limitations on the inflammation inhibition of marine compounds (Figure 4C and D). Siphonolol A, isolated from *C. siphonella*, has also been reported as an inhibitor of protein tyrosine kinase 6 (PTK6) identified for its role in promoting growth factor signaling and migration in MDA-MB-231 breast cancer cells.<sup>17,18</sup> Despite previous reviews suggesting limited inflammation inhibition of marine



**Figure 6** The summarized pathway mechanisms for *C. aerizusa* extract (CAe) in various signaling pathways used in this research on A549 cells.

compounds, this research showed that A549 cells ability for inflammation inhibition and wound healing assay weakened during treatment with *C. aerizusa* extract.

Invasion assay results showed a reduction of A549 cells capable of traversing the matrigel membrane, decreasing by up to 77.8% (Figure 5A and B). Moreover, *HIF-1α* mRNA expression levels were decreased during 24-hour treatment with *C. aerizusa* extract (Figure 5C and D). The results showed that the extract has an effect on reducing the migration and invasion abilities of A549 cells. A similar conclusion about the reduction of the abilities was reported by Foudah et al, where siphonolol A potently inhibited MDA-MB 231 cells invasion at 10  $\mu$ M.<sup>17</sup>

In cancer cells, various signaling pathways play crucial roles. Cells progression and angiogenesis could be regulated through the PI3K/Akt/mTOR or the IL-6/STAT-3 pathway. Within the PI3K/Akt/mTOR pathway, PTEN negatively regulated Akt by inhibiting the conversion of phosphatidylinositol bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3).<sup>19</sup> While mTOR, up-regulated by Akt, contributed to transcription of HIF-1 $\alpha$  which induced pro-angiogenic factors such as VEGF.<sup>20</sup> Apart from being positively regulated by mTOR,<sup>21</sup> STAT-3 could be activated by IL-6.<sup>22</sup> Similar to Akt, it has the capacity to regulate VEGF through up-regulating transcription factor HIF-1 $\alpha$ .<sup>23</sup> The secretion of VEGF could reactivate cells and trigger cells progression.<sup>24</sup> Therefore, *C. aerizusa* extract was proposed to reduce cells migration and invasion by suppressing the expression of Akt, mTOR, STAT-3, VEGF, IL-6, or HIF-1 $\alpha$ , while increasing the expression of PTEN (Figure 6).

This research continued to the growing body of evidence supporting the inhibitory effects of *C. aerizusa* extract on cells malignancy, migration, and invasion by downregulating the PI3K/Akt signaling pathway in A549 cells. Ongoing analysis comprised scaling up *C. aerizusa* source and extract production, while future plans included in vivo experiments to further validate the promising results.

## Conclusion

In conclusion, the presence of ergosteroids in marine sponge *C. aerizusa* extract showed a significant reduction in malignancy, migration, and invasion abilities in human lung carcinoma cells A549. The combination of key factors, including the up-regulation of PI3K/Akt/STAT-3 signaling pathway, coupled with the downregulation of IL-6, VEGF, and HIF-1 $\alpha$ , consistently supported the weakening of A549 cells ability in wound healing and invasion assays. The compounds extracted from *C. aerizusa* showed promising potential as candidates for future anti-angiogenesis in anticancer therapy.

## Abbreviations

*C. aerizusa*, *Callyspongia aerizusa*; RT-PCR, reverse transcription-polymerase chain reaction; PTEN, phosphatase and tensin homolog; mTOR, mammalian target of rapamycin kinase; STAT-3, signal transducer and activator of transcription 3; HIF-1 $\alpha$ , hypoxia-inducible factor 1-alpha; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBST, phosphate-buffered saline Tween-20.

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## Disclosure

The authors declare no conflicts of interest in this work.

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