RESEARCH ARTICLE

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Anticancer Activity of *Aaptos suberitoides* on Glioblastoma Multiforme Cell Line

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Abstract

Objective: Glioblastoma Multiforme (GBM) poses a significant challenge due to its high aggressiveness and unfavorable prognosis, with existing treatments demonstrating limited efficacy in prolonging survival rates. This study aimed to assess the anticancer properties of *Aaptos suberitoides* extracts and fraction on the U87 cell line, serving as a representative model for GBM. **Methods:** U87 cells were treated with ethanol extracts derived from *Aaptos suberitoides*, specifically two extracts (OAA-1 and OAA-2) and one ethyl acetate fraction (EA) isolated from specimens collected on Pramuka Island and Tinjil Island. The evaluation encompased microscopic observation and MTT assay to determine the IC50. Subsequently, antiproliferative effects were investigated through apoptosis and cell cycle assays. **Results:** The extract demonstrated cytotoxic activity against U87 cells, with OAA-1 and OAA-2 exhibiting IC_{50} values of 35.78 µg/mL and 25.38 µg/mL, respectively. OAA-1 notably induced apoptosis at 50 µg/mL and induced cell cycle arrest. On other hand, OAA-2, while also inducing apoptosis significantly, had a lesser impact on cell cycle arrest. In contrast, EA induced significant apoptosis at a concentration of 100 µg/mL. **Conclusion:** The ethanol extracts and the ethyl acetate fraction of *Aaptos suberitoides* emerged as a promising candidate for Glioblastoma Multiforme cancer therapy, showing potential in inhibiting cell proliferation and inducing apoptosis.

Keywords: *Aaptos suberitoides*- Antineoplastic agents- glioblastoma- marine substance- phytogenic

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Introduction

Glioblastoma multiforme (GBM) is grade IV glioma with massive proliferation of glial cell [1]. It poses a significant challenge, with a mere 5% of patients achieving a 5-year survival rate [2]. The annual incidence of this aggressive cancer ranges from 0.59 to 3.69 per 100.000 individuals, according to the Central Brain Tumor Registry of the United States (CBTRUSt) [3]. Glioblastoma can be categorized into 4 categories based on histopathological appearance: Isocitrate dehydrogenase (IDH)-wild type, IDH-mutant, Glioblastoma not otherwise specified, and not-elsewhere-classified. IDH-wild type is the major type of GBM, approximately 90% of GBM cases [4]. Despite current treatments, including craniotomy for tumor removal, chemotherapy, and drugs like temozolomide and bevacizumab, median overall survival remains below 15 months [1, 5]. These drugs have been reported to become resistant because of Methyl guanine methyl transferase (MGMT) regulation and other proangiogenic factors [6, 7]. Because of GBM aggressiveness, a drug with the potential to suppress GBM progressivity is needed to increase patient's quality of life.

Advancements in cancer therapy are being made through the exploration of natural substances, which assessed both as monotherapy or complementary treatments [8, 9]. Notably, Trogrlić et al. (2018) conducted a study involving five herbal mixtures administered as daily supplementary treatment to glioblastoma patients. This research demonstrated the therapeutic effect of these substances, revealing increased survival rates and suppressing the development of tumor [10].

Recently, approvals by the Food and Drug Administration (FDA) have recognized the efficacy of substances derived from sponges, such as Cytarabin and Eribulin, as cancer therapy [11]. This underscores the growing utilization of natural substances in cancer therapy. *Aaptos suberitoides*, a sponge found abundantly in Indonesian seas and oceans [12], has exhibited potential anticancer activity, primarily through a substance isolated from it called aaptamin. Aaptamin acts as antioxidant and induces cell death in cancer cells [13, 14]. It has demonstrated anti-cancer effects in breast cancer cell lines and fibrosarcoma-induced rats [15, 16]. Furthermore, it exhibits an anti-migration effect in colorectal cancer cell lines by increasing cofilin-1 concentration [17].

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While *Aaptos suberitoides* has been extensively studied in various cell lines, research on its impact on GBM cells is lacking. Therefore, our study aimed to evaluate the anticancer activity of both the extract and fraction of *Aaptos suberitoides* on GBM cell lines.

Materials and Methods

Sample preparation

Aaptos suberitoides specimens were collected from Tinjil Island, Banten, and Pramuka Island, Jakarta, Indonesia in November 2022. The The ethanol extract of *Aaptos suberitoides* contains primarily compounds with a molecular mass of 229ES+. Data analysis indicated that the most prevalent molecular weight in the extract was m/z 229.81 [16]. The sponges were harvested through diving at a depth of approximately 20 meters, followed by chopping and maceration with 96% ethanol. After 2-3 days, the solutions underwent filtration using filter paper and subsequent evaporation with rotary evaporator to yield a concentrated paste extract. The ethanol extracts of *Aaptos suberitoides* were then dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma, cat.no. D8418, USA) to achieve a concentration of 40,000 µg/mL, and the resulting solution was stored in a 4°C chiller. For subsequent experiments, the stock should be appropriately diluted with a complete culture medium to obtain the required concentrations. The stock that was taken from Tinjil Island and Pramuka Island was coded OAA-1 and OAA-2 respectively. To achieve ethyl acetate fraction from OAA-2, coded as EA, the extract was then added by ethyl acetate. Ethyl acetate selectively dissolves specific compounds from the mixture based on their solubility characteristic and affinities. The ethyl acetate fraction of *Aaptos suberitoides* was dissolved in 100% DMSO to achieve a concentration of 40,000 µg/mL.

Cell line

The U87 glioblastoma cell line was cultured using RPMI1640 (Gibco, cat.no. 11875-093, USA) medium supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, cat.no.10270-106, USA), 1% Penicillin/ Streptomycin (Sigma, cat.no. 15140122), and Phosphate Buffered Saline (PBS) (Gibco, cat.no. 70011044, USA) within a cell culture incubator (Thermo Scientific model 3429, USA, 2014) set to 37°C temperature and 5% CO2. Cell detachment was achieved with Trypsin-EDTA (Gibco, cat.no. 25200056, USA). Tissue Culture flask 25 cm2 (TPP, cat.no.90025, USA) were employed for cultivation, 96-Well Tissue Culture Plates (TPP, cat. no.92096, USA) for the MTT assay, and 6-Well Tissue Culture Plates (TPP, cat.no.92006, USA) was used for cell cycle and apoptosis assay. The study was conducted at Cell Culture and Cytogenetic laboratory, Faculty of Medicine, Universitas Padjadjaran.

Cytotoxic activity with MTT assay

3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, commonly known as MTT (cat.no M6494, Sigma Aldrich, USA), serves as an assay for assessing cell viability and proliferation [18]. This in vitro assay proves instrumental in evaluating the anticancer activity of drug in cell lines, offering a gold standard for assessing drugs under controlled conditions and procedures. MTT assay is particularly valuable in oncology study, providing a method to predict drug response in malignancies [19]. The conversion of MTT to formazan crystals by NAD(P)H-dependent oxidoreductase imparts a violet color when DMSO is added. The intensity of the violet color, measued by photometric micro plate reader [20, 21], proportionally represents the number of viable cells. Absorbance measurements from each concentration were adjusted by subtracting the value from the blank well containing DMSO to eliminate absorbance contribution from medium.

The cytotoxic activity of *Aaptos suberitoides* extracts and fraction in U87 cells was assessed using MTT assay, following the previously described protocol. This assay was done 3 repetitions with triplicate samples. U87 cells were cultured for 24 hours, followed by a 72-hours incubation with the extracts and fraction. Cellular observation was made using inverted microscope (Olympus CK40, Japan) equipped with a microscope camera (C-mount camera, China). Subsequently, the MTT assay solution was added 4 hours before termination with DMSO. Samples were read at 550 nm with a photometric micro plate reader (Thermo Scientific® Multiscan EX, Singapore). A comparison of cell appearance and absorbance values served as confirmation of the result.

Apoptosis assay

Apoptosis assay in this study employed the AnnexinV/ PI evaluation. The efficacy of this evaluation relies on integrity and permeability of plasma membrane.[23] U87 cells, initially cultured in 6-well plate with 100,000 cells, underwent a 24-hour incubation in cell culture incubator. subsequently, they were subjected to treatment with 50 µg/mL and 100 µg/mL concentration of OAA-1, OAA-2, and EA. After another 24-hour incubation and PBS wash to remove dead cells, the cells were incubated for 30 minutes at 37°C with AnnexinV-FITC/PI kit (Invitrogen, V13242). Following this, the cells were analyzed using BD FACSFlow (Geneaid, EH22202-03901) and processed using FlowJo (BD, v10.9.0).

Cell cycle assay

In this study, a cell cycle assay was conducted using PI (Invitrogen, P3566) in U87 cells. Cell cycle phases, including G1, S, G2, and M, were characterized by different amounts of DNA. PI served as a marker to indicate the amount of DNA due to its ability to bind with DNA. The U87 cells were cultured and treated similarly to the apoptosis assay. Before applying the PI marker, RNase (Geneaid, EH22202-03901) and Triton X-100 (Sigma, T8787) were added to ensure that only DNA was measured, excluding other protein in the cells. The cells were then analyzed using BD FACSFlow and processed with FlowJo.

Evaluation

Data processing and dose-response curves were conducted using GraphPad Prism v9.0 (GraphPad

software, San Diego, CA). Sigmoidal 4-parameter logistic regression with concentration x, was employed for the dose-response curve and 50% inhibition concentration, or IC50. Statistical analyses for apoptosis and cell cycle assay were carried out using one-way ANOVA in GraphPad Prism v9.0 (GraphPad Software, v9.0). For all analysis, a P-value < 0.05 was considered statistically significant. Symbols "*", "**" and "***" were used to indicate P-value < 0.05, < 0.01 and <0.001, respectively.

Results

Cytotoxic assay

The cytotoxic effect of *Aaptos suberitoides* extracts was assessed using the MTT assay. Eleven concentrations were tested in U87 cells, ranging from 1 µg/mL to 100 µg/mL, with 0 µg/mL serving as the control. Absorbance from each concentration was measured, with the lower absorbance indicating a higher percentage of cytotoxic. Subsequently, the dose-response curve and IC_{50} were evaluated using 4-parameter logistic regression, as illustrated in Figure 1a.

Before adding the MTT reagent, we observed the cell conditions under the inverted microscope. Microscopic observation revealed proper growth of the control U87 cells. At higher treatment doses, almost the entire cells population on the plate underwent cell death. We also noted that increasing the treatment dose leads to a decrease in cell confluence (Figure 1a). Despite the different effects observed from both extracts and fractions, as depicted on figure 1b, their IC_{50} values were low. The IC_{50} values for OAA-1 and OAA-2 were 35.78 µg/mL and 25.95 µg/mL, respectively. The ethyl acetate fraction also induced cell death in glioblastoma multiforme cell line with an IC_{50} of 25.38 µg/mL.

Apoptosis assay

We utilized an Annexin V/PI reagent for apoptosis assay, as previously mentioned. Annexin V binds to phosphatidylserine during early apoptosis when phosphatidylserine migrates to external membrane. In late apoptosis, PI enters the cell, attaching to nucleic acid, while annexin V continuous to bind to phosphatidylserine. In the case of cells undergoing necrosis, the cell membrane becomes more permeable, lacking phosphatidylserine on the external side, allowing PI to enter intracellularly abundantly [22].

As illustrated in Figure 2, *Aaptos suberitoides* induced early and late apoptosis in U87 cells. At concentration of 50 µg/mL, OAA-1 and OAA-2 predominantly exhibited significant results in late and early apoptosis. While EA also induced early and late apoptosis, the result for EA

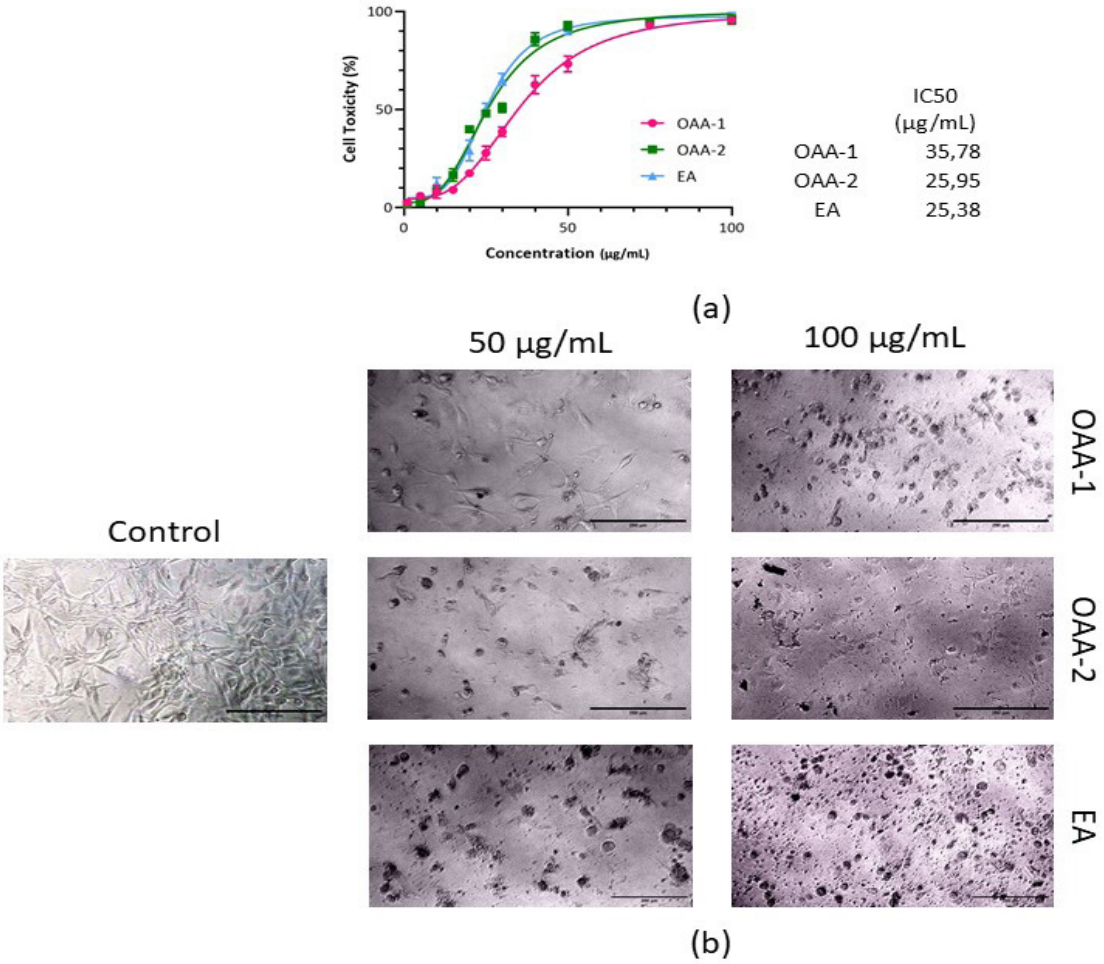

Figure 1. Cytotoxic Curve and Microscopic (magnification 100x) Appearance of U87 Cell by Treatment of extract of Aaptos suberitoides. (a) Curve of percentage of cells death and dose of treatment. (b) Microscopic appearance of U87 cell after 72-h treatment.

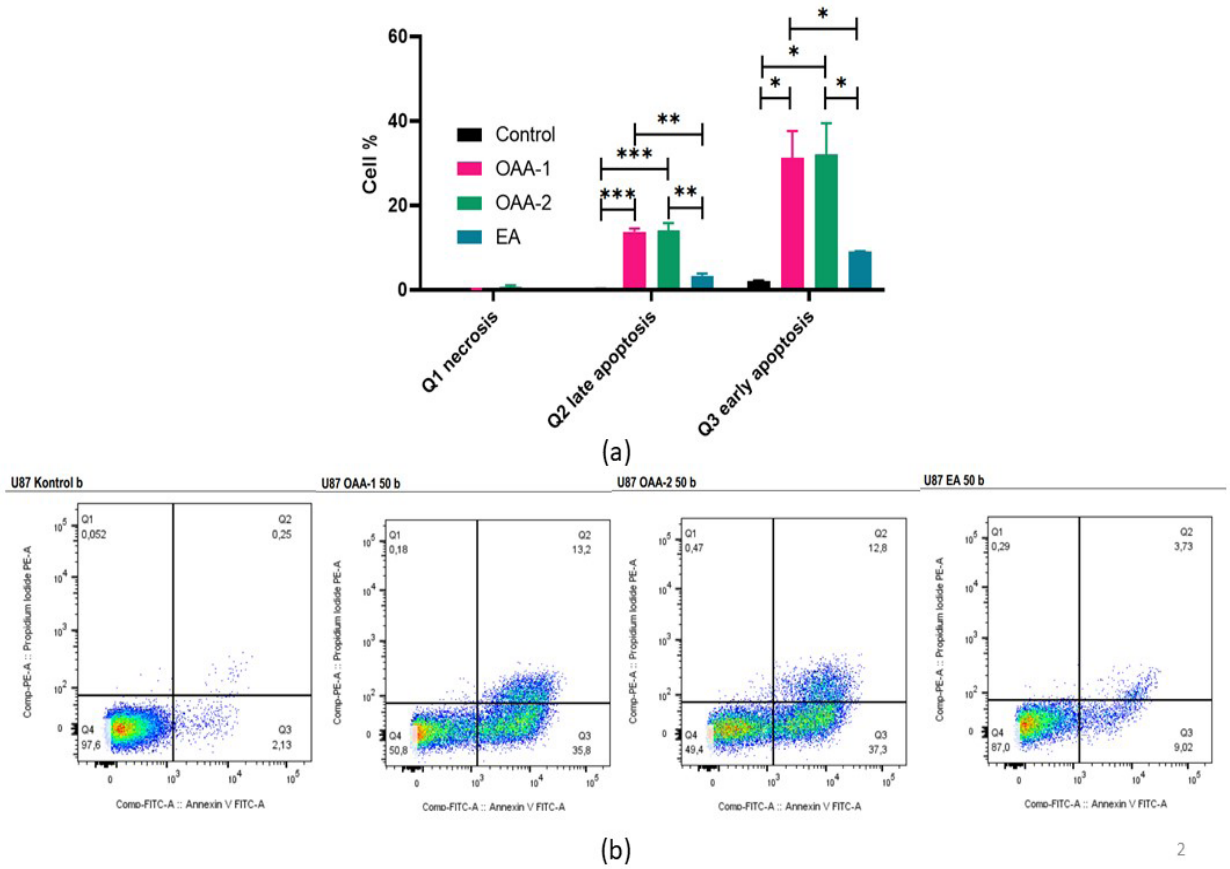

Figure 2. Apoptosis Analysis Using Flowcytometer. (a) Statistical analysis of Aaptos suberitoides inducing apoptosis on U87 cels with AnnexinV/PI marker. (b) Pseudocolor density plot of U87

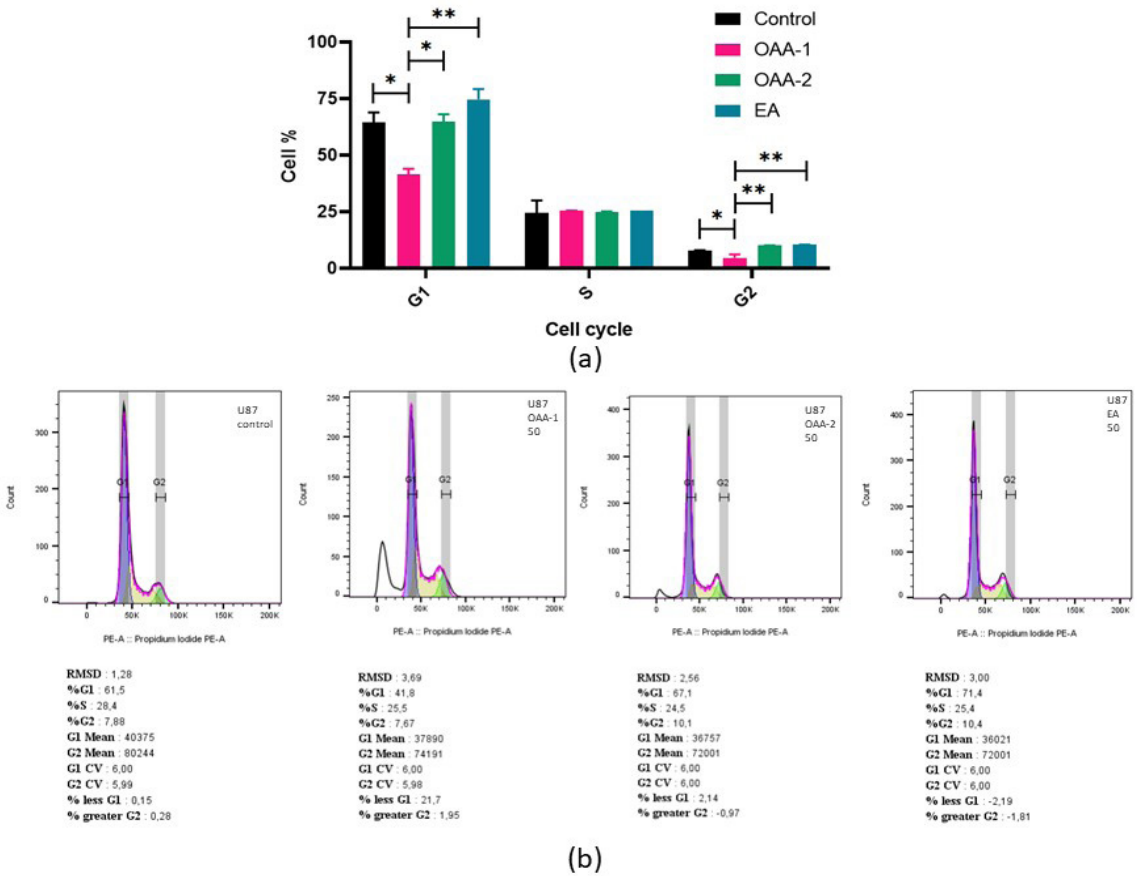

Figure 3. Cell Cycle Analysis Using Flowcytometry. (a) Statististical analysis of U87 cell line and (b) Cell cycle curve of U87

was not significant at 50 µg/mL concentration. However, OAA-2 and EA significantly demonstrated early and late apoptosis conditions at 100 µg/mL concentrations, while OAA-1 did not.

Cell cycle assay

As depicted in Figure 3, *Aaptos suberitoides* exhibits a notable antiproliferative effect in U87 cells. both 50 µg/mL and 100 µg/mL concentrations of OAA-1 significantly hindered cells from progressing through G1 phase. Particularly, the G1 phase under 50 µg/mL concentration of OAA-1 also was significantly impeded compared to OAA-2 and EA cell concentrations. Additionally, OAA-1 prevented cells from entering G2 phase of cell cycle in 50 µg/mL concentrations. While OAA-2 and EA also have an antiproliferative activity, it was less significant than that observed with OAA-1.

Discussion

U87 is well known as representative model of glioblastoma multiforme. Glioblastoma can manifest through various pathways, including isocitrate dehydrogenase mutation, vascular Endothelial Growth Factor, PI3K/AKT/mTOR, and Epidermal Growth Factor Receptor (EGFR). These inctricate pathways contribute to resistance againts cell death and the maintenance of proliferative signaling, influencing the host vasculature to facilitate the nutrition acquisition [23].

During treatment, *Aaptos suberitoides* exhibited the ability to induced cell death in U87 cells. The marine sponge, known for its anti-cancer activity in vitro and in vivo, demonstrated noteworthy potential [15–17]. Through the analysis of cell death using photometric micro plate reader in this study, OAA-2 emerged as more potent than OAA-1 in inducing cell death, with an IC_{50} of 25.95 µg/mL. Further assessment of the OAA-2 fraction revealed a comparable potency in inducing cell death, with an IC50 of 25.35 µg/mL. These findings suggested that the ethyl acetate fraction of *Aaptos suberitoides* holds promise for in-depth exploration in U87 cell studies.

Moreover, additional analyses encompassed apoptosis and cell cycle assay. The result revealed that OAA-1 induced apoptosis U87 cell line to apoptosis, with the assay marker indicating early and late apoptosis. This effect was observed only at concentration of 50 µg/mL, not at 100 µg/mL. Both concentrations of OAA-2 yielded significant results in apoptosis, demonstrating early and late apoptosis in U87 cell line. conversely, the *Aaptos suberitoides* fraction exhibited apoptosis activity solely at concentration of 100 µg/mL. Beyond inducing early and late apoptosis, the fraction also significantly demonstrated an ability to induce necrosis in U87 cell.

Furthermore, we also investigated the impact of *Aaptos suberitoides* extract and fraction on cell cycle regulation. OAA-1 exhibited significantly greater efficacy in inducing cell cycle arrest compared to OAA-2 and EA. Specifically, OAA-1 halted the progression of G1 phase at both concentrations and the G2 phase at concentration of 50 µg/mL. The distinct effect with the *Aaptos suberitoides* extract and fraction could be attributed to variations in ecosystem condition, leading to difference in nutrition and physical environment [24, 25]. This diversity induced varying concentration of active substances and metabolites in each *Aaptos suberitoides* samples.

Aaptos suberitoides demonstrates a multifaceted approach to induce cell death and curtail proliferative activity, by inhibiting the proteasome, which regulates cell communication, cell cycle, and transcription, *Aaptos suberitoides* disrupts the delicate balance maintained by the ubiquitin system. The 19s and 20s protein subunits within the proteasome facilitate the activity of ubiquitin system a cascade of 76 amino acids that collaboratively degrade non-functional proteins [26–28]. This inhibition prompts cell death by elevating oxidative protein levels, inducing excessive Reactive Oxygen Specieces (ROS) in cancer cell [29]. Even when inducing reactive oxygen species (ROS) in cancer cells, extracts of Aaptos sp. also exhibit antioxidant activity as demonstrated by the DPPH assay [30, 31]. The results revealed a low IC_{50} of 27.42 µg/mL for Aaptos sp [31]. Furthermore, another study found that the IC50 of the *Aaptos suberitoides* extract was lower than that of ascorbic acid [30]. Notably, proteasome inhibition also triggers apoptosis and cell cycle arrest [28]. Another mechanism involves the inhibition of NF-kB, a transcription factors influencing celluler immunity, inflammation, stress responses, cell differentiation, and cell proliferation [32–35]. The suppression of NF-kB can impede cancer cell progression [34].

Aaptos suberitoides also exerts a cytotoxic effect by inhibition of MAPK enzyme, crucial for cells to survive, proliferate, and growth. Uncontrolled interaction between MAPK and EGFR can increase the progression of cell cancer. MAPK also induces metastatis of cell cancer and activates HIF-1α, fostering angiogenesis [32, 36]. The reported anti-photoaging effect involves the inihibition of AP-1 protein by aaptamine in human dermal fibroblasts and epidermal keratinocytes. This protein, activated by MAPK enzyme, contributes to cell proliferation and the transformation of cell cancer [32, 37, 38]. Beyond pathway inhibition, *Aaptos suberitoides* components induces p21 protein, stalling cell proliferation by inihibiting cyclin-dependent kinases. Additionally, p21 also interacts with proliferating cell nuclear antigen, effectively impeding DNA replication [26, 39, 40]. Building on previous research highlighting *Aaptos suberitoides*'s anticancer potential, treatment with *Aaptos suberitoides* extracts and fraction resulted in cell death in U87 cells.

In conclusion, the ethanol extracts of *Aaptos suberitoides* induced cell death in GBM cell line, demonstrating anticancer activity. However, this research was not delved into the mechanism of cell death with the treatment. This study involved a one-time trial without positive control, potentially affecting the data. Further experiments are crucial to identify active compounds in extract and fraction. Further research should explore the extract and fraction's mechanisms inducing cell death in U87 cells.

Author Contribution Statement

The authors confirm contribution to the paper as

follows: study conception and design: FH MHB TP; data collection: FH RH MHB; analysis and interpretation of results: RH, RGD, AB; funding: FH, RGD, AB, MHB; draft manuscript preparation: RH FH MHB; final draft and revision: FH, RH. All authors reviewed the results and approved the final version of the manuscript.

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Ethical issues and approval

The ethical aspects of this research were reviewed and approved by Universitas Padjadjaran Research Ethics Committee.

Data availability

Not applicable as we used information from previously published articles.

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Approved by any scientific body

Not applicable as the manuscript is not a part of any student thesis or study.

Conflict of Interest

No potential conflict of interest was reported by the authors.

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