



# Antiproliferative Role of Secondary Metabolites From *Aspergillus unguis* AG 1.1 (G) Isolated From Marine Macroalgae *Enteromorpha* sp. by Inducing Intracellular ROS Production and Mitochondrial Membrane Potential Loss Leading to Apoptosis

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Drug resistance to the classically used chemotherapeutic drugs is the major challenge in their treatment of cancer needing the discovery of novel anticancer drugs. In terms of finding novel therapeutics, endophytes are quite promising as they are an excellent source of novel structures, which exhibit bioactivity. The present study demonstrated a dose-dependent antiproliferative activity of mycelial-derived secondary metabolites from a macroalgae associated endophyte *Aspergillus unguis* AG 1.1 (G). The antiproliferative activity of *A. unguis* mycelial extract (AUME) was observed on different human cancer cell lines. PI live/dead assay further confirmed the cytotoxic potential of the mycelial extract. Furthermore, AUME caused mitochondrial membrane aberration and generated ROS production as well indicating its potential to induce cell death by apoptosis. The metabolic profiling of the mycelial extract using GC-MS and LC-MS/MS revealed the presence of fatty acids, a benzoquinolinone derivative, imidazolidinedione derivative, diethyl phthalate and phthalate acid ester, a difuraxanthone, two prenylxanthone analogs and a phthalide derivative and some unknown metabolites. Presence of 4-(4-Hydroxy-3,5-dimethoxy-phenyl)-3,4-dihydro-1H-benzo[h]quinolin-2-one, 1-hydroxy-3,5-dimethoxy-2-prenylxanthone, 1,6-dihydroxy-3-methoxy-2-prenylxanthone and 3-butylidene-7-hydroxyphthalide in AUME could be correlated to the notable cytotoxicity exhibited by the endophyte. The additional presence of many unidentified compounds heightened the prospects of finding some novel bioactive metabolites. Our results indicated that secondary metabolites produced by *A. unguis* AG 1.1 (G) have therapeutic potential as anticancer agents.

**Keywords:** secondary metabolites, anticancer, *Aspergillus unguis*, GC-MS, LC-MS/MS, endophytes

## INTRODUCTION

Cancer is the major cause of morbidity and mortality worldwide. Global statistics estimated the death of 9.6 million people due to cancer in 2018 (Bray et al., 2018). Survival rate and quality of life of cancer patients are drastically improved by anticancer drugs, thanks to advances in cancer biology and medical biotechnology (Ma and Adjei, 2009). A major challenge in the successful treatment of cancer is the drug resistance to the chemotherapeutic drugs due to the various intrinsic and extrinsic factors such as drug inactivation by cancer cells, apoptosis suppression and epigenetic modification (Mansoori et al., 2017). Hence, the discovery of novel anticancer drug targets is an utmost priority.

Natural products derived from plants, marine organisms and microorganism contribute to more than 60% of the currently used anticancer agents. The bioactive metabolites such as alkaloids, terpenoids, flavonoids, sterols and polysaccharide from natural sources are widely reported for their anticancer activity (Seelinger et al., 2012; Rayan et al., 2017). Endophytes are the promising tools for red biotechnology as they are the rich source of bioactive compounds of therapeutic value. Some of these compounds are identical natural products synthesized by the associated host plant. The importance of endophytes was brought into the limelight by the discovery of taxol producing endophyte *Taxomyces andreanae*, isolated from the bark of *Taxus brevifolia* (Kusari et al., 2014). The genetic possibility for the secondary metabolite production and the stimuli of the production basically determine the biotrophic relationship between fungi and host plant as the fungal partner has to alter its lifestyle to assimilate within a plant host. Secondary metabolites serve complex roles; some are involved in cross-kingdom talk, others are in conjunction with fungal life cycle such as sporulation, while some suppress the host immune system (Pusztahelyi et al., 2015; Zeilinger et al., 2016).

Considering the high diversity of marine ecosystem, the marine fungi still remain untapped. Endophytic fungi are ubiquitous in all marine habitats such as marine plants, marine vertebrates and invertebrates. There is a huge knowledge gap in the marine derived endophytes and their secondary metabolite production. Bioactive metabolites produced by macroalgal endophytes play a role in chemical defense of the host by conferring protection against the various pathogens, which explains their heterogeneity and functionality (Vallet et al., 2018).

*Enteromorpha* species are widely distributed green algae in the saline coastal region. They were reported to produce bioactive carotenoids, fucoidans, phlorotannins and bioactive polysaccharide with potential application as functional ingredients in pharmaceuticals, nutraceuticals and cosmeceuticals industries (Zhao et al., 2016). *Wardomyces anomalus* isolated from *Enteromorpha* species produced potent antibacterial and antioxidant molecules (Abdel-Lateff et al., 2003). *Aspergillus* genus is an excellent chemical factory that is capable of producing biotechnologically important compounds such as enzymes, organic acid and bioactive metabolites of therapeutic potential. Endophytes belonging to the *Aspergillus* genus were reported to produce pharmaceutically relevant

compounds such as deoxy podophyllotoxin and taxol (Kusari et al., 2009; Zhao et al., 2009). In our previous study, we reported that *Aspergillus* species, the predominantly present endophytes in marine macroalgae isolated from the Konkan coast are producing secondary metabolites with potential antibacterial, antioxidant and cytotoxic activities. One of the isolate, *A. unguis* AG 1.2 exhibited excellent cytotoxicity against various human cancer cell lines by inducing apoptosis. The total culture crude extract of another isolate, *A. unguis* AG 1.1 (G) demonstrated significant cytotoxic activity and antibacterial activity (Kamat et al., 2020). In the present study, we investigated whether the cytotoxic secondary metabolites produced by the potential isolate, *A. unguis* AG 1.1 (G) were mycelial bound or cell-free. We aimed to study the mechanism behind the cytotoxic activity and secondary metabolite profiling of *A. unguis* AG 1.1 (G).

## MATERIALS AND METHODS

### Chemicals

Potato dextrose agar and broth (PDA and PDB) were procured from Himedia, India. Dulbecco's modified Eagle's medium (DMEM) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich, United States. Fetal Bovine Serum (FBS) and trypsin-EDTA were obtained from GIBCO-BRL. Antibiotics and 3-(4, 5-dimethylthiazole-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) reagent were procured from SRL-Ranbaxy. TLC plates and organic solvents like ethyl acetate and methanol were obtained from Merck-Millipore. All other chemicals and reagents used were of analytical grade.

### Endophytic Fungus

The endophytic fungus, *A. unguis* AG 1.1 (G) was isolated from a green macroalga, *Enteromorpha* sp. The preservation and maintenance of stock cultures were carried in PDA slants at 4°C and for long-term storage as spores and mycelium in 15% (v/v) glycerol at -70°C. Phylogenetic analysis was performed for the strain identification. The cytotoxic activity of the total culture crude extract was initially screened by MTT assay on different human cancer cell lines (Kamat et al., 2020).

### Screening of Mycelial Extract and Culture Broth for Antiproliferative Activity

To determine whether the cytotoxic compounds produced by *A. unguis* AG 1.1 (G) are mycelial bound or extracellular, ethyl acetate extract of the mycelium and culture filtrate were tested separately for cytotoxicity by MTT assay on different human cancer cell lines- HeLa, MCF-7, A-431, and COLO 205 as described below. Cytotoxicity of fungal extracts was also tested on normal human embryonic kidney cell line (HEK 293). For the preparation of crude extracts, *A. unguis* AG 1.1 (G) was grown on PDA plates at 25°C for 7 days. Two agar discs (0.5 × 0.5 cm) were used as inoculum. *A. unguis* AG 1.1 (G) was then cultivated in potato dextrose broth (PDB) by incubation for 28 days in dark and static conditions. At the end of the incubation period, the fungal mycelia were separated from the culture medium

by filtration through cheesecloth. The biomass was dried in an oven for 48 h. The dried mycelial biomass was ground in liquid nitrogen, and extracted twice with double volume of ethyl acetate. Culture filtrate was also extracted twice with double volume of ethyl acetate. The ethyl acetate fraction of mycelial and culture filtrate extracts was concentrated in a rotary evaporator under reduced pressure at 40°C. The extracts were further concentrated using a speed vacuum chamber. For bioactivity assays, the extracts were dissolved in dimethyl sulfoxide.

Cells were cultured in DMEM supplemented with 10% FBS, 100 mg/l penicillin, 250 mg/l streptomycin and 2 mM glutamine and incubated at 37°C in a humidified chamber with 5% CO<sub>2</sub>. 100 µl of cells at a density of 1 × 10<sup>4</sup> cells/well were seeded in 96-well plates and grown for 24 h at 37°C in a 5% CO<sub>2</sub> incubator. When cells reached ~70% confluency, fungal extracts were added at various concentrations, ranging from 10 to 100 µg/ml. After 48 h, MTT solution (10 µl of 5 mg/ml stock prepared in PBS) was added to each well and incubated for 90 min. Thereafter, the media containing MTT was removed, and DMSO (100 µl) was added to each well to dissolve the formazan crystals. The absorbance was measured at 595 nm using a microplate reader (Infinite 200Pro, Tecan Life Science). Cells treated with 0.1% DMSO were kept as a control group.

### Live/Dead Cell Viability Assay Using Propidium Iodide (PI)

For viability assay, HeLa cells were seeded at a density of 1 × 10<sup>5</sup> cells/well in 24 well plates and allowed to grow for 24 h. HeLa cells were then treated with different concentrations of *A. unguis* mycelial extract (AUME) (10–100 µg/ml) and incubated for 48 h at 37°C. Then cells were harvested using trypsin-EDTA, washed with PBS, and suspended in PBS. 10 µl PI (10 µg/ml) was added to cell suspension just prior to analysis (Kumari et al., 2018). Cells treated with paclitaxel (100 nM) were taken as positive control and untreated cells were taken as negative control. PI fluorescence was acquired with CytoFLEX S flow cytometer (Beckman Coulter, USA) and analyzed by CytExpert 2.0 software.

### Cell Cycle Analysis With PI Staining

For cell cycle analysis, HeLa cells (1 × 10<sup>6</sup>) were seeded into 6-well plates in 1 ml DMEM with 10% FBS and allowed to grow for 24 h at 37°C. Then, AUME was added at different concentration (10–100 µg/ml) and incubated for 48 h. Cells were harvested and fixed in cold 70% ethanol for overnight. Then, cells were treated with RNase A for 5 h and stained with PI (50 µg/ml) (Crowley et al., 2016). Cells treated with paclitaxel (100 nM) were taken as positive control and untreated cells as negative control. Cells in different phases were investigated by CytoFLEX S flow cytometer (Beckman Coulter, United States) and data was analyzed by CytExpert 2.0 software.

### Measurement of Mitochondrial Membrane Potential (MMP)

HeLa cells (1 × 10<sup>4</sup> cells) were seeded in 24 well plates, incubated for 24 h and treated with AUME at the concentrations of 10, 25, 50, and 100 µg/ml for 48 h. Further, cells were washed with

PBS and stained with 2.5 µg/ml of JC-1 dye for 15 min at 37°C in dark and analyzed by flow cytometry (CytoFLEX S, Beckman Coulter, United States). The emission of JC-1 monomers peak at 530 nm (FL-1 channel -green fluorescence) and J-aggregate peaks at 590 nm (FL-2 channel- red fluorescence) were recorded and the data was analyzed by CytExpert 2.0 software. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP 50µM) treated cells were served as positive control and untreated cells were served as negative control (Chazotte, 2011).

### Measurement of Intracellular Reactive Oxygen Species

Reactive oxygen species (ROS) generated after treatment with AUME were quantified with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma Aldrich, United States). In this assay, HeLa cells (1 × 10<sup>4</sup> cells) were seeded in 24 well plates, incubated for 24 h and treated with different concentrations of 10, 25, 50, and 100 µg/ml AUME for 48 h. Further, cells were washed with PBS and stained with 50 µM of DCFH-DA for 15 min at 37°C in dark. Fluorescence generated due to oxidation of DCFH was measured by flow cytometry at 500 nm using CytoFLEX S (Beckman Coulter, United States) and analyzed by CytExpert 2.0 software. H<sub>2</sub>O<sub>2</sub> (100 nM) treated cells served as positive control and untreated cells served as a negative control (Eruslanov and Kusmartsev, 2010).

### Antioxidant Activity

Antioxidative activity of AUME was determined by its ability to scavenge stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical following the protocol of scavenging activity (Herald et al., 2012). Hundred microliter of fungal extract at different concentration (10–100 µg/ml) prepared in methanol was added to 100 µl of 100 µM DPPH prepared in methanol. The mixture was mixed well and incubated in dark for 30 min at room temperature. Absorbance values were measured at 517 nm using a microplate reader (Infinite 200Pro, Tecan Life Science). The scavenging activity of DPPH radicals was calculated by assessing the decrease in purple color of DPPH. Ascorbic acid (10–100 µg/ml) was taken as a positive control and methanol as a negative control.

### GC-MS Analysis of AUME

The GC-MS analysis of bioactive compounds of AUME was done using Agilent Technologies GC systems with GC-7890A/MS-5975C (Agilent Technologies, Santa Clara, CA, United States) equipped with HP-5MS column (30 m length × 250 µm internal diameter × 0.25 µm film thickness). Spectroscopic detection by GC-MS involved electron impact ionization system which utilized high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with a flow rate of 1 ml/min. The initial temperature was set at 50–150°C with an increasing rate of 3°C/min and holding time of about 10 min. Finally, the temperature was increased to 250°C at 10°C/min. For derivatization, 20 µl sample was taken in a glass vial and dried with pure N<sub>2</sub> gas. 100 µl derivatizing reagent [BSTFA: TMCS (99:1)] was added and incubated at 60°C again for 60 min,



derivatized samples were diluted and subjected (1  $\mu$ l) for GC-MS analysis. All the compounds obtained are trimethylsilyl aducts/derivatives. Derivatized samples were diluted 100 times with ethyl acetate and 1  $\mu$ l of the derivatized extract was injected into the GC-MS using a microsyringe. The scanning was done at 30–600  $m/z$  range. The compounds were identified by mass library search (AMDIS and NIST, 2011) with their relative retention indices. The relative quantity of the chemical compounds present in the extracts of endophytic fungi was expressed as a percentage based on peak area observed in the chromatogram.

## LC-MS/MS Analysis of AUME

AUME was subjected to liquid chromatography-mass spectrometry (LC-MS/MS) for secondary metabolites profiling. The analysis was performed using Dionex Ultimate 3000 Micro LC instrument fitted with an analytical column, Agilent poroshell 120 (4.6\*150 mm) SB-C18, 2.7  $\mu$ m particle size and a guard column, Agilent Zorbax Eclipse Plus C8 (4.6\*12.5 mm). The mobile phase consisted of solvent A: Solvent B. Solvent A was 0.2% formic acid in 5 mM ammonium acetate and solvent B was 100% acetonitrile. The column temperature was set to 40°C. The separation was performed using gradient elution with a flow rate of 0.3 ml/min. MS/MS was performed using the instrument ESI-Qtof (Impact HD from Bruker) in the negative mode. Acquisition range was from 50 to 1700  $m/z$  at the spectral Rate of 1 Hz. LC-MS interface used was electrospray ionization (ESI). The sample and mobile phases were filtered through 0.22  $\mu$ m polyvinylidene fluoride (PVDF) filters before injecting into the column. Metabolites were identified using Metfrag<sup>1</sup> and Metlin tandem MS/MS<sup>2</sup> databases.

## Statistical Analysis

All experiments were carried out at least in triplicates and data obtained presented as means  $\pm$  SD. All the data were subjected to one-way ANOVA *post hoc* analysis using the SPSS 11.0 Software. Results were considered significant at  $p < 0.05$  throughout the present study.

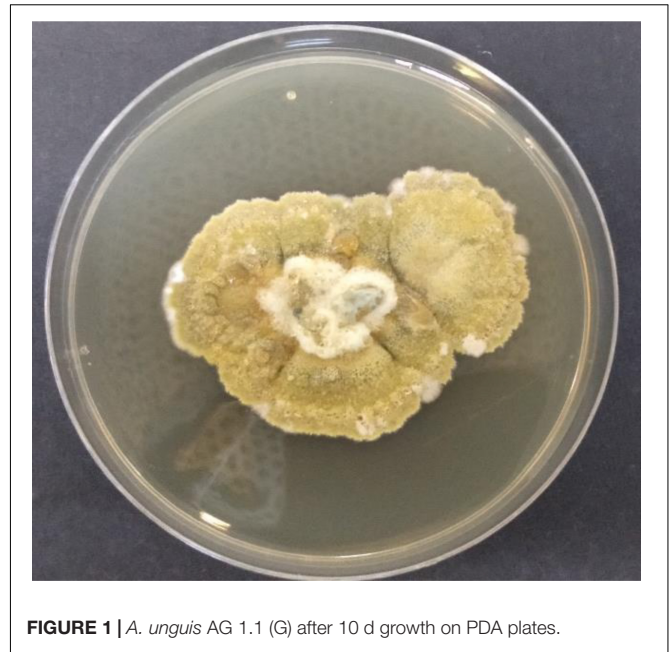
## RESULTS

### Endophytic Fungus *A. unguis* AG 1.1 (G) From Macroalgae *Enteromorpha* sp.

**Figure 1** shows the growth of *A. unguis* PDA. On the phylogenetic tree, the isolate was positioned close to *A. unguis* with 99% similarity on BLAST search. The partial gene sequence has been submitted to GenBank with accession number MH654997 (Kamat et al., 2020).

### Antiproliferative Activity of *A. unguis* Mycelial Extract (AUME)

The MTT assay is based on the cellular reduction of tetrazolium salts to their intensely colored formazans and the amount of



**FIGURE 1** | *A. unguis* AG 1.1 (G) after 10 d growth on PDA plates.

formazan produced is proportional to the number of living cells present in culture (Florento et al., 2012). AUME exhibited significant antiproliferative activity against tumor cell lines HeLa, MCF-7, A-431, and COLO-205 in a dose-dependent manner (**Figure 2**), while culture filtrate had not exhibited any activity (data not shown). This suggested that the bioactive metabolites are mycelial bound. Furthermore, the mycelial extract showed negligible cytotoxicity against normal cell line, HEK 293. The calculated IC<sub>50</sub> values ranged from  $13.46 \pm 0.89$  to  $19.97 \pm 0.21$  for different cancer cell lines and  $> 100 \mu\text{g/ml}$  for HEK 293 cells, respectively (**Table 1**). The relative low cytotoxicity of *A. unguis* derived secondary metabolites against normal cell line HEK 293 makes it an attractive source for an anticancer drug candidate. Since the proliferation of HeLa cells was inhibited to a greater extent, further investigation was performed on the same cell line.

### Live/Dead Cell Viability Assay Using PI

To evaluate the performance of secondary metabolites, quantification of the viability of the cell population after treatment with the mycelial extract is necessary. Propidium Iodide is a membrane impermeant dye that stains dead cells. In the presence of AUME, cell viability decreased significantly and followed a dose-response relationship (**Figure 3**). The dead cell percent raised from 6% in untreated cells to 90% in treated cells with 100  $\mu\text{g/ml}$  AUME, which confirmed the concentration dependent cytotoxicity of AUME.

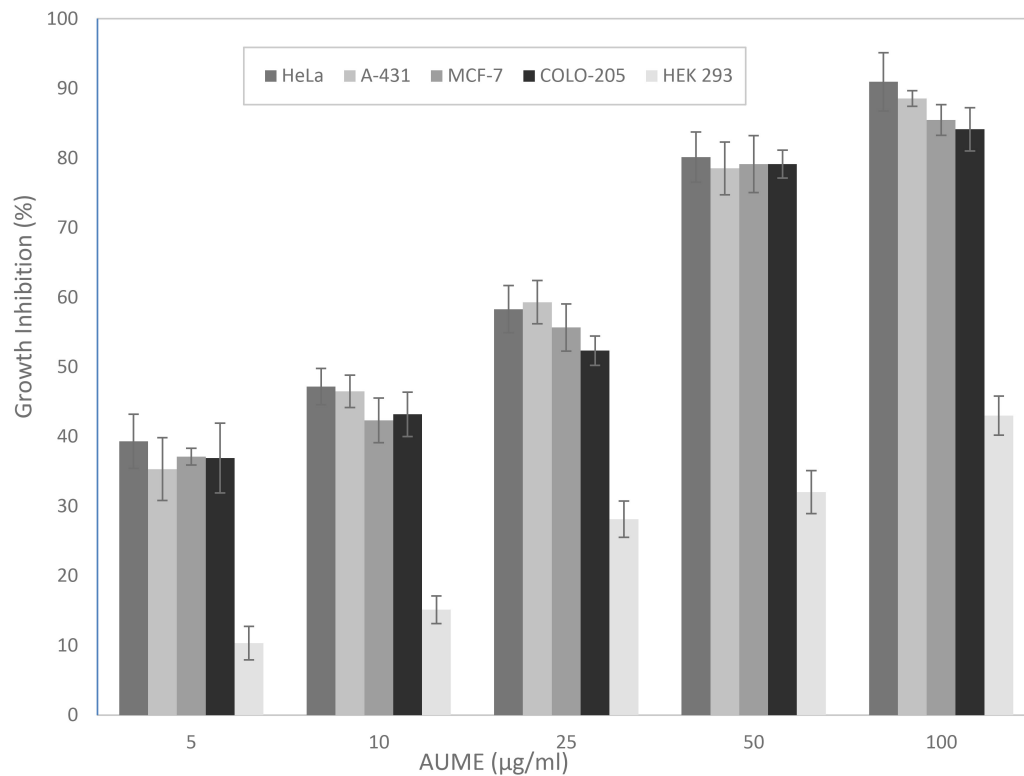
### Cell Cycle Analysis With PI Staining

Cell cycle phase arrest is an extensively observed phenomenon in response to treatment with cytotoxic drugs (Kamat et al., 2020). **Figure 4** illustrates the cell cycle distribution of HeLa cells treated with different concentration of AUME. This was then quantified with PI staining and analyzed by FACS. Treatment with AUME resulted in a dose-dependent increase of G<sub>0</sub> peak (also called

<sup>1</sup><https://msbi.ipb-halle.de/MetFragBeta/>

<sup>2</sup><https://metlin.scripps.edu/>





**FIGURE 2** | Antiproliferative activity of *A. unguis* AG 1.1 (G) mycelial extract (AUME) on different cell lines estimated using MTT assay, expressed in percentage growth inhibition. The data shown are average  $\pm$  SD of values obtained from three replicates,  $p < 0.05$ .

**TABLE 1** |  $IC_{50}$  values of AUME calculated for multiple human cell lines.

Cell line	$IC_{50}$ ( $\mu$ g/ml)
HeLa	$13.46 \pm 0.89$
MCF-7	$18.75 \pm 0.43$
A-431	$15.04 \pm 0.91$
COLO 205	$19.97 \pm 0.21$
HEK 293	$> 100 \mu$ g/ml

sub- $G_1$ ), which represents the apoptotic cell population. The percentage of cells in  $G_0$  increased from 8% in control group to 9, 31, 44, and 46% in cells treated with 10, 25, 50, and 100  $\mu$ g/ml AUME, respectively. There was a corresponding decrease in the cell population of other cell cycle phases  $G_1$ , S, and  $G_2$ . Hence, the results indicated that AUME induced apoptosis in HeLa cells to a significant extent. In cells treated with paclitaxel, there was a remarkable increase in the  $G_2$  phase and  $G_0$  phase.

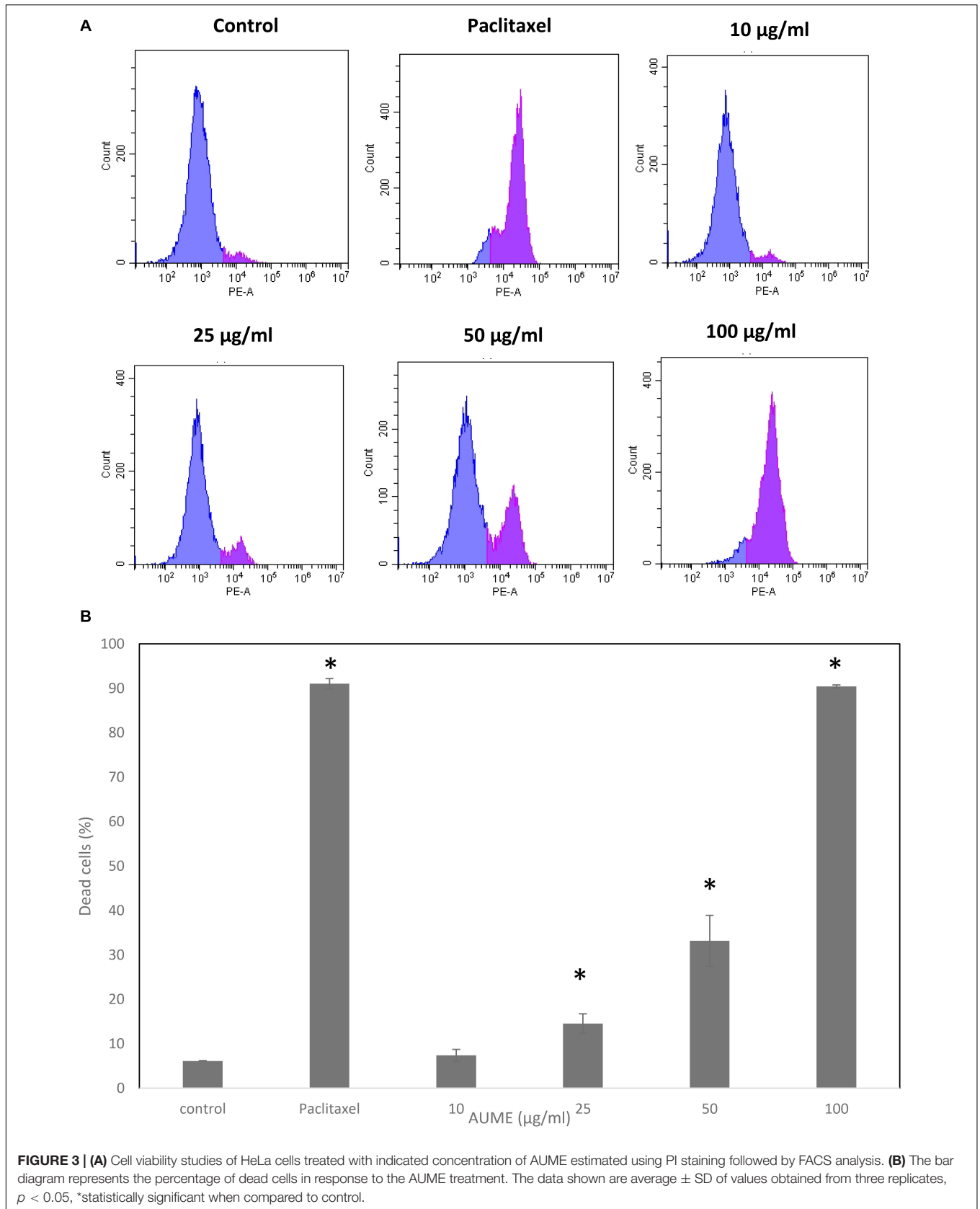
### Induction of Mitochondrial Membrane Depolarization by AUME

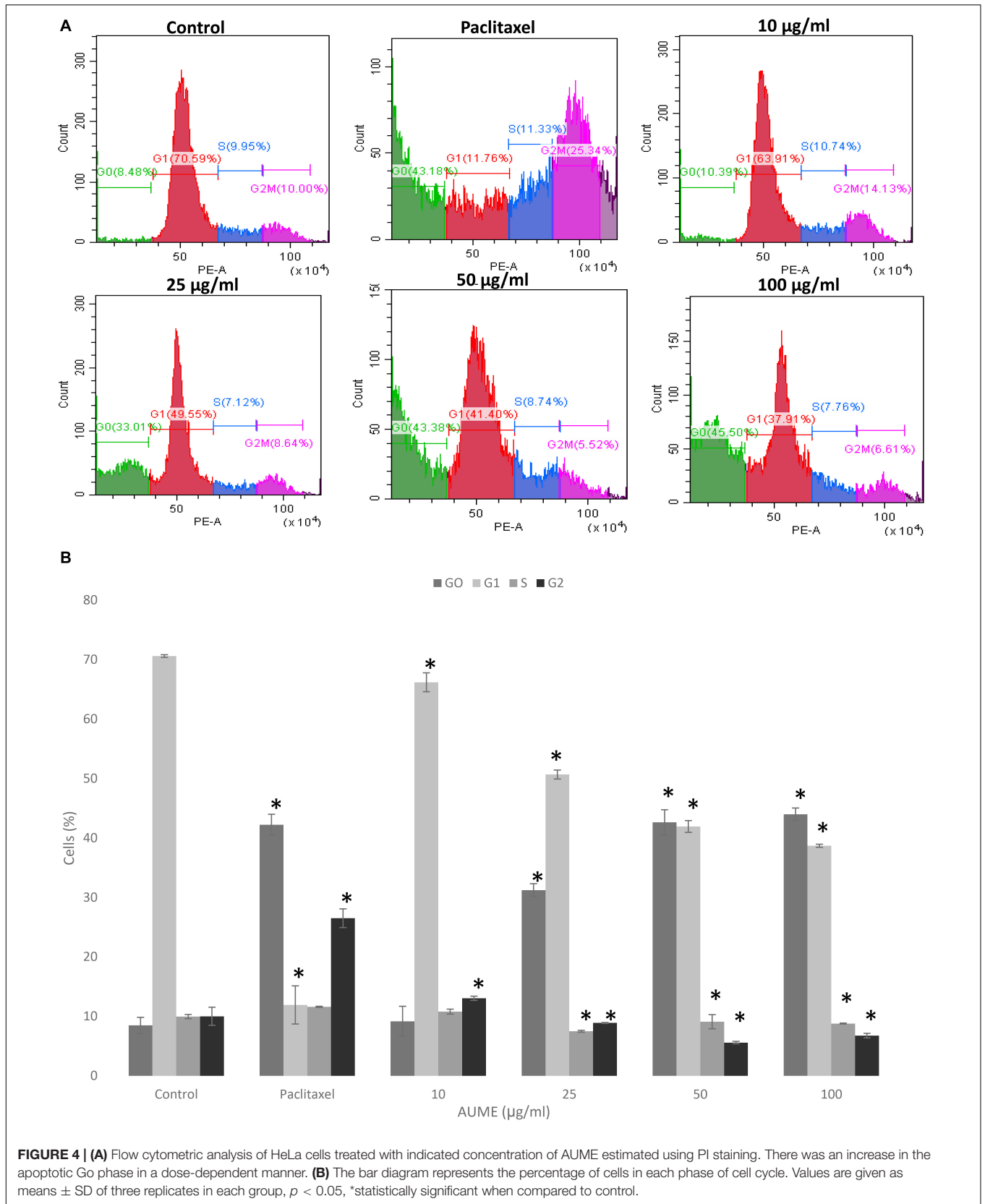
One of the significant events that happens during apoptosis is the loss of MMP. Hence, measuring the dynamics of MMP is an effective way to determine apoptosis induction. JC1 is a cationic, lipophilic dye which can be used to assess the mitochondrial membrane potential ( $\Delta\Psi_m$ ). In

healthy cells, JC1 enters negatively charged and polarized mitochondria and forms red fluorescent aggregates whereas JC1 monomers do not aggregate and retain their original green fluorescence in apoptotic cells as a result of the loss of MMP (Sivandzade et al., 2019). Treatment with AUME resulted in the loss of mitochondrial membrane potential as measured by the green fluorescence of JC1 monomers, which is shown in the **Figure 5**. Dose-dependent increase in cells with collapsed mitochondria when treated with AUME revealed the its induction of mitochondria-mediated apoptosis.

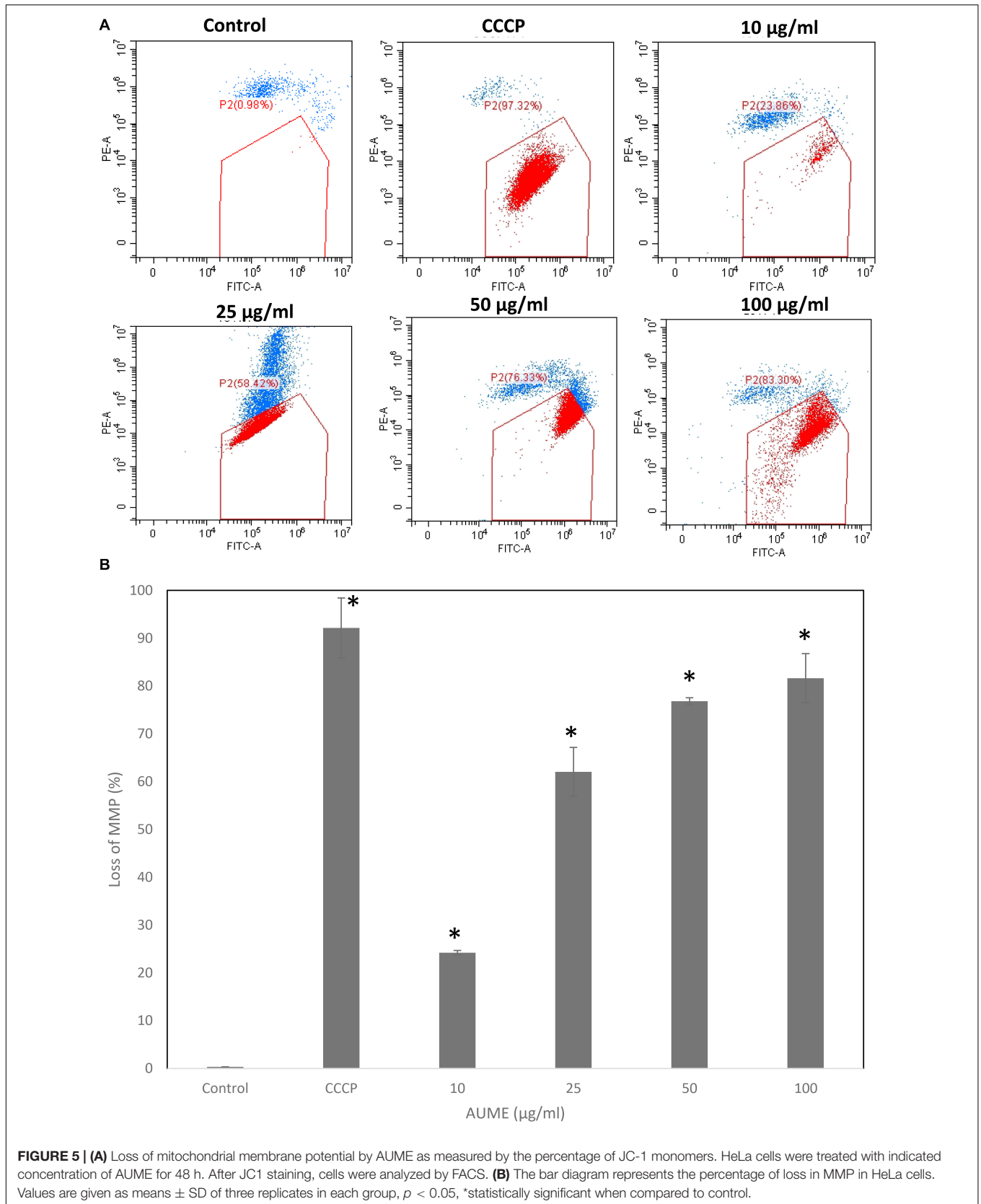
### Induction of Intracellular Reactive Oxygen Species by AUME

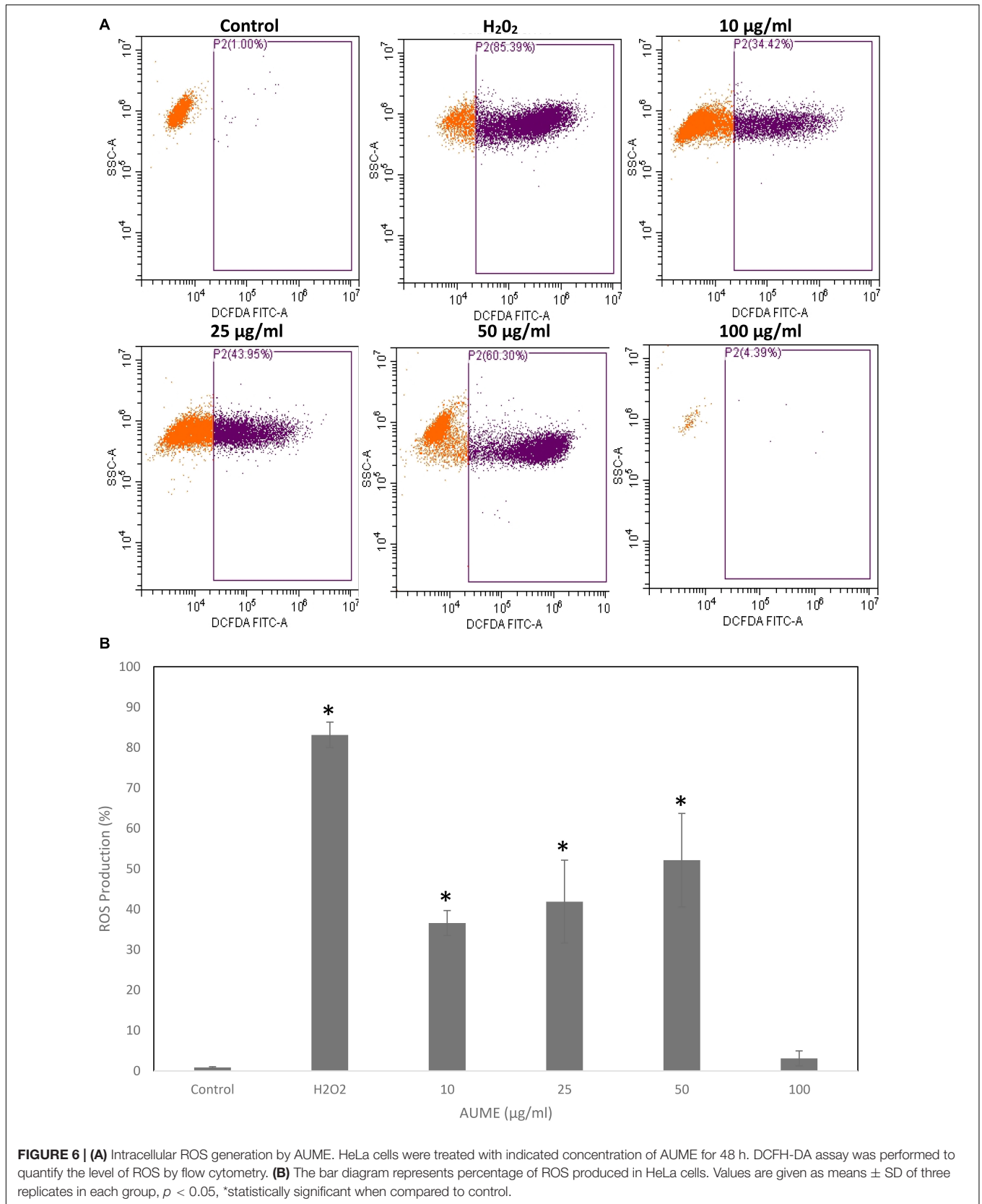
Quantification of oxidative stress by DCFH makes use of oxidation of non-fluorescent 2',7'-dihydrodichlorofluorescein diacetate (DCFH-DA) to fluorescent DCF by intracellular ROS (Jarolim et al., 2018). FACS analysis revealed a steady increase in DCF fluorescence in a dose-dependent manner on treatment with AUME at concentrations up to 50  $\mu$ g/ml (**Figure 6A**). The ROS production increased from 36.60 to 52.12% at a concentration range from 10 to 50  $\mu$ g/ml (**Figure 6B**). However, at higher concentration (100  $\mu$ g/ml), fluorescence reduced, which could be due to cell death. This revealed that treatment with AUME induced ROS production.

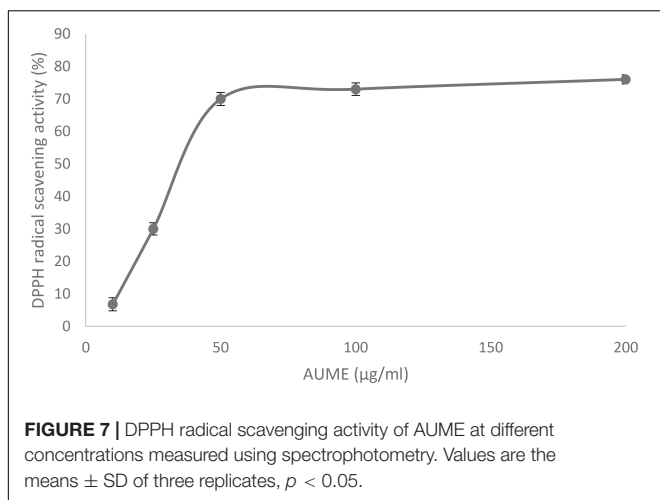












## Antioxidant Activity of AUME Using DPPH Assay

Antioxidant activity of AUME was evaluated by its ability to reduce the free radical DPPH. AUME showed moderate antioxidant activity with an  $IC_{50}$  value of  $73.91 \pm 0.91 \mu\text{g/ml}$  (Figure 7). The  $IC_{50}$  value of ascorbic acid was found to be  $28.01 \pm 0.5 \mu\text{g/ml}$  (data not given). AUME contains a number of bioactive compounds and some of which could have moderate free radical scavenging activity.

## GC-MS Analysis of AUME

The GC-MS analysis was performed to investigate the bioactive compounds present in AUME (Figure 8). Out of 11 compounds detected, an unknown compound had the highest peak area, which did not have a significant match in the NIST database. Other major constituents were fatty acids such as *trans*-9-octadecenoic acid, hexadecanoic acid and octadecanoic acid, *cis*-4,7,10,13,16,19-docosahexaenoic acid and oleic acid. Apart from fatty acids, 4-(4-Hydroxy-3,5-dimethoxy-phenyl)-3,4-dihydro-1H-benzo[h]quinolin-2-one and imidazolidinedione derivative were also present. A prostaglandin, prosta-5,13-dien-1-oic acid was also detected on GC-MS chromatogram. Remaining compounds were glycerol and 1,4-benzenedicarboxylic acid. List of metabolites with retention time and area percentage of total are summarized in Table 2.

## LC-MS/MS Analysis of AUME

Figure 9 represents the base peak chromatogram of AUME and fragmentation pattern of the peaks and representative metabolites. Mass-based search and MS/MS spectra of each peak analyzed using MetFrag revealed the presence of some known compounds such as 01-ethyl 04-(2-hydroxyethyl) benzene-1,4-dicarboxylate, diethyl phthalate, asperxanthone, 1-hydroxy-3,5-dimethoxy-2-prenylxanthone, 3-butylidene-7-hydroxyphthalide, and 1,6-dihydroxy-3-methoxy-2-prenylxanthone. Only highest scoring MetFrag candidates were chosen, which were further cross-checked by Metlin database search. 13 out of 19 peaks were not tentatively identified as they had come up with zero

hits of natural products in mass search. Table 3 summarizes LC-MS/MS result.

## DISCUSSION

The endophytic community of macroalgae is underexplored due to the difficulty in sampling and isolation. The complex and dynamic relationship between algicolous endophytic fungi and its host determines the production of secondary metabolites. There is a large number of secondary metabolites discovered so far serving many roles. These compounds contribute to the adaptive or defensive mutualism against biotic and abiotic stress incurred by algae. In addition to this, horizontal gene transfer between algae and endophytic fungi can result in the production of host secondary metabolites by endophytes. Secondary metabolites possess unique structural features, which contribute to the various bioactivities such as antibacterial, antifungal, antiviral, antitumor, antioxidant, anti-inflammatory, anticoagulant, antidiabetic activity and so forth (Tadjch and White, 2012; Barzkar et al., 2019). Furthermore, these molecules can act as the structural scaffolds for synthetic chemistry to develop more potent and efficient therapeutics.

The chemotherapeutic potential of the compounds can be evaluated by *in vitro* cell-based cytotoxicity assay such as MTT, which is quite rapid and reliable. The potency and effectiveness of anticancer agents are determined by half-maximal inhibitory concentration ( $IC_{50}$  value) derived from the dose-response curve. Furthermore, the anticancer compound should be selective between cancerous and non-cancerous cells in the context of cytotoxicity (Popiołkiewicz et al., 2005). AUME treatment demonstrated significantly higher cytotoxicity ( $IC_{50}$ -13.46  $\mu\text{g/ml}$ ) in HeLa cells in comparison with other tested cell lines, while exhibiting low cytotoxicity on normal cells. Kamat et al. (2020) reported that total culture crude extract of *A. unguis* AG 1.2 exhibited high cytotoxicity in A431 cell with an  $IC_{50}$  value of 5.94  $\mu\text{g/ml}$ . Both studies inferred that macroalgal derived *A. unguis* could be a principal source of cytotoxic secondary metabolites.

Cytotoxicity of AUME was further validated by PI live dead assay. Estimation of cell survival using flow cytometry to assess the effect of the chemotherapeutic agent on the cancer cells is a facile and relatively rapid method and is based on the cellular fluorescence after staining with fluorescent dyes (Ross et al., 1989). PI live dead assay result confirmed the cytotoxic potential of AUME. To elucidate the cellular mechanism by which extract exerts anticancer activity, the effect of AUME on cell cycle progression, mitochondrial membrane potential and intracellular ROS production was also studied.

The noteworthy observation on cell cycle analysis was the presence of dose-dependently increasing  $G_0$  or sub- $G_1$  peak, which indicated the occurrence of apoptosis brought about by the treatment with AUME. Darzynkiewicz et al. (2010) reported that apoptotic cell population is manifested as the sub- $G_1$  peak on DNA content histogram, due to the lowest DNA content. The cell cycle arrest at  $G_2/M$  phase leading to apoptosis is one of the mechanisms by which paclitaxel exerts



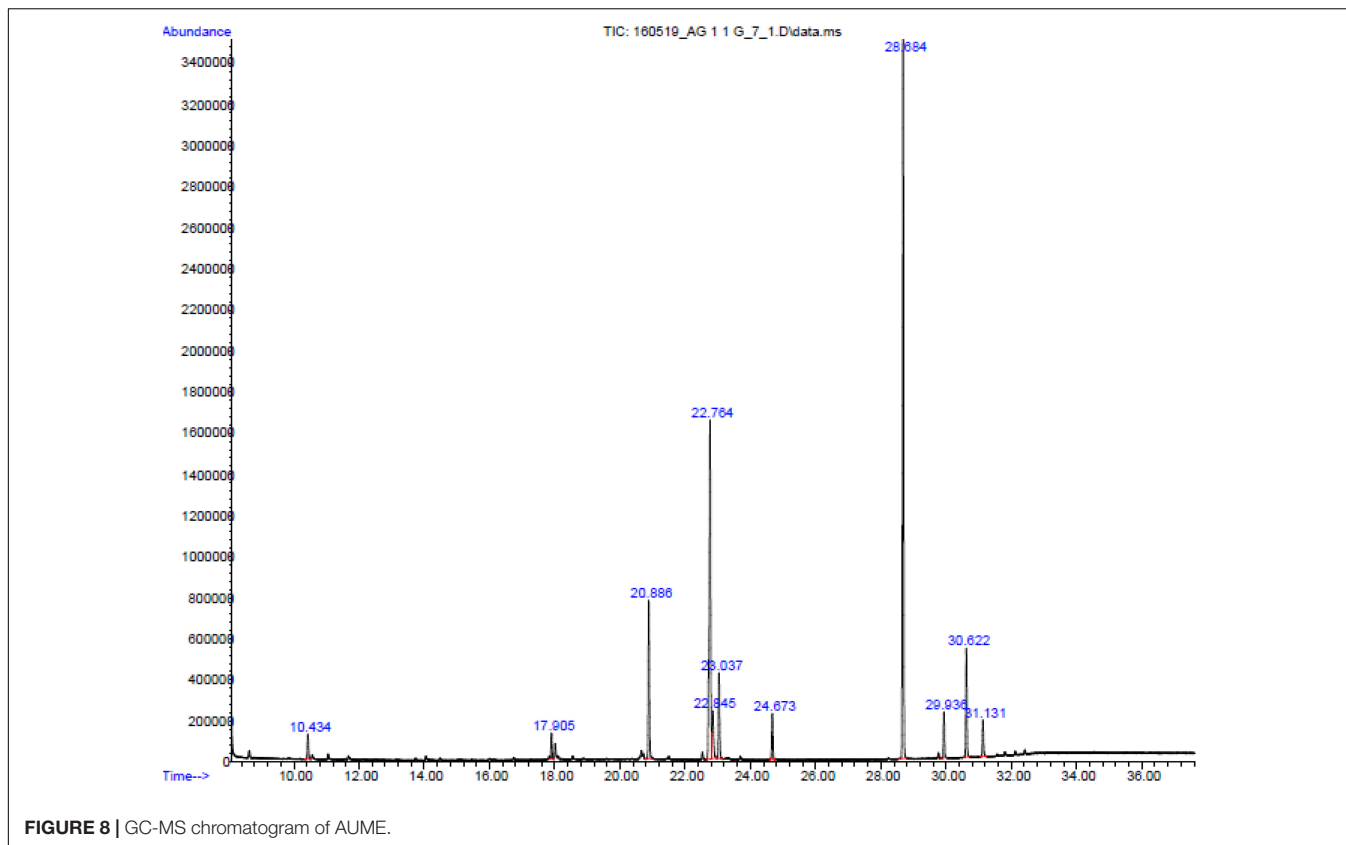


FIGURE 8 | GC-MS chromatogram of AUME.

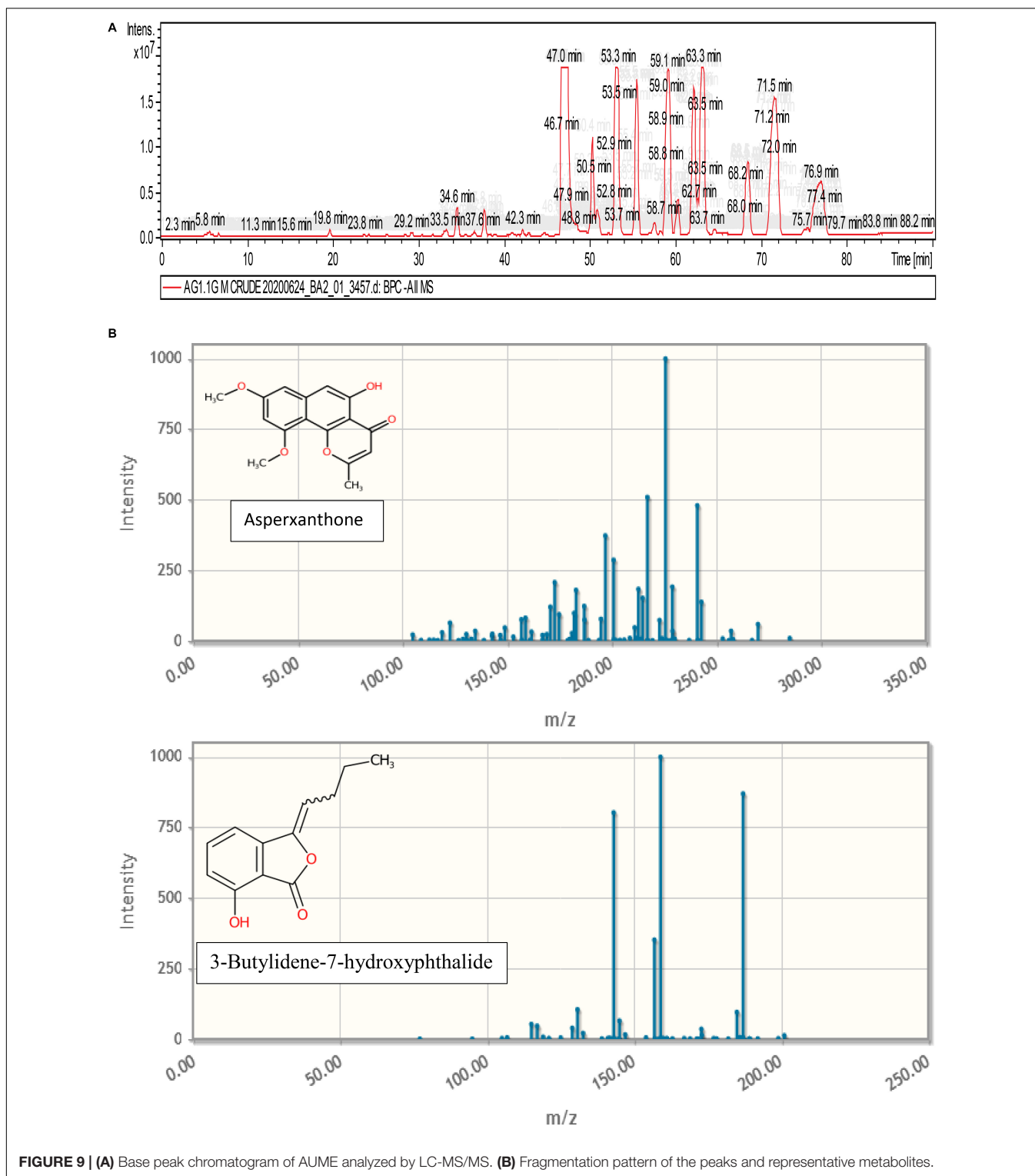
TABLE 2 | List of secondary metabolites in AUME analyzed by GC-MS.

Peaks	Retention time (min)	% of total	Metabolite	Nature of the compound
1	10.434	1.4	Glycerol, tris(trimethylsilyl) ether	Polyol
2	17.905	0.86	1,4-Benzenedicarboxylic acid, bis(trimethylsilyl) ester	Ester compound
3	20.886	8.9	Hexadecanoic acid, trimethylsilyl ester	Fatty acid
4	22.764	24.4	<i>trans</i> -9-Octadecenoic acid, trimethylsilyl ester	Fatty acid
5	22.845	3.9	Oleic acid, trimethylsilyl ester	Fatty acid
6	23.037	5.3	Octadecanoic acid, trimethylsilyl ester	Fatty acid
7	24.673	2.7	<i>cis</i> -4,7,10,13,16,19-Docosahexaenoic acid, tert-butylidimethylsilyl ester	Fatty acid
8	28.684	40	Unknown	
9	29.936	2.5	Prosta-5,13-dien-1-oic acid, 9,11,15-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester	Prostaglandin
10	30.622	6	4-(4-Hydroxy-3,5-dimethoxy-phenyl)-3,4-dihydro-1H-benzo[h]quinolin-2-one	Heterocyclic aromatic compounds
11	31.131	2.1	2,4-Imidazolidinedione, 5-[3,4-bis[(trimethylsilyl)oxy]phenyl]-3-methyl-5-phenyl-1-(trimethylsilyl)-	Nitrogenous heterocyclic compounds

cytotoxicity (Wang et al., 2000). In comparison with paclitaxel, it was observed that AUME treatment increased the number of cells in G<sub>0</sub> phase, while decreased the number of cells in G<sub>1</sub>, S, and G<sub>2</sub>/M phases. A similar observation of apoptotic phase increase was reported in leukemic cells when treated with Greensporone C, a fungal derived secondary metabolite (Prabhu et al., 2018). *A. unguis* AG 1.2 total culture crude extract exhibited G<sub>1</sub> phase cell cycle arrest in A431 cells (Kamat et al., 2020). Anticancer agents induce cell death by either causing DNA lesions or hamper cell division mechanisms, leading to the activation of DNA

damage checkpoints, eventually causing cell cycle arrest followed by cell death (Visconti et al., 2016).

Our results suggest that AUME exerts the cytotoxic effect on HeLa cells by inducing mitochondrial-mediated apoptosis. The role of MMP in the induction of apoptosis is still unclear as there are a lot of conflicting results suggesting that loss of  $\Delta\Psi_m$  can trigger apoptosis or  $\Delta\Psi_m$  can be dissipated as a consequence of apoptosis (Ly et al., 2003). Still, loss of  $\Delta\Psi_m$  is a key step in apoptosis. Our results confirmed that AUME was causing loss of  $\Delta\Psi_m$  and induced apoptosis.



We have further demonstrated that AUME triggered oxidative stress. Most of the anticancer agents induce ROS, which leads to oxidative-stress mediated cell death (Singh et al., 2016). ROS are highly reactive molecules, serving a number of crucial roles at a low to moderate concentration, particularly in

regulations of physiological functions and maintaining a normal immune system. Excess intracellular ROS induces damage to nucleic acids, proteins, lipids, membranes and organelles leading to the activation of apoptosis, which is mediated by mitochondria, death receptors and the ER signaling pathway

**TABLE 3** | List of secondary metabolites in AUME with retention time and MetFrag score identified by LC-MS/MS analysis.

Peaks	Retention time	m/z [M-H]	Metabolite	MetFrag score
1	5.8 min	265.09083	No hit	
2	19.8 min	237.07701	O1-ethyl O4-(2-hydroxyethyl) benzene-1,4-dicarboxylate	0.84
3	33.5 min	221.0831	Diethyl phthalate	0.6425
4	34.6 min	271.06216	No hit	
5	37.6 min	285.07694	Asperxanthone	0.91
6	42.3 min	375.05988	No hit	
7	47 min	651.21552	No hit	
8	50.4 min	339.12390	1-Hydroxy-3,5-dimethoxy-2-prenylxanthone	0.921
9	53.3 min	361.0808	No hit	
10	55.5 min	759.3066	No hit	
11	58.7 min	311.1669	No hit	
12	59.1 min	393.0278	No hit	
13	60.1 min	295.1324	No hit	
14	62.5 min	339.1228	No hit	
15	63.3	203.0731	3-Butylidene-7-hydroxyphthalide	1
16	64.6	325.1068	1,6-Dihydroxy-3-methoxy-2-prenylxanthone	0.8218
17	68.2 min	373.0838	No hit	
18	71.9 min	407.0434	No hit	
19	76.9 min	441.0038	No hit	

(Redza-Dutordoir and Averill-Bates, 2016). Additionally, cancer stem cell maintains a low level of intracellular reactive oxygen species (ROS) level to maintain its survival and proliferation during tumorigenesis. Increasing oxidative stress to cause ROS-mediated cell injury by the exogenous agent is an effective anticancer therapy, which selectively kills cancer cells without affecting normal cells (Liu and Wang, 2015). The molecular mechanism of antiproliferative activity of endophytic fungal derived cytotoxic secondary metabolites could be by the induction of apoptosis through ROS dependent MMP loss. Similar observations were reported from macroalgal associated endophytes isolated from the Indian coastline (Kumari et al., 2018; Kamat et al., 2020). *Aspergillus protuberus* and *Talaromyces flavus*, marine derived fungi isolated from Indian coastline also showed cytotoxicity and antibacterial activity (Mathan et al., 2011; Anand et al., 2016).

Though reactive oxygen species (ROS) are an integral part of the intracellular signaling pathway, high ROS production is usually deleterious and involved in inflammatory diseases (Henrotin et al., 2003). It has been shown that antioxidant supplementation can alleviate oxidative damage caused by high ROS production (Liu et al., 2018). Metabolites produced by endophytic fungi can be a potential source of novel natural antioxidants (Huang et al., 2007). Many synthetic analogs of natural compounds show potent antioxidant activity. The synthetic analog of the metabolite derived from fungus *Colletotrichum gloeosporioides* exhibits excellent free radical scavenging activity which is comparable to protocatechuic acid (Femenía-Ríos et al., 2006). Various biotic and abiotic stresses in plants generate ROS and adversely affect plant growth. Endophytes have the ability to alleviate the drought and metal stress by modulating ROS scavenging system and thus improving the plant viability (Ma et al., 2019; Sadeghi

et al., 2020). A diverse array of plants has been in a symbiotic relationship with endophytes. Secondary metabolites produced by endophytes heavily contribute to this mutualistic relationship through herbivore and oxidative stress protection (White and Torres, 2010). In comparison with the antioxidant activity of the total culture crude extract reported by Kamat et al. (2020), a better antioxidant activity of AUME revealed the mycelial origin of antioxidant metabolites.

Metabolic profiling is critical to identify the bioactive compounds contributing for the interesting biological activities exhibited by *A. unguis* AG 1.1 (G). GC-MS and LC-MS/MS are robust dereplicative tools for metabolite identification. GC-MS analysis performed on AUME revealed the presence of therapeutically important compounds such as *trans*-9-Octadecenoic acid, hexadecanoic acid, octadecanoic acid, 4-(4-Hydroxy-3,5-dimethoxy-phenyl)-3,4-dihydro-1H-benzo[h]quinolin-2-one, imidazolidinedione derivative, 1,4-benzene dicarboxylic acid and a prostaglandin, prosta-5,13-dien-1-oic acid. A critical observation was the presence of a major peak of an unknown compound. Octadecanoic acid and hexadecanoic acid exhibit antiproliferative activity, antioxidant activity, and anti-microbial activities (Habib et al., 1987; Ravi and Krishnan, 2017; Ashraf et al., 2018). In our study, the presence of above-mentioned fatty acids in *A. unguis* mycelium could be greatly contributing to its antioxidant activity. A number of compounds belonging to 5-hydroxy-4-aryl-quinolin-2(1H)-one alkaloids are reported from *Aspergillus* and *Penicillium* species. A prenylated quinoline, aspoquinolones produced by *Aspergillus* species exhibited good cytotoxicity due to isoprenyl derived sidechain in the structure (Uchida et al., 2006; Simonetti et al., 2016). 5-methyl-2,4-imidazolidinedione was detected in the bioactive fraction of *Lactobacillus plantarum* VTTE-78076, contributing to its antibacterial activity against Gram negative



test organism (Niku-Paavola et al., 1999). In our previous study, we observed that *A. unguis* AG 1.1 (G) ethyl acetate extract demonstrated antimicrobial activity against Gram negative bacterial pathogens, *E. coli*, *P. aeruginosa*, and *X. campestris* (Kamat et al., 2020). A high concentration of 1,2-benzene dicarboxylic acid mono(2-ethylhexyl) ester contributed to the antifungal activity of the endophytic fungi, *Aspergillus flavipes* strain (Verma et al., 2014). Many pathogenic fungi have the ability to produce prostaglandins that act as virulence factors (Noverr et al., 2002). Due to the fact that bioactive compounds such as *trans*-9-Octadecenoic acid, hexadecanoic acid, and octadecanoic acid are present at relatively low concentration and are less cytotoxic, we hypothesize that the bioactive compounds, 4-(4-Hydroxy-3,5-dimethoxy-phenyl)-3,4-dihydro-1H-benzo[h]quinolin-2-one or the unknown compound present in the mycelial extract of *A. unguis* may be directly involved in the strong anticancer activity that we have observed. GC-MS analysis of cytotoxic *A. unguis* AG 1.2 total culture crude extract detected the presence of azelaic acid, azetidine and furoprans, which could be contributing to the excellent cytotoxicity (Kamat et al., 2020). The metabolic profile of *A. unguis* AG 1.1 (G) was quite distinct from that of *A. unguis* AG 1.2, though both belong to the same species, demonstrated significant cytotoxicity and were isolated from the same algal sample. This reveals that secondary metabolite diversity of *A. unguis* species is strain specific. Therefore, it is necessary to investigate the natural productome of each fungal isolate with respect to its potential bioactivity.

LC-MS/MS analysis revealed that *A. unguis* AG 1.1 (G) is capable of producing another phthalate acid ester, O1-ethyl O4-(2-hydroxyethyl) benzene-1,4-dicarboxylate and diethyl phthalate, apart from 1,2-benzene dicarboxylic acid mono(2-ethylhexyl) ester. A few filamentous fungi have been reported to produce phthalate acid esters (Tian et al., 2016). Asperxanthone, a difuraxanthone reported from marine-derived *Aspergillus* species exhibited moderate activity against tobacco mosaic virus (Wu et al., 2009). Some prenylxanthone analogs with various bioactive functions such as antibacterial, antifungal and cytotoxic activities have been reported from the *Aspergillus* species (Zhu et al., 2018). Butylidenephthalide, present in many medicinal herbs possesses various bioactivities including antitumor activity (Chang et al., 2020). A cytotoxic 3-butyl-7-hydroxyphthalide was reported from *Penicillium vulpinum* (Ohseki and Mori, 2003). The production of 3-butylidene-7-hydroxyphthalide has not been reported from a fungal source to the best of our knowledge. Based on LC-MS/MS results, 1-hydroxy-3,5-dimethoxy-2-prenylxanthone, 1,6-dihydroxy-3-methoxy-2-prenylxanthone and 3-butylidene-7-hydroxyphthalide in AUME are presumed to contribute to the cytotoxic activity exhibited by AUME, owing to the known bioactivity of their related metabolites. Additionally, many unknown metabolites are also present, which may be contributing to the cytotoxicity of AUME. Hence, bioassay-guided purification followed by the experimental verification can only pinpoint the molecule possessing excellent cytotoxic activity of AUME in view of developing an anticancer drug candidate. Though numerous bioactive secondary metabolites have been identified from endophytic fungi, lack of their MS spectra in the public databases is a major constraint in the dereplication

of fungal secondary metabolite. Availability of an open natural product database with all relevant chemical and taxonomic information could facilitate the bioprospecting of novel natural products (Nielsen and Larsen, 2015; Zhou et al., 2013).

## CONCLUSION

In conclusion, the mutualistic relationship of an endophytic fungus with its host influences the secondary metabolite diversity of the fungus. Macroalgal derived *A. unguis* could be a principal source of cytotoxic secondary metabolites. Even the identical endophytic fungal species isolated from a common source can have a unique set of therapeutically significant compounds, which empowers the diversity of marine natural productome. Induction of apoptosis by markedly increasing the intracellular ROS production and severely aberrating the mitochondrial function could be the major molecular mechanism behind the cytotoxic activity of *A. unguis* derived secondary metabolites. The strong cytotoxicity of AUME warrants further investigation to purify and characterize the cytotoxic compounds with the prospect of developing novel anticancer therapeutics. Our study highlights the importance of bioprospecting of macroalgal derived fungal endophytic community for the discovery of novel anticancer therapeutics.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in GenBank with accession number MH654997.

## AUTHOR CONTRIBUTIONS

KS carried out the designed research, collected, analyzed, and interpreted the scientific data, wrote the article. SK did the sampling, isolation and identification of the fungus, analyzed and interpreted the data, made critical revisions, and edited the article. CJ designed the study and did the supervision, project administration, and funding acquisition. All authors read and approved the final manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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