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IN VITRO EVALUATION OF ANTIOXIDANT AND ANTICANCER PROPERTIES OF METHANOLIC EXTRACTS OF *Ulva paschima* AGAINST A549 AND H1299 LUNG CANCER CELL LINES

Digvijay Singh Yadav[1], Alka Rani[1], Amit Kumar[2], Vikas Jaitak[2] and Felix Bast[1]*

[1]*Department of Botany,* [2]*Laboratory of Natural Products, Department of Pharmaceutical Sciences and Natural Products, Central University of Punjab, Ghudda, Bathinda – 151 401, Punjab (India)*
*****e-mail: *felix.bast@cup.edu.in*

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ABSTRACT

The present study focused on the antiproliferative effect of methanolic extracts of *Ulva paschima* (MEUP) on lung cancer cell lines A549 and H1299. The superoxide dismutase, catalase and glutathione reductase activities in MEUP were assayed. The intracellular reactive oxygen species, antioxidant potential, mitochondrial membrane potential and cell proliferation ability were also determined using standard methods while the extracts were analysed by GCMS. The study showed that MEUP had selective antiproliferative effect on cancer cell lines with IC_{50} 0.187 ± 0.120 µg µL^{-1} for A549 and 0.130 ± 0.031 µg µL^{-1} for H1299 cells as determined by 3-(4,5-dimethylthiazol-2-cyl)-2,5-diphenyltetrazolium bromide (MTT) assay. MEUP induced oxidative stress in dose-dependent manner as measured by dihydro-ethidium (DHE) and 2′,7′-dichloro-dihydro-fluorescein diacetate (H_2DCFDA) assays. Due to the induced oxidative stress by MEUP treatment, a rise in intracellular levels of antioxidant enzymes (glutathione reductase, superoxide dismutase, and catalase) was observed. A dose-dependent depolarization of mitochondrial membrane potential by 38% for A549 and 41% for H1299 cell lines was observed. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was 61.69% as compared to the ascorbic acid with EC_{50} of 176.7 ± 0.09 µg mL^{-1}. GC-MS analysis of MEUP showed the presence of 1-dodecanol, 5-eicosene, neophytadiene, stearic acid, lauric acid, hexadecanoic acid, margaric acid, linoleic acid, L-α-terpineol, oleic acid, 9-octadecenamide, myristic acid and phytol known for their anticancerous properties. The phytochemical profile was evaluated for potential for serving as novel anticancer drug candidates.

Keywords: Crude extract, GC-MS, mitochondrial membrane potential, seaweed, *Ulva paschima*

INTRODUCTION

Algal metabolites have recently attracted a lot of attention as potential anticancer drugs due to their structural uniqueness and diversity (Lefranc *et al.*, 2019). Marine organisms contain phycocolloids, polyunsaturated fatty acid, phycobilin, sterol, tocopherol, terpene, halo-terpene, phycocyanin, brominated phenol, polysaccharide, and carotenoid compounds that provide unique bioactive, health-promoting and medicinal properties (Motallebi, 2020; Pradhan *et al.*, 2021; Akram and Suleria, 2022). International Agency for Research on Cancer (IARC) has reported 19.29 million cases and 9.95 million deaths due to cancer in 2020, out of which lung cancer claimed 18% whereas, in India, 72510 new cases of lung cancer with 91% deaths have been reported (Sung *et al.*, 2021). The high mortality rate was attributed to late detection during distant metastatic stages (Mathur *et al.*, 2020).

Ulva genera (Ulvales, Chlorophyta) consists of cosmopolitan green seaweeds with known immuno-modulating, antiviral, antihyperlipidemic, antioxidant, and anticancer properties (De Jesus Raposo *et al.*, 2015; Matloub *et al.*, 2015; Kidgell *et al.*, 2019). Focusing on antitumour activity, Kosanić *et al.* (2015) noted the cytotoxicity of *U. lactuca* and *U. intestinalis* against lung, colon, leukaemia, and malignant melanoma cells. The isolated polysaccharides from *Ulva* known as ulvans were reported to have immunomodulatory effects (Jiao *et al.*, 2010) The ethanolic extract of *U. lactuca* reportedly possess antiproliferative, antioxidant and cytotoxic potential against blood cancer cell lines, MOLT-3 (Chidambarajan *et al.*, 2019). The selective cytotoxicity has been demonstrated by Nazarudin *et al.* (2020), when HEPG2 liver cancer cell lines were effectively killed by the methanolic extract of *U. intestinalis* but no effect was seen on normal embryonic fibroblast (3T3) cells. Studying the underlying mechanism, ROS-induced apoptosis and p53 mediated cell-death was caused by the crude extracts of *U. lactuca* studied on HCT-116 colon cancer cell line (Acharya *et al.*, 2021; Monla *et al.*, 2021). Pal *et al.* (2021) have reported that the methanolic fraction of *U. intestinalis* and *U. lactuca* damage the nuclei and enhance autophagic proteins causing death to cervical cancer (SiHa) cells but not to human peripheral blood mononuclear cells (PBMCs). Hence, in present study the methanolic extract of *Ulva paschima* Bast was assessed on A549 and H1299 lung cancer cells for their antioxidant and anticancer properties and also the underlying mechanism was studied.

MATERIALS AND METHODS

Extract preparation and phytochemical assessment

Ulva paschima Bast (Bast *et al.*, 2014) was collected from Somnath and Dwarka coasts of Gujarat, India. The identification was got authenticated at the Herbarium, Central University of Punjab, India. The collected specimens were shade-dried and powdered. The powder was extracted in methanol for 72 h with continuous stirring with a Teflon rod. The solvent was filtered using Whatman paper No. 4 and concentrated using a rotary evaporator (Cole Parmer, US). Then, 50 mg methanolic extract of *U. paschima* (MEUP) was mixed with 1 mL culture-grade dimethyl sulfoxide (DMSO) to prepare the working stock and stored at -20˚C till use. The presence of phytochemical metabolites in the algal extract was tested as per Mehdinezhad *et al.* (2016).

Cell line cultures and equipment

Two lung carcinoma cell lines, A549 and NCI-H1299, were purchased from NCCS, Pune. Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI-1640) (Gibco, Thermo Fischer Scientific) supplemented with 4.5 g L^{-1} glucose, 10% FBS (fetal bovine serum), 100 U mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin. The cells were incubated at 37˚C, 5% CO_2, and 95% relative humidity. Cell seed stocks were preserved in a cell freezing medium (HiMedia) at -80˚C.

Enzyme assays

For superoxide dismutase (SOD) assay, the pyrogallol method was followed (Marklund and Marklund, 1974). Cell lysate (5 µL) were separately added to 10, 50, and 100 µg $µL^{-1}$ MEUP and mixed with 50 µL Tris (0.1 M), 16 µL EDTA (6 mM), and 34 µL pyrogallol (6 mM). The absorbance was recorded at 420 nm using a Synergy™ H1 microplate reader (Agilent, USA). For catalase (CAT) assay, 10, 50, and 100 µg $µL^{-1}$ MEUP was separately mixed with 0.05 M H_2O_2 prepared in 0.05 M phosphate buffer at 25°C. Absorbance at 240 nm was measured for 3 min on a Synergy™ H1 microplate reader (Aebi, 1984). For glutathione reductase assay, 5 µL cell lysate was separately added to 10, 50, and 100 µg $µL^{-1}$ MEUP. Then, 50 µL assay buffer, 5 µL GSSG, 5 µL NADPH, and 35 µL double distilled water were added, and absorbance was recorded on Synergy™ H1 microplate reader (Carlberg *et al.*, 1985).

Measurement of intracellular reactive oxygen species

For dihydroethidium (DHE) assay the method of Mehra *et al.* (2019) was followed. The cells were treated with 0.1, 0.2, 0.5, 1.0, and 1.5 μg μL^{-1} of MEUP for 24 h. The cells were rinsed with 1XPBS (phosphate-buffered saline) and stained with 10 μM DHE. The absorbance was measured at 535/635 nm (excitation/emission) on a Synergy™ H1 microplate reader (Agilent, USA). For 2',7'-dichloro-dihydrofluorescein diacetate (H_2DCFDA) assay (Gill *et al.*, 2017), the cells were treated with 0.1, 0.2, 0.5, 1.0, and 1.5 μg μL^{-1} MEUP in a 96 well plate for 48 h. Then the cells were rinsed with 1XPBS and 10 μM H_2DCFDA added. The plates were incubated in dark and washed with PBS after 30 min. The fluorescence intensity for excitation/emission at 485 nm/535 nm were recorded on a Synergy™ H1 microplate reader (Agilent, USA).

Antioxidant assay

MEUP extracts (2 mL) at a concentration varying from 5 to 15625 μg mL^{-1} were added separately to DPPH (1,1-diphenyl-2-pikryl-hydrazin) methanol (0.1 mM) solution were mixed. The mixture was vortexed and left in the dark for 30 min. The decline in the absorbance was measured on a Synergy™ H1 microplate reader (Agilent, USA) for 20 min at 517 nm with methanol taken as blank. The percent radical scavenging activity (%RSA) was calculated (Yen and Chen, 1995).

Mitochondrial membrane potential (Δψm) and cell proliferation assay

Cells (1×10^4) were seeded on 96-well. After treatment with MEUP at sublethal doses, the cells were rinsed with 1X PBS and stained with 20 μM 5,5'6,6'-tetraethylbenzimidazolyl carbocyanine iodine (JC-1) dye for 30 min at 37˚C. A fluorescence intensity ratio of 485/535 and 550/600 nm was read on a Synergy™ H1 microplate reader (Agilent, USA) (Alex *et al.*, 2014). For antiproliferative assessment, the A549, NCI-H1299, and hBMCs (human peripheral blood mononuclear cells) were grown to 10,000 cells per well and then serum-starved for 12 h. After MEUP treatment, the wells were washed twice with 1XPBS, and 0.5 mg mL^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added. The plates were incubated for 4 h in dark, and then 100 μL DMSO was added to each well. The plates were placed in Synergy™ H1 microplate reader on orbital shaker mode for 20 min, and then the absorbance was recorded at 570 nm (Joshi *et al.*, 2017).

Gas chromatography-mass spectrometry (GC-MS) analysis

The extracts were analysed on GC-MS (Shimadzu QP 2010 Ultra). The oven temperature was from 150 to 300˚C with a gradient of 10˚C min^{-1}. The 1 μL samples were injected in a spitless mode at 220˚C with helium gas as a carrier at 1 mL min^{-1}. The data was acquired in electron impact (EI) mode (70 eV) with ion source at 200˚C and scan-interval of 0.5 sec. The chromatogram peaks were identified and compared with the data on Wiley8.Lib (Konda *et al.*, 2017) and National Institute of Standards Technology (NIST) MS library in (NIST11s.lib) (Konda *et al.*, 2017).

Statistical analysis

The experiments on enzymes, ROS, DPPH, MTT and JC-1 assays were performed in triplicate (n = 3) in a completely randomized design. The values were mean values and standard deviation (SD) as error bars. GraphPad Prism (V6.01) was used to test one-way analysis of variance followed by Dunnett's test for multiple comparison and significance was tested for each analysis where statistical significance was denoted by P-value of *$p<0.001$, **$p<0.005$ and ***$p<0.001$.

RESULTS AND DISCUSSION

Cancer is a major health issue late detection, relapse and side-effects of chemotherapy and surgery make it difficult to treat cancer effectively (Mao *et al.*, 2022). Plant phytochemicals are under a continuous scrutiny to find novel potential anticancer agents due to their effect on involved signalling

pathways, apoptosis induction and ability to induce oxidative stress selectively in cancer cells (Singh *et al.*, 2016). *Ulva* is an edible green algae genus abundantly found in intertidal and subtidal habitats (Bast and Rani, 2019), discovery of any significant therapeutic activity may lead to an economical and widely available source for novel drug candidate.

Phytochemical and GC-MS profiling

The chemical characterization was done using phytochemical tests and GC-MS analysis. The algal biomass yielded up to 6 ± 1% of methanolic fractions. The methanolic extract of *U. paschima* (MEUP) was studied for the first time for its phytochemical constituents and it showed the abundance of carbohydrates, saponins, terpenoids, quinones, and phenols. GC-MS chromatogram detected 37 phytoconstituents from MEUP. The major chemical components present in MEUP with anticancer effects are given in Table 1. For example, terpenoids act as natural antioxidants and reduce reactive species of oxygen, nitrogen, sulphur, carbon or selenium (Huang *et al.*, 2012). The GC-MS analysis showed the presence of palmitic acid from MEUP which reportedly targets DNA topoisomerase I to induce cancer cell deaths (Jóźwiak *et al.*, 2020). Similarly, the detected polyunsaturated fatty acid (PUFA), α-linoleic acid and mono-unsaturated fatty acids (MUFA), palmitoleic acid and oleic acid show cytotoxic effects on breast, cervical, prostate and colon cancer cell lines (Wei *et al.*, 2022). PUFAs block the Akt/mTOR pathway to induce cancer death in tongue squamous cell carcinoma TSCC cells (Jiang *et al.*, 2017). The lauric acid, tetra-cosane, azelaic acid and eicosanoid, have *in vivo* and *in vitro* induction of caspase mediated cell apoptosis, protease mediated cell degradation, cell cycle arrest and prevention of angiogenesis on several cancer cell lines (Gomes *et al.*, 2018). Phytol detected is used to commercially produce vitamin E, known for reducing prostate cancer incidences (Alkhenizan and Hafez, 2007).

Table 1: Details of anticancer phytochemicals in *Ulva paschima* identified by GC-MS analysis

Name	Molecular formula	Retention time (min)	% Peak area
2,4-Ditert-butylphenol	$C_{14}H_{22}O$	14.517	5.12
Methyl stearate	$C_{19}H_{38}O_2$	21.492	0.55
1-dodecanol	$C_{12}H_{26}O$	8.333	0.73
5-eicosene	$C_{20}H_{40}$	16.683	0.78
Neophytadiene	$C_{20}H_{38}$	18.492	1.61
Stearic acid	$C_{18}H_{36}O_2$	18.558	8.91
Lauric acid	$C_{12}H_{24}O_2$	18.958	2.96
Hexadecanoic acid	$C_{16}H_{32}O_2$	19.440	8.17
Margaric acid	$C_{17}H_{34}O_2$	19.450	8.17
Linoleic acid	$C_{18}H_{32}O_2$	19.550	0.80
L-α-terpineol	$C_{10}H_{18}O$	21.242	0.53
Oleic acid	$C_{18}H_{34}O_2$	21.317	1.25
9-octadecenamide	$C_{18}H_{35}NO$	22.175	0.57
Myristic acid	$C_{14}H_{28}O_2$	24.842	3.53

Disruption of antioxidant machinery and mitochondrial membrane potential

DHE and H_2DCFDA dyes indicate the changes in intracellular superoxide and hydroxyl radicals, respectively (Gill *et al.*, 2017). An increase in internal ROS levels by 5.4- and 6.8-fold for A549 and H1299 cells, respectively, was observed (****= $p<0.0001$) [Fig. 1 and 2]. Compared to the normal cells, cancer cells are usually under oxidative stress and any increase in ROS levels disrupts the homeostasis (Gill *et al.*, 2017). Treatment with phycochemicals boosts the mitochondrial metabolism process, so increase the intracellular ROS levels (Hansel and Diaz, 2021). The treatment with MEUP raised superoxide levels by 1.8 to 3.4 U mL^{-1} for A549. The increase in oxidative stress is managed by the antioxidant enzyme machinery of the cell and explains the rise in superoxide dismutase, catalase and glutathione reductase activities in A549 and H1299 cells by two times in both cell lines as compared to the untreated control cells. Similar results were observed when breast cancer cells were treated with methanolic extract of *Caulerpa taxifolia* (Mehra *et al.*, 2019). The superoxide dismutase, catalase, and glutathione reductase enzyme activities in A549 and H1299 cells increased on treatment with extracts in a dose-dependent manner as compared to the untreated control cells. A 1.5-fold increase in SOD was observed in both the cells (Fig. 3). For catalase and glutathione reductase activities, a 2-fold upsurge was observed in A549 and 1.8-fold increase in H1299 (Fig. 3).

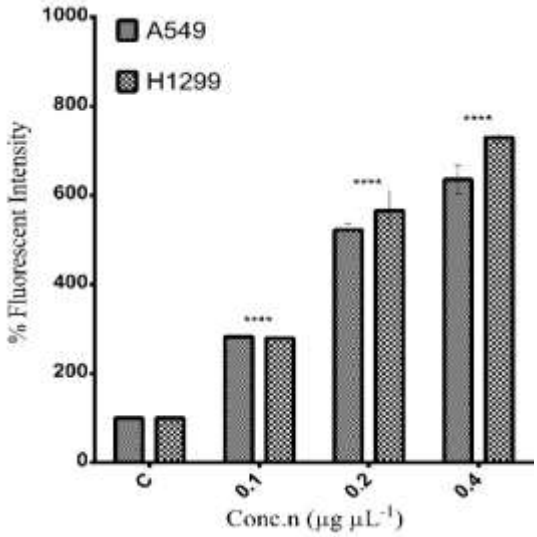

Fig. 1: ROS levels detected in A549 and H1299 cells using dihydroethidium (DHE). Graph shows mean percentage of fluorescent intensity change in comparison to control. Data values are mean ± SD (n = 3) (* = p <0.05, **** = p < 0.0001)

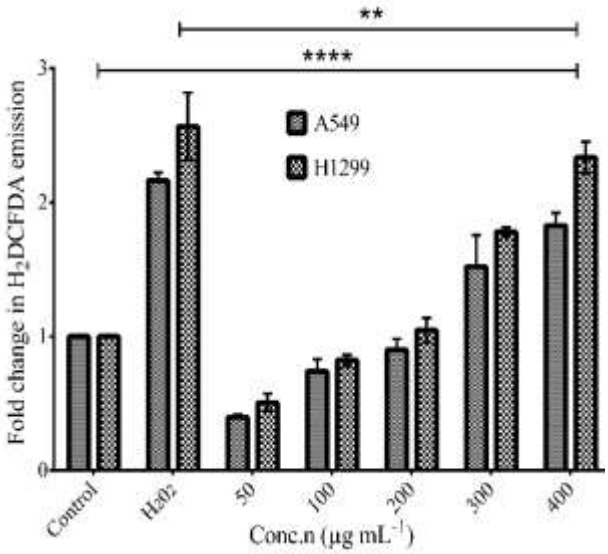

Fig. 2: Change in H_2DCFDA (2',7'-dichloro-dihydrofluorescein diacetate) emission as compared to the untreated cells and H_2O_2 @ 400 µg mL^{-1} for A549 and H1299 cells. Data values are mean ± SD (n = 3), (= p < 0.05, **** = p < 0.0001)**

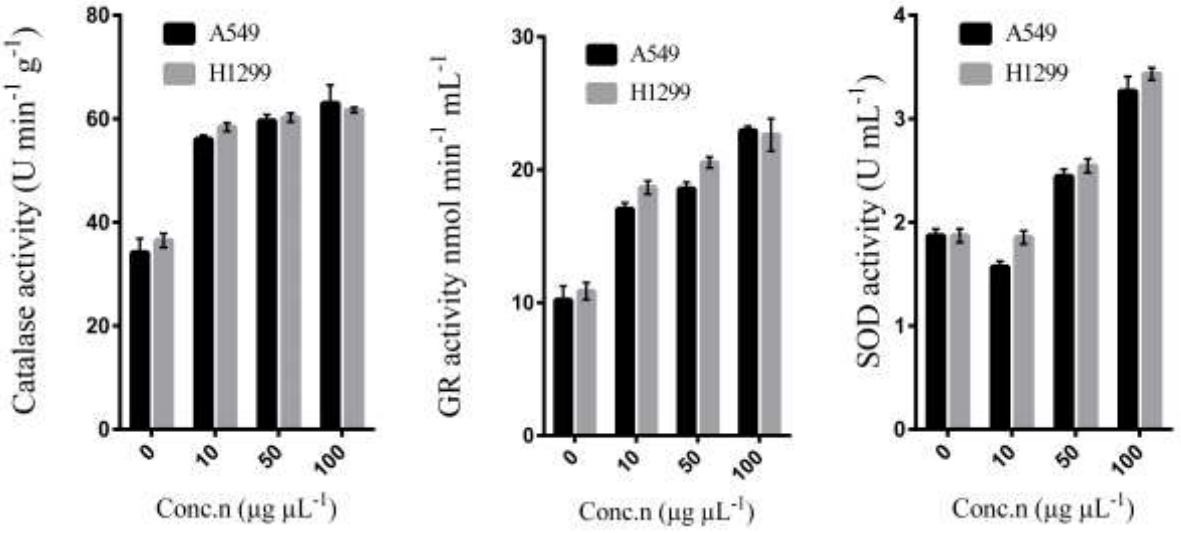

Fig. 3: The enzyme activities of A549 and H1299 cells treated with 10, 50 and 100 µg mL^{-1}; a) catalase activity, b) glutathione reductase activity, and c) superoxide dismutase activity. The values are mean ± SD (n = 3), (p < 0.05).

The results for MEUP showed a dose-dependent depolarisation of mitochondrial membrane potential (MMP) of up to 38.2 ± 3.04 and 41.15 ± 2.09% ($p < 0.001$) for A549 and H1299 cells, respectively, at 300 µg mL^{-1} after 48 h of treatment (Fig. 4). The 100 µM H_2O_2 was taken as a positive control. JC-1 dye detects intracellular change in monomeric and oligomeric mitochondrial aggregates (Mehra *et al.*, 2019). On depolarization of mitochondrial membrane potential, Bax oligomerizes lead

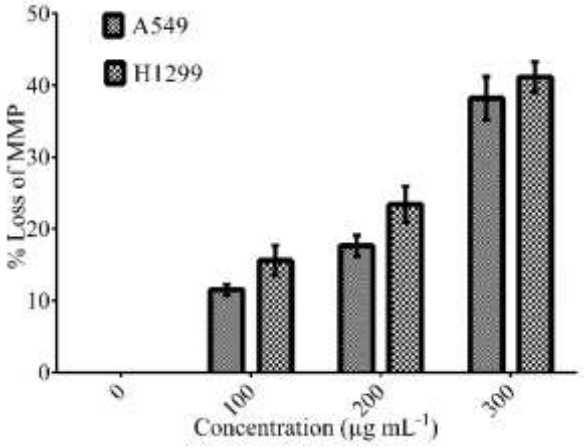

Fig. 4: The percent loss of mitochondrial membrane potential (MMP) in relation to extract concentration. The values are mean ± SD (n = 3), p < 0.001 with respect to the control group

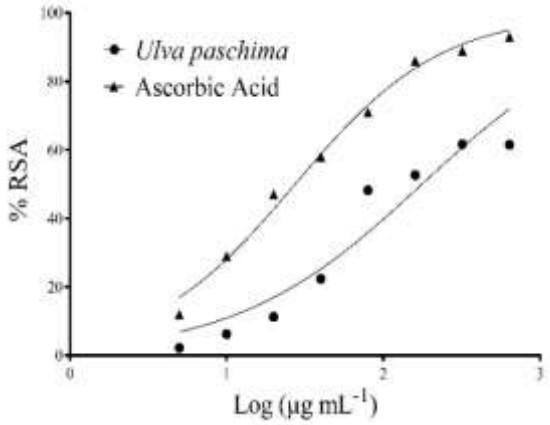

Fig. 5: DPPH radical scavenging activity (% RSA) of *Ulva paschima* and ascorbic acid

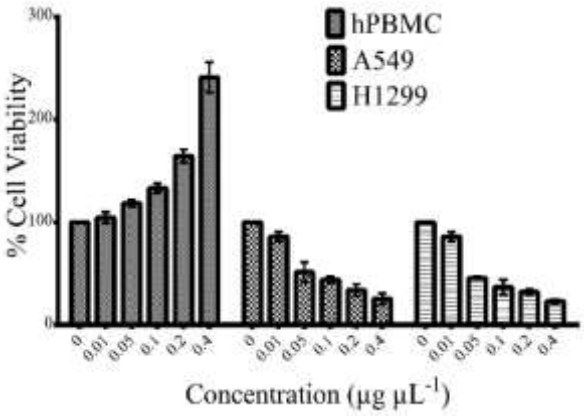

Fig. 6: Cell viability as determined by MTT assay on hPBMCs, A549 and H1299 cells after 48 h of MEUP treatment. Data values are mean cell viability ± SD (n = 3)

to the formation of voltage-dependent anionic channels and release cytochrome *c* which further activates terminal caspases and cause apoptosis (Wang *et al.*, 2014). DNA damage and oxidative stress also trigger the same pathway for cell apoptosis (Adrain and Martin, 2001). In non-apoptotic cells or control, JC-1 aggregates showed a red fluorescence peak at 590 nm, and apoptotic cells showed a fluorescence peak at 527 nm. After treatment with MEUP, a decrease in red and an increase in green fluorescence, indicated membrane depolarization. The *Ulva* extracts and the isolated polysaccharides from *U. lactuca* and *U. intestinalis* reportedly cause cell death in human hepatoma (HepG2) and colon cancer (HCT116) cell lines in a similar fashion (Ryu *et al.*, 2013; Wang *et al.*, 2014).

Antioxidant and antiproliferative potential

DPPH free radical scavenging capacity in a cell- free system was analysed as compared to the ascorbic acid (Fig. 5). The median effective concentration EC_{50} value was calculated as 176.7 ± 0.09 µg mL^{-1} after converting the used concentration to a logarithmic scale. A reaction plateau was attained at a concentration > 320 µg mL^{-1}, and the highest effective radical scavenging activity was 61.69%. Ascorbic acid used as positive control showed EC_{50} of 27.41 ± 0.03 µg mL^{-1}. The antioxidant potential of *U. paschima* extract in a cell-free system was tested by DPPH, a free radical compound. The extracts exhibited a moderate radical-scavenging effect. The results were comparable to the studies on other members of the genus (Shao *et al.*, 2013; Rodeiro *et al.*, 2015; Olasehinde *et al.*, 2019).

The cytotoxicity of MEUP was tested by MTT assay (Fig. 6). A dose and time-dependent decrease in cell viability was observed with the maximum effect after 48 hours of treatment. IC_{50} values were calculated from dose-response curves for A549 (0.187 ± 0.120 µg $µL^{-1}$) and H1299(0.130 ± 0.031 µg $µL^{-1}$) cell lines exhibiting a potent *in vitro* anticancer activity of *U. paschima* methanolic extract. MEUP had no significant toxic effect on hPBMCs.

The results were comparable to the chloroform- hexane, aqueous, and methanolic extracts of *U. intestinalis*, *U. tubulosa*, and *U. lactuca*, that showed significant cytotoxicity against liver, breast, ovarian, and colon cancer cell lines without any cytotoxic effect against tested normal cell lines (Abd-Ellatef *et al.*, 2017; Matloub *et al.*, 2018; Nazarudin *et al.*, 2020).

Conclusions: The study established a relationship between antioxidant and cytotoxic potential of *Ulva paschima*. The data suggests that *U. paschima* extracts disrupted mitochondrial membrane potential, stopped cell proliferation, induced intracellular reactive oxygen species and modulated the antioxidant enzyme defence system in a dose-dependent manner in A549, and H1299 lung cancer cell lines. *U. paschima.* exhibited the presence of beneficial phytoconstituents like phytol, α-linoleic acid, palmitoleic acid, oleic acid, stearic acid, tetracosane, azelaic acid and eicosanoid known for their anticancerous potential. The present study would contribute to understand the vast diversity of bioactive compounds from *Ulva* genera particularly to explore their anticancer potential. *U. paschima* may be considered as a potent source of therapeutic compounds with potential against lung cancer.

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Conflict of Interests: All the authors declare that they have no competing interests.

Ethical statement: This study does not involve any active human participants.

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