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Research Article

Phytochemical Screening and *In-vitro* Anticancer Activity of Ethyl Acetate Fraction of Seagrass *Halodule uninervis* from Mandapam Coastal Region, Rameswaram, Gulf of Mannar, India

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ABSTRACT

Seagrasses are one of the groups of marine angiosperms widely distributed all over the coastal regions. The seagrass ecosystem is very productive and traditionally used for a variety of therapeutic purposes. The present study aimed to investigate the *in-vitro* anticancer activity against malignant melanoma (A375), lung carcinoma (A549), cervix adenocarcinoma (HeLa) and colorectal adenocarcinoma (HT29) cell lines and phytochemical screening of ethyl acetate fraction of *Halodule uninervis* (EAF) from Mandapam coastal region, Rameswaram, Gulf of Mannar, India. The qualitative phytochemical analysis of EAF was performed as followed by standard methods. EAF showed the presence of alkaloids, steroids, terpenoids, flavonoids, phenols and quinones. The total flavonoid and phenolic contents of EAF were determined using the spectrophotometric method. The total flavonoid and phenolic contents showed 67.33 ± 1.52 mg QE/g dried fraction and 87.75 ± 1.39 mg GAE/g dried fraction, respectively. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of EAF shows the presence of 23 phytochemical compounds. The *in-vitro* anticancer activity of EAF was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, AO/EtBr staining and DNA fragmentation assay for 24 hours treatment. The IC_{50} values of EAF showed 91.64 ± 1.61 µg, 269.16 ± 5.17 µg, 845.16 ± 36.09 µg and >1000 µg against A549, HeLa, HT29 and A375 cells, respectively. Followed by reference standard, Doxorubicin showed IC_{50} values of 17.53 ± 0.07 µg, 14.12 ± 0.30 µg, 17.44 ± 0.25 µg and 21.44 ± 0.40 µg against A549, HeLa, HT29 and A375 cells, respectively. AO/EtBr and DNA fragmentation assay confirmed the occurrence of apoptosis in the A549 cell line after the treatment of EAF. These results suggest that *H. uninervis* is a promising source of cytotoxicity against A549 cells and could be considered as a source for new lead structures to the development of new anticancer drugs.

Introduction

Globally, Cancer is the second leading cause of death that is characterized by the uncontrolled growth of human cells; World Health Organization (WHO) estimates that the deaths due to cancer are higher in developed countries compared to developing countries.[1,2] In the year 2050, the number new cases of cancer will increase by 24 million and the number of cancer-related deaths will increase by 17 million worldwide.[3]

Over the past century, the available option for cancer treatment involves surgery, radiation therapy, immunotherapy and chemotherapy.[4] However, these therapies induced severe side effects that have been identified.[5] The current scenario for cancer researches and the development of a new anticancer drug are from naturally derived bioactive produces.[6] At present, 60% of drugs used for cancer treatment have been derived from natural products.[7]

Marine natural products including seagrasses, micro and macroalgae, sponges and corals are the important source which plays a major role in the discovery of novel biologically active compounds.[8] Marine-derived natural products have been reported with various

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pharmacological activities including antibacterial, antifungal, antiviral, analgesia, anticoagulant, antimalarial, allergy, anthelmintic, antiprotozoal, anti-inflammatory, antituberculosis and strong anticancer activity against a variety of cancer cells.[9-11]

Seagrasses are one of the groups of marine angiosperms which often live entirely submerged and are capable of completing their life cycle in a coastal environment.[12] In traditional medicine, seagrasses have been frequently used for a variety of therapeutic purposes such as wound healing, fever, stomach aches, muscle pains and skin diseases; in addition to that, seagrasses have been used for various biomedical applications such as anticancer, antidiabetic, anti-inflammatory, antifungal, antibacterial, anti-viral activities.[13-19]

Halodule uninervis (Forssk.) Boiss. is an abundant seagrass species that belongs to the family of Cymodoceaceae and widely distributed in the Indo-Western Pacific region.[20] The characteristic features of *H. uninervis* with leave flat, narrow, short; shoots short, erect; rhizomes creeping, white to pale brown.[21] Moreover, *H. uninervis* can generate phytoconstituents including phenols, flavonoids, tannins, steroids and alkaloids, which were reported to possess promising biological applications including antibacterial and antidiabetic activities.[22,23] The present study aims to evaluate, for the first time, *in-vitro* anticancer activity of ethyl acetate fraction of *H. uninervis* against various human cancer cell lines including malignant melanoma (A375), lung carcinoma (A549), cervix adenocarcinoma (HeLa) and colorectal adenocarcinoma (HT29) cells.

Materials and Methods

Collection, Identification, and Authentication of the Sample

H. uninervis (Forssk.) Boiss. was collected from the Mandapam coastal region, Rameswaram, Tamil Nadu, Gulf of Mannar, India. The seagrass was identified and authenticated by the Botanical Survey of India, Southern Regional Centre, Coimbatore, India, and a reference number BSI/SRC/5/23/2019/Tech./286 was deposited.

Extraction of Seagrass Sample

The collected seagrass was washed well with running water followed by distilled water. The sample was allowed to be shade-dried and powdered using a grinder. The powder was fractionated with ethyl acetate by gentle mixing on a shaker for 72 hours. The ethyl acetate fraction of *H. uninervis* (EAF) was filtered through Whatman No. 1 filter paper and the filtrate was dried and the extract residue was stored at -20°C until use.

Qualitative Phytochemical Screening

The ethyl acetate fraction of *H. uninervis* (EAF) was phytochemically evaluated to determine the presence of alkaloids, steroids, terpenoids, flavonoids, and phenols according to standard methods.[24]

Test for Alkaloids

The EAF (10 mg) was mixed with few drops of Mayer's reagent. A yellowish-white precipitate indicated the presence of alkaloids.

Test for Steroids

The EAF (10 drops) was added into 2 mL of chloroform following the addition of 1-mL of concentrated sulfuric acid from the sides of the test tube. The interface turns into dish-brown color indicates the presence of steroids.

Test for Terpenoids

To 2 mL of EAF was mixed with 1-mL of anhydrous acetic acid and concentrated sulfuric acid was added carefully. After 5 minutes, a red coloration of the interface showed the presence of terpenoids.

Test for Flavonoids

To 1-mL of absolute ethanol and three drops of concentrated hydrochloric acid were added to 0.5 mL of EAF. The formation of magenta coloration indicated the presence of flavonoids.

Test for Phenols

To 1-mL of EAF was mixed with 4 drops of lead acetate (10% w/v) aqueous solution. The formation of a white precipitate indicated the presence of phenols.

Quantitative Phytochemical Analysis

Determination of Total Flavonoid Content

The total flavonoid content was estimated by spectrophotometric assay.[25] A total of 1-mg of EAF was diluted with 500 µL of distilled water. Next, 300 µL of sodium nitrite (5% w/v) solution was added followed by the addition of 300 µL of aluminum chloride (10% w/v) solution and incubated for 6 minutes. Then, 2 mL of sodium hydroxide (4% w/v) solution was added and made up to 5 mL with distilled water. The mixture was shaken well and incubated for 15 minutes at 37°C. The absorbance was measured at 510 nm. The total flavonoid content was expressed as quercetin equivalent mg QE/g fraction on a dry weight basis using the standard curve in the range of 10 to 320 µg/mL.

Determination of Total Phenol Content

The total phenolic content was determined using the spectrophotometric method.[26] In brief, 1-mg of EAF was mixed with 1 mL of distilled water. Then, 500 µL of Folins-Ciocalteau reagent was added and kept for 5 min at 25°C. After 5 min, 3 mL of sodium carbonate (7.5% w/v) solution was added to the mixture and incubated for 2 hours at 37°C. After incubation, the absorbance was read at 725 nm. The calibration curve for total phenolic was made using gallic acid standard solution (2–64 µg/mL) under the

same procedure as earlier described. The total phenolic content was expressed as gallic acid equivalent mg GAE/g of dried fraction.

Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis

The GC-MS analysis of EAF was done using Clarus 500 Perkin–Elmer (Auto system XL) Gas Chromatograph equipped and coupled to a mass detector Turbo mass gold – Perkin Elmer Turbomass 5.2 spectrometer with an Elite – 5MS (5% Diphenyl/95% Dimethyl poly siloxane), 30 m x 0.25 µm DF of the capillary column. The instrument was set to an initial temperature was set at 110°C and maintained at this temperature for 2 min. At the end of this period, the oven temperature was increased to 280°C, at 5°C/min. Injection port temperature was ensured to be 200°C and Helium flow rate as 1 mL/min. The ionization voltage was 70eV. The EAF was injected in split mode as 10:1. Relative quantity of the phytochemical compounds present in EAF was expressed as percentage based on peak area produced in the chromatogram.

In-vitro Anticancer Activity

Cell Lines and Culture Condition

Human cancer cell lines A375, A549, HeLa and HT29 (Table 1) were obtained from National Center for Cell Science (NCCS), Pune, India. The cells were grown in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin and maintained in a humidified incubator at 37ºC with 5% CO_2 in the air.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell proliferation assay

A375, A549, HeLa and HT29 cells (5×10^4 cells/mL) were seeded in 96-well plates and exposed to different concentrations (25, 50 and 100 µg) of EAF for a period of 24 hours. After the treatment period, the cells were allowed to react with MTT for a period of 4 hours in dark at 37°C. After the incubation period, dark purple formazan crystals were formed. These crystals were solubilized with DMSO and absorbance at 595 nm was measured, microplate reader. The experiment was repeated at least three times. Doxorubicin reference standard was used as a positive control for this experiment. The cell viability was calculated using the formula, cell viability (%) = [(Optical density {OD} of treated cell – OD of blank)/(OD of vehicle control – OD of blank) × 100].[27]

Table 1: Human cancerous cell lines used for the study

Cell line	*Type*
A375	Malignant melanoma
A549	Lung carcinoma
HeLa	Cervix adenocarcinoma
HT29	Colorectal adenocarcinoma

Acridine Orange/Ethidium Bromide (AO/EtBr) Staining

The AO/EtBr double staining test was employed to analyze the morphological changes of apoptosis. Briefly, A549 cells were seeded at a density of 2×10^5 cells in each well of a 24 well plate and incubated for 24 hours. The cells were treated with different concentrations (22.84, 45.68 and 91.36 µg) of EAF for 24 hours at 37°C. Untreated cells were used as control. Afterward, cells were washed twice with 1x PBS for further staining with 5 µL of AO/EtBr. Finally, the morphological changes of the A549 cells were analyzed using Nikon (Japan) bright-field inverted light microscopy at 20 × magnification.

DNA Fragmentation Assay

A549 cells were seeded at a density of 1×10^6 cells/well in a 24 well tissue culture plate. After 24 hours of culture, cells were incubated with different concentrations (22.84, 45.68 and 91.36 µg) of EAF for 24 hours at 37°C. Untreated cells were used as control. Cells were lysed after 24 hours of treatment in 100 µL of digestion buffer (100 mM NaCl; 10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0; 0.1% SDS and 0.1 mg/mL Proteinase-K) per sample, incubated at 50°C for 3 hours, followed by DNA extraction with a phenol:chloroform:isoamyl alcohol solution (25:24:1). The DNA was treated with DNase-free RNase at a concentration of 20 mg/ml at 4°C for 45 minutes and precipitated with 100 µL of 2.5 M sodium acetate and 3 volumes of ethanol. The samples were then centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatants were collected in fresh centrifuge tubes, treated with RNase for 20 minutes on ice. The DNA pellets were electrophoresed in a 2% agarose gel and photographed.

Results

Qualitative Phytochemical Analysis

The preliminary phytochemical analysis of EAF was given in Table 2. The results showed the presence of alkaloids, steroids, terpenoids, flavonoids, phenols and quinones.

Quantitative Phytochemical Analysis

Total Flavonoid Content

The total flavonoid content of EAF was determined from the regression equation of the calibration curve

Table 2: Qualitative phytochemical screening of ethyl acetate fraction of *H. uninervis*

Phytoconstituents	*Ethyl acetate fraction*
Alkaloids	+
Steroids	+
Terpenoids	+
Flavonoids	+
Phenols	+
Quinones	+

+; Detected

(Y = 0.001x; R2 = 0.998) and expressed as mg quercetin equivalents (QE) per gram of fraction in dry weight (mg/g). The total flavonoid content was found to be 67.33 ± 1.52 mg QE/g dried fraction (Table 3).

Total Phenolic Content

The total phenolic content of the EAF was quantified from the regression equation of the calibration curve (Y = 0.004x; R2 = 0.989) and expressed as mg gallic acid equivalents (GAE) per gram of fraction in dry weight (mg/g). The total phenolic was found to be 87.75 ± 1.39 mg GAE/g dried fraction (Table 3).

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Te GC-MS chromatogram of EAF recorded a total of 23 peaks corresponding to the bioactive compounds (Fig. 1). The phytoconstituents of ethyl acetate fraction were found to be Dipropyl propylphosphonate (0.70%), Benzeneethanamine, 2-fluoro-beta.,3,4-trihydroxy-N-isopropyl- (0.92%), (S)-6,6-Dimethyl-2-azaspiro[4.4] non-1-ene (1.25%), Pyrimidine-2,4,6(1H,3H,5H)-trione, 5-[1-(2-diethylaminoethylamino)propylidene]-(2.02%), 9-Octadecenamide, (Z)- (28.48%), Methyl 6,11-octadecadienoate (1.56%), 9,12-Octadecadien-1-ol, (Z,Z)- (18.03%), 9-Tetradecenal, (Z)- (17.85%), n-Octylsuccinic anhydride (2.03%), 3-Methyl-cyclopentadecylamine (0.71%), Methyl 5,9,12-octadecatrienoate (0.93%), Methyl 5,6-octadecadienoate (1.70%), 9,12-Octadecadienoic acid (Z,Z)- (0.65%), Pyrrolidin-2,5-dione-1-yl 6-oxo-3-thiaoctate (0.65%), Cholan-24-oic acid, 3,6-bis(acetyloxy)-, methyl ester, (3.alpha.,5.beta.,6.alpha.)- (0.65%), Methyl 5,13-docosadienoate (0.67%), Pyridine, 2-tridecyl- (3.20%), Pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta.)- (4.38%), Ofurace (10.29%), 4,5-Methylenedioxy-2-bromo-N-[1-methyl-4-diethylaminobutyl]aniline (1.07%), 9,12,15-Octadecatrienal, dimethyl acetal (0.71%), 2,3-Cyclododecenopyridine (0.92%), Acetic acid, 3-cyano-2-cyclohexyl-6-methyl[1,2]oxazinan-6-ylmethyl ester (0.62%), respectively (Table 4).

Table 3: Total flavonoid and phenolic content of ethyl acetate fraction of *H. uninervis*

Sample	*Flavonoid content (mg QE/g dried fraction)*	*Phenolic content (mg GAE/g dried fraction)*
EAF	67.33 ± 1.52	87.75 ± 1.39

The data were performed in three independent experiments and represented as mean ± SD.

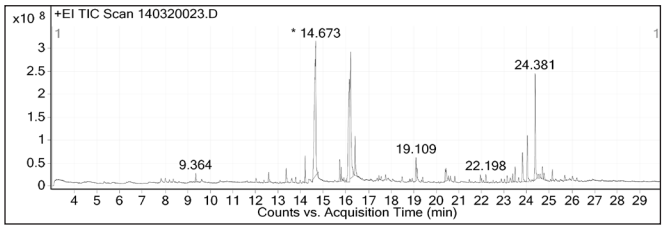

Fig. 1: GC-MS chromatogram of ethyl acetate fraction of *H. uninervis* (EAF)

In-vitro Anticancer Activity

Screening of Cytotoxic Effects of Ethyl Acetate Fraction of H. uninervis on various Cancer Cell Lines

The anticancer activity of ethyl acetate fraction of *H. uninervis* (EAF) and Doxorubicin as a reference standard was evaluated based on its protective effects on cell viability was shown in Table 5. EAF showed an IC_{50} value of 91.64 ± 1.61 µg, 269.16 ± 5.17 µg, 845.16 ± 36.09 µg and >1000 µg against A549, HeLa, HT29 and A375 cells, respectively. Followed by the reference standard, Doxorubicin showed an IC_{50} value of 17.53 ± 0.07 µg, 14.12 ± 0.30 µg, 17.44 ± 0.25 µg and 21.44 ± 0.40 µg against A549, HeLa, HT29 and A375 cells, respectively.

Table 4: The major components found in the ethyl acetate fraction of *H. uninervis* based on GC-MS analysis

S. No.	*Constituents*	*RT min*	*Area%*	*Mass*	*Formula*
1	Dipropyl propylphosphonate	9.36	0.70	208.1	$C_9H_{21}O_3P$
2	Benzeneethanamine, 2-fluoro-.beta.,3,4-trihydroxy-N-isopropyl-	12.59	0.92	229.1	$C_{11}H_{16}FNO_3$
3	(S)-6,6-Dimethyl-2-azaspiro[4.4]non-1-ene	13.37	1.25	151.1	$C_{10}H_{17}N$
4	Pyrimidine-2,4,6(1H,3H,5H)-trione, 5-[1-(2-diethylaminoethylamino)propylidene]-	14.20	2.02	282.2	$C_{13}H_{22}N_4O_3$
5	9-Octadecenamide, (Z)-	14.67	28.48	281.3	$C_{18}H_{35}NO$
6	Methyl 6,11-octadecadienoate	15.73	1.56	294.3	$C_{19}H_{34}O_2$
7	9,12-Octadecadien-1-ol, (Z,Z)-	16.16	18.03	266.3	$C_{18}H_{34}O$
8	9-Tetradecenal, (Z)-	16.22	17.85	210.2	$C_{14}H_{26}O$
9	n-Octylsuccinic anhydride	19.11	2.03	212.1	$C_{12}H_{20}O_3$
10	3-Methyl-cyclopentadecylamine	19.15	0.71	239.3	$C_{16}H_{33}N$
11	Methyl 5,9,12-octadecatrienoate	20.40	0.93	292.2	$C_{19}H_{32}O_2$
12	Methyl 5,6-octadecadienoate	20.44	1.70	294.3	$C_{19}H_{34}O_2$

S. No.	*Constituents*	*RT min*	*Area%*	*Mass*	*Formula*
13	9,12-Octadecadienoic acid (Z,Z)-	21.95	0.65	280.2	$C_{18}H_{32}O_2$
14	Pyrrolidin-2,5-dione-1-yl 6-oxo-3-thiaoctate	22.20	0.65	261.1	$C_{10}H_{15}NO_5S$
15	Cholan-24-oic acid, 3,6-bis(acetyloxy)-, methyl ester, (3.alpha.,5.beta.,6.alpha.)-	23.14	0.65	490.3	$C_{29}H_{46}O_6$
16	Methyl 5,13-docosadienoate	23.38	0.67	350.3	$C_{23}H_{42}O_2$
17	Pyridine, 2-tridecyl-	23.82	3.20	261.2	$C_{18}H_{31}N$
18	Pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta.)-	24.03	4.38	372.2	$C_{22}H_{33}BO_4$
19	Ofurace	24.38	10.29	281.1	$C_{14}H_{16}ClNO_3$
20	4,5-Methylenedioxy-2-bromo-N-[1-methyl-4-diethylaminobutyl]aniline	24.70	1.07	356.1	$C_{16}H_{25}BrN_2O_2$
21	9,12,15-Octadecatrienal, dimethyl acetal	24.77	0.71	308.3	$C_{20}H_{36}O_2$
22	2,3-Cyclododecenopyridine	25.14	0.92	217.2	$C_{15}H_{23}N$
23	Acetic acid, 3-cyano-2-cyclohexyl-6-methyl[1,2]oxazinan-6-ylmethyl ester	25.69	0.62	280.2	$C_{15}H_{24}N_2O_3$

Table 5: The IC_{50} values of ethyl acetate fractions of *H. uninervis* against various human cancer cell lines

	Human Cancer cell lines IC_{50} (µg)			
Samples	*A375*	*A549*	*HeLa*	*HT29*
EAF	>1000	91.64 ± 1.61	269.16 ± 5.17	845.16 ± 36.09
Doxorubicin	21.44 ± 0.40	17.53 ± 0.07	14.12 ± 0.30	17.44 ± 0.25

The data were performed in three independent experiments and represented as mean ± SD.

Further, the treated cells exhibited obvious morphological features such as shrinkage, distortion and rounded which was consistent with the MTT assay (Fig. 2).

AO/EtBr Dual Staining Cell Morphological Analysis

The induction of apoptosis, after the treatment of EAF against A549 cells, was assessed by fluorescence microscopy after staining with acridine orange/ethidium bromide (AO/EtBr). The EAF treated cells showed yellow-orange and red fluorescent and thus confirmed apoptotic cells compared to untreated cells exhibited green fluorescent due to the presence of viable cells (Fig. 3A).

DNA Fragmentation Assay

The evaluation of apoptosis was further conducted by determining the DNA fragmentation patterns; the EAF was able to induce apoptosis in the A549 cell line with different concentrations (22.84, 45.68 and 91.36 µg). The result showed after 24 hours the number of apoptotic cells was higher and most of the nuclei exhibited condensed chromatin (Fig. 3B).

Discussion

Marine natural products have a rich source of therapeutically active compounds that are universally dispensed all over the coastal regions. Today, the pharmaceutical industries worldwide continue to rely

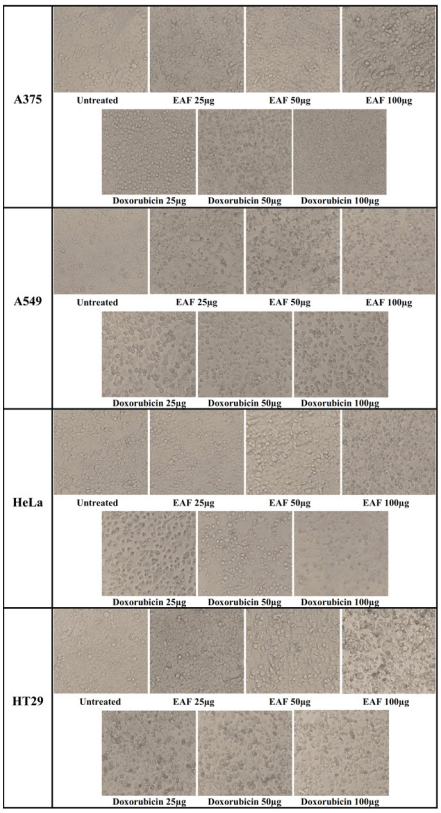

Fig. 2: Morphological changes were captured at various doses (25, 50 and 100 µg) with EAF and Doxorubicin for 24 hours at 20 × magnification

on several pharmacologically approved marine-based products for the development of new drug programmes.[28] In recent years, the uses of marine-derived bioactive components are responsible for cancer control and treatment have been increasingly concerned because of the influence of phytoconstituents with various biological properties.[29] The authors Baehaki *et al.,*[30] Karthikeyan and Sundarapandian[23] studied, the preliminary phytochemical analysis made for *H. uninervis* contains phenols, alkaloids, flavonoids, steroids, glycosides and saponins was present in the methanol extract. This result is similar to the present study, EAF was illustrated that contains alkaloids, steroids, terpenoids, flavonoids, phenols and quinones.

These secondary metabolites were reported to have the treatment of various human diseases from ancient times.[31] Among the phytoconstituents, flavonoids and phenolic compounds that hold an aromatic ring[32] have been shown variety of biological applications including Alzheimer's disease, atherosclerosis and strong anticancer activities.[33,34] In this study, the EAF was determined total flavonoid and phenolic contents using the colorimetric method based on the quercetin and gallic acid standard, respectively. The result indicated that EAF showed the total flavonoid content (TFC) and total phenolic content (TPC) with 67.33 ± 1.52 mg QE/g dried fraction (Fig. 4A) and 87.75 ± 1.39 mg GAE/g dried fraction (Fig. 4B), respectively. This study also confirms the GC-MS analysis of *H. uninervis* revealed the presence of some the phytoconstituents that have been documented pharmacological activities. Among the bioactive components, some fatty acids have cancer-preventive, antibacterial, antioxidant, anti-inflammatory properties.[35] However, these phytoconstituents may be responsible for the anticancer properties against A375, A549, HeLa and HT29 cell lines.

The anticancer activity of EAF was investigated through MTT assay on various human cancer cells such as A375, A549, HeLa and HT29. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is used for detecting the cell viability of the products. MTT assay is based on the reduction of MTT by mitochondrial dehydrogenase by conversion of colorless tetrazolium to the purple-colored formazan product. The present study reveals that the anticancer activity of ethyl acetate fraction of *H. uninervis* against various human cancer cell lines such as malignant melanoma (A375), lung carcinoma (A549), cervix adenocarcinoma (HeLa) and colorectal adenocarcinoma (HT29) cells for 24 hours of treatment. The EAF showed significantly decreased cell viability at different doses (25, 50 and 100 µg) against A549 cell line (65.33 ± 0.31%, 58.90 ± 0.21% and 48.27 ± 0.16%) followed by in the order of other cell lines HeLa (80.60 ± 0.11%, 70.74 ± 0.21% and 64.51 ± 0.13%), HT29 (88.71 ± 0.14%, 83.76 ± 0.20% and 64.51 ± 0.13%) and A375 (90.14 ± 0.11%, 85.49 ± 0.20% and 80.21 ± 0.11%), respectively. Followed by reference standard, Doxorubicin showed A549 cell line was 39.64 ± 0.09%, 22.59 ± 0.20% and 11.28 ± 0.41%, HeLa cell line (31.70 ± 0.19%, 18.17 ± 0.28% and 5.39 ± 0.40%), HT29 cell line (38.21 ± 0.33%, 21.53 ± 0.37% and 8.48 ± 0.34%) and A375 cell line (43.43 ± 0.39%, 26.18 ± 0.82% and 8.06 ± 0.48%), respectively (Fig. 5).

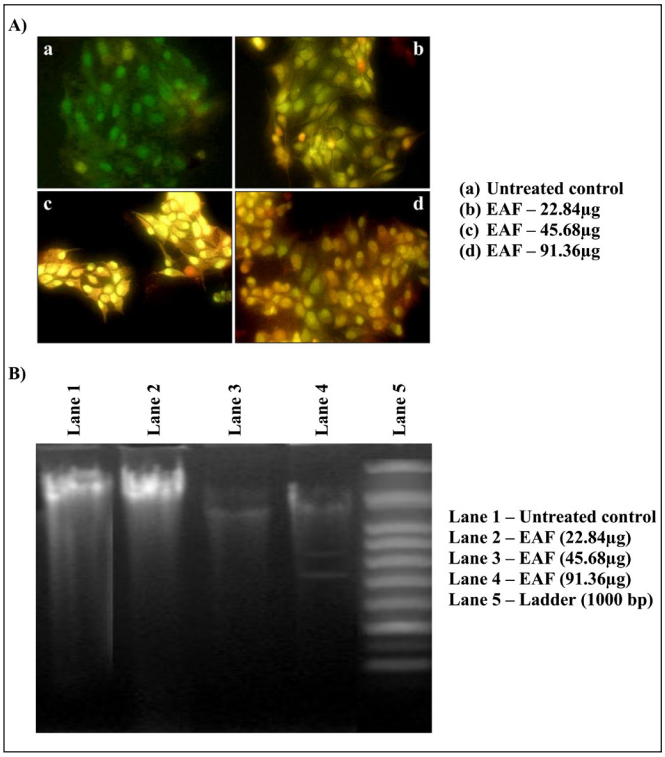

Fig. 3. (A) AO/EtBr double staining on A549 human cells for 24 h. Note: (a) untreated control cells; (b) cells treated with 22.84µg EAF; (c) cells treated with 45.68µg EAF; (d) cells treated with 91.36µg EAF. (B) DNA fragmentation assay on A549 human cancer cells for 24 h. Lane 1 (untreated control); Lane 2 (cells treated with 22.84µg EAF); Lane 3 (cells treated with 45.68µg EAF); Lane 4 (cells treated with 91.36µg EAF; Lane 5 (1000 bp ladder).

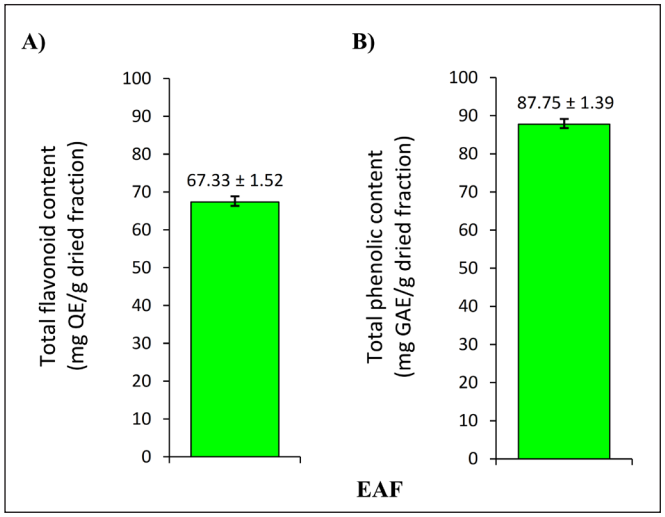

Fig. 4: (A) Total flavonoid content and (B) total phenolic content in EAF of ***H. uninervis***. The data were performed in three independent experiments and represented as mean ± SD.

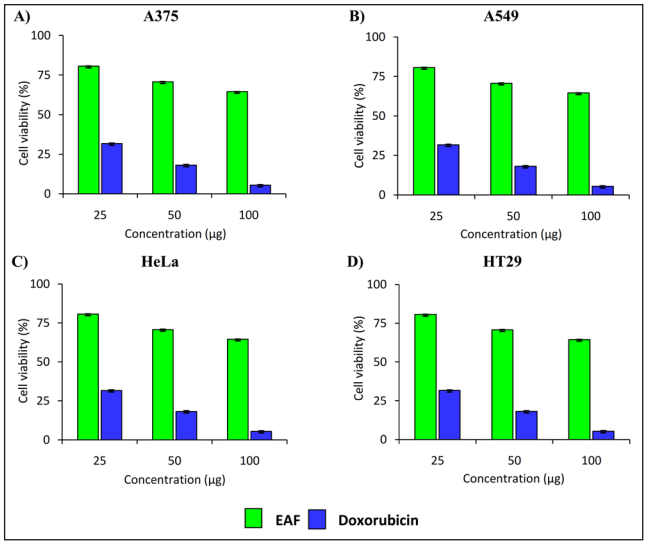

Fig. 5: MTT assay to evaluate the cytotoxicity of EAF and Doxorubicin on various human cancer cell lines A) melanoma (A375) cell line, B) lung carcinoma (A549) cell line, C) cervix adenocarcinoma (HeLa) cell line and D) colorectal adenocarcinoma (HT29) cell line. The data were performed in three independent experiments and represented as mean ± SD.

A dual staining method of acridine orange (AO) and Ethidium Bromide (EtBr) is considered as an authentic tool to ascertain the mechanism of apoptotic cells. AO is the green fluorescence dye that stains with cytoplasm and nucleus of the entire viable cells, whereas EtBr is the red fluorescence dye that preferentially stains only with apoptotic cells.[36,37] The present study confirms that the increasing concentration of EAF (22.84, 45.68 and 91.36 µg) induced apoptosis on A549 cells through modifications in cell morphology and apoptotic cells after 24 hours of treatment. Fig. 3A revealed that the formation of nuclear shrinkage, blebbing and yellow-orange and red colored fluorescence shows the presence of apoptotic cells, green fluorescence shows the presence of viable cells. Therefore, it is noticeable that the strong anticancer activity of EAF is accompanied by the induction of apoptosis in A549 cells.

The present study also indicated that the appearance of ladder-like DNA observed in the A549 cell line treated with different concentrations (22.84, 45.68 and 91.36 µg) of EAF is shown in Fig. 3B. DNA fragmentation occurred during apoptosis, the chromatin DNA is degrading into short fragments by activated endonuclease, which could be visible through gel electrophoresis. In summary, these findings suggest that the seagrass *H. uninervis* showed a variety of phytoconstituents thus played a crucial role in the cytotoxic and apoptosis of the A549 cells compared to other cells lines.

Conclusion

A marine natural product plays a vital role in the treatment of various ailments including Cancer. The discovery of new bioactive natural products is one of the most important and current challenges for the pharmaceutical industry and academic research groups. Our present study, for the first time, the seagrass *H. uninervis* contains a wide variety of secondary metabolites that hold strong anticancer activity against the A549 cell line. Further, purification of the specific active phytoconstituents and preclinical studies needs to be conducted, that can be used for the discovery of novel anticancer drugs to treat lung cancer and reduced side effects.

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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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