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A comparative study of the bioavailability of Red Sea seagrass, *Enhalus acoroides* (L.f.) Royle (leaves, roots, and rhizomes) as anticancer and antioxidant with preliminary phytochemical characterization using HPLC, FT-IR, and UPLC-ESI-TOF-MS spectroscopic analysis

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Abstract

Background Seagrasses are unique marine flowering plants. *Enhalus acoroides* (L.f.) Royle (family Hydrocharitaceae), a new record for the Egyptian coast of the Red Sea, was the grass of choice. A comparative study on *Enhalus acoroides* (L.f.) Royle (leaves, roots and rhizomes) was done to determine the plant organ that shows the highest antiproliferative and antioxidant activities. The total phenolic content was determined using the Folin–Ciocalteu method. The total flavonoid content was estimated by the aluminum chloride assay. Fourier transform infrared (FT-IR) analysis was performed to detect the chemical functional groups in the extract. High-performance liquid chromatography (HPLC) was done for the qualitative and quantitative evaluation of phenolic compounds. UPLC-ESI-TOF-MS was performed for metabolomics profiling of the extract. Antioxidant activity was determined using the DPPH scavenger percentage method. Antiproliferation assay against hepatocellular carcinoma HepG2, human breast cancer cell lines MCF-7, MDA-MB-231 was performed for the three seagrass organs. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured after treatment with three extracts against MCF-7 cell line.

Results The highest phenolic content is found in the leaves, while roots exhibited the highest DPPH scavenger percentage. The total concentration of phenolic compounds detected by HPLC was leaves > rhizomes > roots. Also leaves exhibit the highest antiproliferative activity and mitochondrial membrane potential depletion effect against MCF-7 cell line tested. UPLC-ESI-TOF-MS metabolite profiling of leaves detected different secondary and primary metabolites to which the activity was retained. Leaves are a new candidate to be used in the treatment of cancer.

Conclusion *Enhalus acoroides* (L.f.) Royle leaves extract is a new nutraceutical candidate. Further in-depth studies are required on *Enhalus acoroides* (L.f.) Royle leaves.

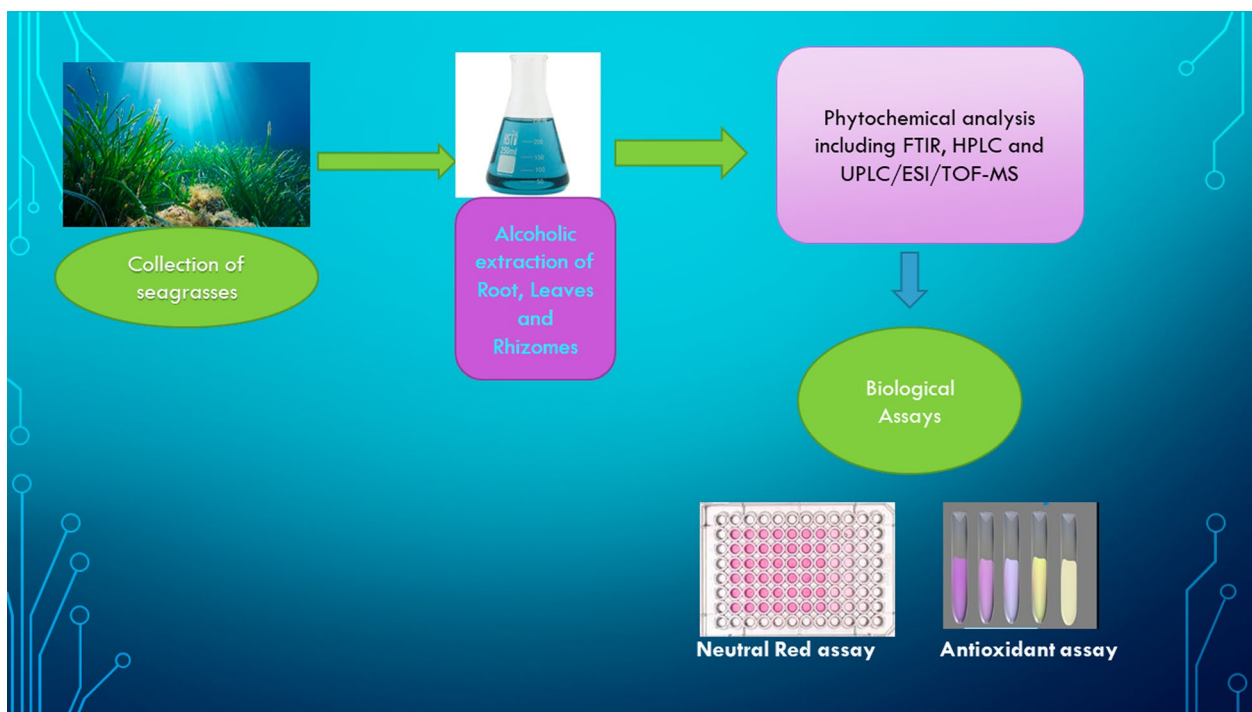
Keywords *Enhalus acoroides*, Red sea, Antiproliferation, Antioxidant, Cancer

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Graphical abstract



1 Background

Nature encompasses a wide variety of species that produce a huge number of primary and secondary metabolites. Traditional healers have used plants and marine organisms to treat diseases for decades [1]. Secondary metabolites are synthesized by the plant through a metabolic pathway. They are a diverse group of chemical classes, viz. phenolic compounds, sterols, alkaloids, terpenes, etc., which are reported to possess a wide variety of biological activities [2]. The phytochemical constituents of marine organisms have been deeply exploited except for the very small taxonomic class of seagrass, which is not well studied.

The tropical region is the most productive seagrass area. The Red Sea encloses eleven seagrass species belonging to two families; family Cymodoceaceae and family Hydrocharitaceae. We continue our exploration of this marvelous marine plant's primary and secondary metabolites [3–6]. *Enhalus acoroides* (L.f.) Royle (family Hydrocharitaceae), a new record for the Egyptian coast of the Red Sea, was the grass of choice [7, 8]. It is an edible plant that is consumed by the coastal population and is used as a traditional medicine to relieve skin disease, enhance indigestion, act as an aphrodisiac and as a contraceptive [9, 10]. The authors reported the presence

of different phytochemical constituents in the seagrass, such as phenols, flavonoids, steroids, tannins, and terpenes [11]. Furthermore, they also discovered that *Enhalus acoroides* (L.f.) has antioxidant, antimicrobial, larvicidal, and recently, anticancer properties [12, 13].

Cancer is a mortal disease resulting from abnormal cell proliferation and propagation. Several studies have shown that phenolic compounds have a prophylactic effect against cancer progression and initiation due to their antioxidant, anti-inflammatory, and antimutagenic activities, as well as their cytotoxic activity via apoptosis and cell cycle arrest [14, 15]. Furthermore, they have an inhibitory effect on tumor invasion and angiogenesis [16]. Flavonoids have the capacity to induce anticancer activities through modulation of ROS-scavenging enzyme activities, arresting of the cell cycle, and induction of apoptosis, as well as suppression of cancer cell proliferation and invasiveness [17]. Phenolic compounds of marine origin are of great interest to authors to explore, although phenolic compound production depends on the environmental conditions around the organism. Nowadays, nutraceuticals are gaining great awareness in the field of cancer treatment due to their low cost and high efficiency [18, 19].

In this work, we divided the seagrass into three parts: leaves, roots, and rhizomes and compared the phenolic and flavonoid content of the three parts of the alcoholic extract using colorimetric assays and spectroscopic analysis (HPLC). Phytochemicals functional groups were detected using FT-IR. Antioxidant and antiproliferative activity was assessed, and the part with higher activity was further analyzed using UPLC-ESI-TOF-MS.

2 Methods

2.1 Seagrass material

Seagrass samples were collected from Wadi El Gemal National Park (WGNP). It is situated in the Red Sea Governorate, approximately 50 km south of Marsa Alam. It encompasses a segment of the Red Sea coastal plain (about 70 km of coastline, including the ecotourism development areas) and mountains extending roughly between 24°52' N in the north and 24°05' N in the south; and between the Red Sea shoreline in the east to about 34°28' E in the west (the Sheikh Shazli road). Samples were collected and identified by Dr. Amgad ElShaffai (Egyptian Environmental Affairs Agency (EEAA), Nature Conservation Sector, Ministry of Environment, Egypt). Voucher specimen was prepared and kept in the Wadi El Gemal National Park. The collection of living plant material from the wild complied with Seagrass Net protocol and as per standards for seagrass collection, identification, and sample design.

2.2 Extraction of seagrass

Fresh samples of *Enhalus acoroides* (Linnaeus f.) Royle (EA) (800 g) were carefully washed under running water and debris was removed. Clean samples were classified to root, rhizomes, and leaves. Each part was separately blended in an electric blender with methanol and then sonicated for 15 min, and the process was repeated until exhaustion. Alcoholic extracts were combined and filtered, and the solvent was evaporated under reduced pressure at 45 °C. The crude methanol extracts of root, rhizomes, and leaves, R, Rh, and L, respectively, were stored for further analysis.

3 Phytochemical assay

3.1 Total phenolic content

The total soluble phenolic content was determined using the Folin–Ciocalteu method [20]. A standard curve was prepared with different concentrations of gallic acid. The total phenolic content was presented as mg of gallic acid equivalents (GAE).

3.2 Total flavonoid content

The total flavonoid content was colorimetrically determined by aluminum chloride assay as described by

[21]. Briefly, add 2 ml methanol to 0.5 ml of extract, and 0.15 ml NaNO₂ (1 mol/l) and vortex for 3.0 min followed by addition of 0.15 ml AlCl₃ (10% w/v), vortex for 3.0 min, add 1 ml NaOH (1 mol/l), adjust the volume to 5 ml using methanol and vortex. Samples were kept in the dark for 40 min and then measured at λ 500 nm using spectrophotometer (Jasco-V-630, UV/Visible). The total flavonoid content was expressed as mg of quercetin equivalents.

4 Spectroscopic analysis

4.1 Fourier transform infrared (FT-IR) analysis

Few mgs of the dried powder of the alcoholic extract of each of roots, rhizomes, and leaves of the seagrass *Enhalus acoroides* (L.f.) Royle relied on a Jasco FT/IR-6100 type A. The sample was mixed with potassium bromide (KBr) and compressed to a disc (10 mm diameter), and the spectra were taken between wave numbers of 4000 and 400 cm⁻¹.

4.2 HPLC analysis of phenolic compounds

Phenolics were detected against authentic samples using HPLC. Thirty microliters of each alcoholic extract (R, Rh, and L, 20 mg/ml) and authentic samples was injection into HPLC 1100 Series Agilent, USA. The Column, C18 Inertsil ODS 3 (4.6 × 250 mm, 5 μ m), was gradient eluted with buffer (0.1% phosphoric acid in water) and methanol at a flow rate of 1 ml/min, ambient temperature, and wave length 280, 320, 360 nm.

4.3 UPLC/ESI/TOF-MS

The alcoholic extract that shows the highest biological activity was defatted. Fifty mg of the defatted sample was used for analysis by UPLC/ESI/TOF-MS (Exion LC analytical UHPLC system (SCIEX, Framingham, USA) according to the procedure described by [22]. The data were interpreted using Peak View 2.2 software (SCIEX, Framingham, MA, USA) and with comparison with previous literature.

5 Biological activities

5.1 Antioxidant activity

RO, RH, and L (10 mg/ml) antioxidant activity was determined by measuring the percentage of DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging using a spectrophotometer (Jasco V-630, UV/Visible) as described by [23]. IC_{50s} were determined from the standard curve of each sample. Briefly, twofold serial dilutions of the stock solution 10 mg/ml (10, 5, 2.5, 0.625, 0.3125 mg) were prepared and the scavenger percentage of each was measured procedure and the curve was plotted concentration against percentage.

5.2 Antiproliferation assay

Hepatocellular carcinoma HepG2, human breast cancer cell line MCF-7, MDA-MB-231 human breast cancer, and normal human skin fibroblast HSF were obtained from ATCC (American Type Culture Collection). Cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Belgium) supplemented by 100 mg/ml of Streptomycin, 100 units/mL of penicillin, and 10% of heat-inactivated fetal bovine serum in humidified incubator and 5% (v/v) CO₂ atmosphere at 37 °C. The cells harvested after trypsinization (0.025% trypsin and 0.02% EDTA) and washed twice with phosphate-buffered saline (PBS). When the cell confluence reached approximately 80%, cells were split for further culture. The experiments were made up when the cells were in the logarithmic growth phase.

Cell viability was assessed by neutral red assay [24]. Aliquots of 200 µl cell suspension, typically containing 5000–20,000 cells/well, were added in 96-well flat-bottom plate and incubated in complete media for 24 h. Cells were treated with another aliquot of 100 µl media containing treatment at serial concentrations (0, 31.25, 62.50, 125, 250, 500, 1000 µl/ml). After 48 h of treatment exposure, cells were washed by 150 µl of PBS and then 100 µl of neutral red media working solution (0.4 µg/ml) (Sigma-Aldrich) was added to each well. Then, the cells were then incubated for 2 h at 37 °C and 5% CO₂. At the end of incubation time, carefully remove the neutral red media and wash with 150 µl of PBS. Add 150 µl of neutral red de-stain solution (1% acetic acid: 50% ethanol (96%): 49% deionized H₂O), followed by rapid shaking for at least 10 min on a micrometer plate shaker. The neutral red color intensity was measured at 540 nm in a microtiter plate reader spectrophotometer (Sorin, Biomedica S.p.A., Milan, Italy), using blanks that contain no cells as a reference. The IC₅₀ value of the tested extract was calculated. Doxorubicin (Dox, Mr=543.5) was used as a positive control. DMSO (dimethyl sulfoxide) was used as a vehicle to dissolve the tested extract, and cell viability % was calculated as follows:

Cell viability (%) = $\frac{\text{OD Treatment} - \text{OD blank}}{\text{OD control} - \text{OD blank}} \times 100$. Each experiment group was repeated three times.

5.3 Assessment of mitochondrial membrane potential

The JC-1 mitochondrial membrane potential assay kit (ab113850, Abcam, Cambridge, UK) was used to determine the mitochondrial membrane potential ($\Delta\psi$ M) in seagrasses-treated MCF-7 cell line since the extracts exhibited the highest inhibition capacity on the cell viability. Attracted by a high $\Delta\psi$ M, the cationic dye JC-1 (tetraethylbenzimidazolylcarbocyanine iodide) accumulates within polarized mitochondria, where it forms red

fluorescent aggregates at high concentrations [25]. Upon $\Delta\psi$ M dissipation, JC-1 stops accumulating within mitochondria, and the JC-1 molecules, present as monomers at low concentration, emit green fluorescence. We performed the assay according to the manufacturer's protocol. Briefly, MCF-7 cells were seeded in a black-walled 96-well plate (15,000 cells/well) and covered with 100 µl of treatment solution. Thirty minutes before completed treatment, we added 2X JC-1 solution (40 µM) to each well (100 µl/well) and incubated for 30 min at 37 °C protected from light. The cells were washed twice with pre-warmed 1X dilution buffer (100 µl/well). The last wash was left on the cells, and the fluorescence of both aggregates ($\lambda_{\text{excitation}}=475$ nm, $\lambda_{\text{emission}}=590$ nm) and monomers ($\lambda_{\text{excitation}}=475$ nm, $\lambda_{\text{emission}}=530$ nm) was measured using the microplate reader. The ratio between the aggregate and monomer fluorescence (JC-1 ratio) was calculated which decreases upon $\Delta\psi$ M depolarization, and then we calculated the fold change relative to the negative control. Both JC-1 staining and final measurement were performed in the presence of test treatment. Therefore, we spiked both staining solution and dilution buffer with the corresponding concentration of extract. As a positive control, we treated the cells for 2 h with the ionophore uncoupler FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, 50 µM), which decreased the JC-1 ratio to ~38% of the control (DMSO 0.1%) levels.

5.4 Statistical analysis

All statistical parameters will be calculated using GraphPad Prism software. Paired Student's t test (two-tailed) and one-way ANOVA will be used to analyze data where appropriate. A probability level of $p < 0.05$ is considered statistically significant.

6 Results

6.1 Phytochemical analysis

6.1.1 Total phenolic and total flavonoid contents

Leaves (L) provides the highest phenolic content 0.136 mg/ml GAE and flavonoid content 0.33 mg/ml QE (Table 1).

6.2 Spectroscopic analysis

6.2.1 Fourier transform infrared (FT-IR) analysis

FT-IR analysis was performed to determine the functional groups of the alcoholic extracts of RO, Rh, and L. The groups were identified by comparing the peak values with previous reports and the value in the region of IR spectrum (Figs. 1, 2, 3, Table 2). The results of FT-IR analysis represent a difference in the functional groups between the three *Enhalus acoroides* (L.f.) Royle organs that may explain their difference in the biological activity.

Table 1 Total phenolic and total flavonoid content RO, Rh and L crude alcoholic extract

	RO	Rh	L
Total phenolic content (mg/ml GAE)	0.117	ND	0.136
Total flavonoid content(mg/ml QE)	0.155	ND	0.333
Antioxidant activity	76%	66%	70%
Scavenger % IC50	0.013 mg		0.05 mg

ND: not detected

Primary amine, carboxylic acid, alkenes, alcohol, and phenol were present only in root (RO), while the rhizome (RH) is characterized by the presence of alkane, aromatic ether, ether (indicates the presence of cellulose and hemicellulose), alkyl aryl ester, and lipids. Moreover, leaves extract (L) spectral data have a similar profile of RO and RH except for abundance of amine and aromatic amine in leaves that revealed presence nitrogenous compounds. The three extracts showed some similar bands representative for polyphenols, C=O stretching vibration in carbonyl compounds revealed a high flavonoid content.

The sharp peak signals in L and RH at $2850\text{--}2924\text{ cm}^{-1}$ correspond to symmetric and asymmetric CH sp^3 stretching, respectively, and are the characteristic signals of polysaccharides, lipids, and carbohydrates. The signal at 2920 cm^{-1} contains a significant overlapping with methylene scissoring vibration; thus, it may be also attributed methoxy compounds [26], while peak at

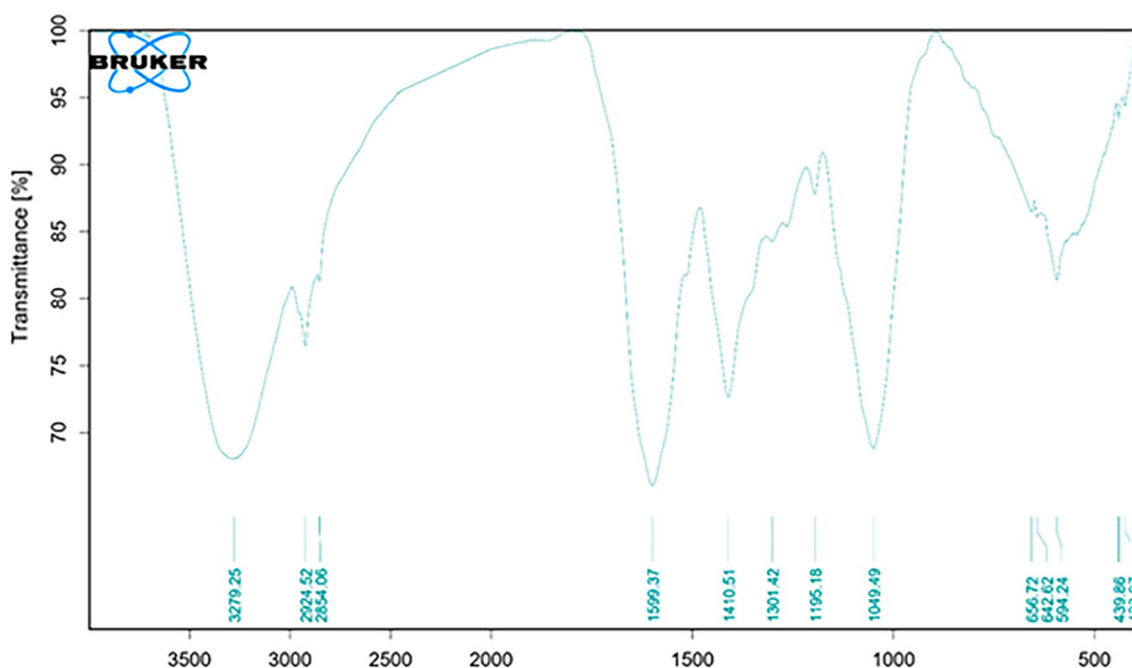
2850 cm^{-1} is considered free of such overlapping; thus, it contributes to CH symmetric stretching vibrations [27, 28]

6.2.2 HPLC analysis of phenolic compounds

HPLC analysis against authentic phenolic acids and flavonoids (Table 3) reveals the abundance of proto-catechuic and p-hydroxybenzoic, cinnamic acid, and rutin in the three organs with highest concentration in leaves (L) and the least in root (RO). The total concentration of phenolic compounds detected by HPLC was leaves > rhizome > root, 3037.64, 547.092, and 122.017 $\mu\text{g/ml}$, respectively. HPLC results support the total phenolic and total flavonoid content as determined by colorimetric analysis, with L exhibiting the highest concentrations of 0.136 mg/ml GAE and 0.333 mg/ml QE, respectively (Additional file 1).

6.2.3 UPLC/ESI/TOF-MS

Metabolite profiling of the L alcoholic extract was performed using UPLC-ESI-TOF-MS. The analysis revealed the detection of both primary metabolites (vitamins, sugars, amino acids, and peptides) and secondary metabolites (polyphenols, terpenes, and alkaloids). The results indicated that the leaves (L) alcoholic extract shows the highest phenolic compound content in comparison with RO and RH. Table 4 represents the phenolic compounds detected in the L extract, flavonoids, and phenolic acids.

**Fig. 1** FT-IR spectrum of crude methanolic extract of *Enhalus acoroides* (L.f.) Royle leaves

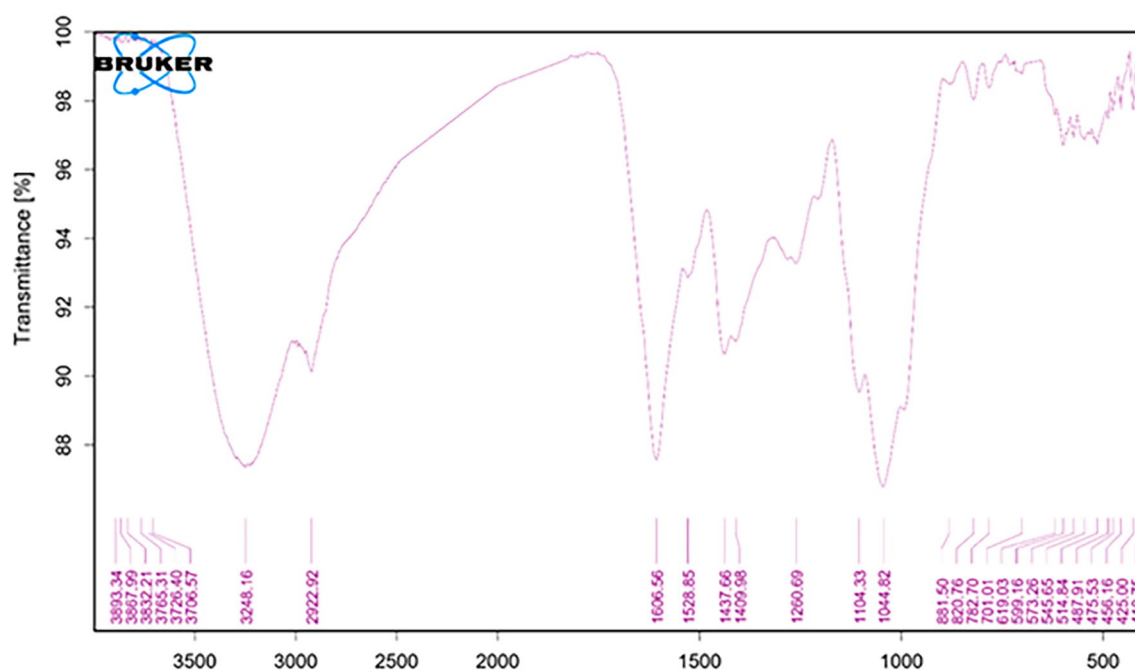


Fig. 2 FT-IR spectrum of crude methanolic extract of *Enhalus acoroides* (L.f) Royle rhizome

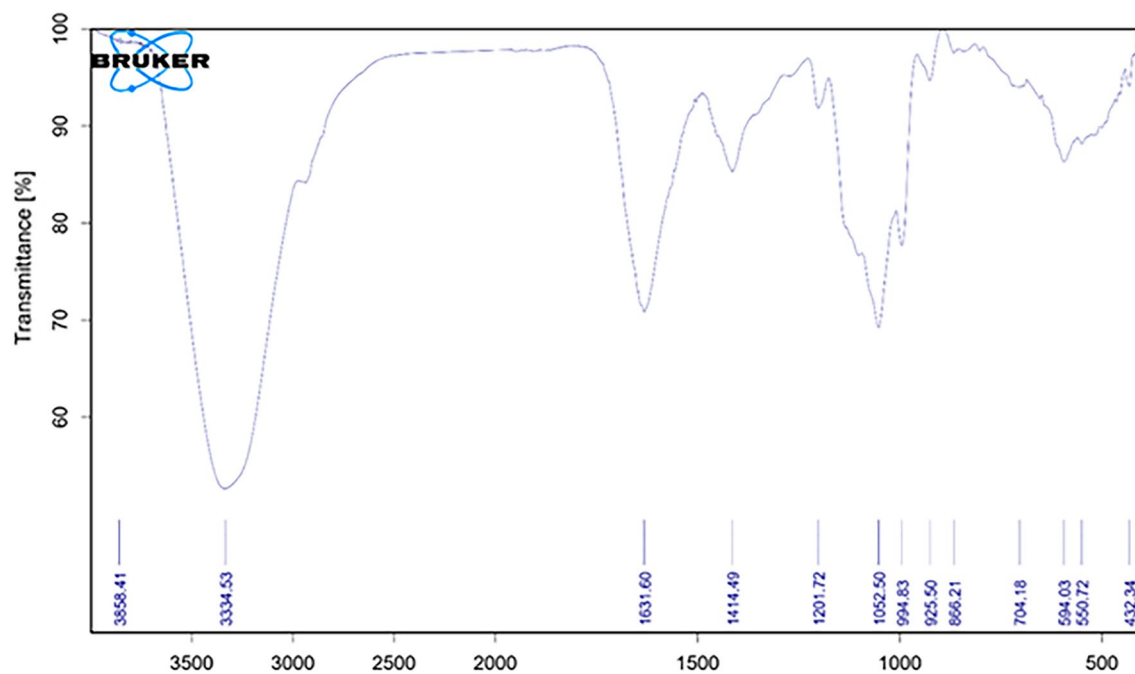


Fig. 3 FT-IR spectrum of crude methanolic extract of *Enhalus acoroides* (L.f) Royle root

Moreover, catalpol iridoids and iridoid glycoside derivatives were detected but not fully identified. Catalpol derivatives can be identified using UPLC-ESI-TOF-MS by their characteristic neutral losses [37]. Catalpol derivatives' characteristic neutral loss is as follows M-182 Da

(aglycone), 162 Da (glucose), 114 Da (two aldehyde groups and cleavage of the rings of the basic skeleton), 68 Da (C₄H₄O), 44 Da (carboxyl unit), and 28 Da (carbonyl unit). Secoiridoids also were detected with

Table 2 FT-IR spectral assignment and functional groups of R, Rh, and L crude alcoholic extract

R	Rh	L		Functional group	References
490–620			C–Br, C–I	Halogen compounds	[29]
–	610–690		C–H wagging vibration	w, Vinyl hydrocarbon compounds	[30]
700–782		–	Deformation out-of-plane vibration of the OH groups	w, Benzene derivative	[29]
–	820.76	–	C–C skeletal vibration	w, Alkane	[30]
866.21	–	–	R–NH ₂	W, primary amine	[26]
–	881.50	–	C–C skeletal vibration	W, Alkane	
925.50	–	–	OH bending	W,s, carboxylic acid	[31]
994.83	–	–	C≡C bending	m,s, Alkene	
–	1044.82	1049.49	C–O–C symmetric stretch	St.br, aromatic ethers	[29]
1052.50	–	–	CH ₂ and CH–O–H	m,s Aromatic alcohols	[26]
–	1104.33	–	C–O–C stretching vibration of the	s, Ether group cellulose and hemicellulose	[29]
–	–	1195.18	C–N stretch	w, amine	
1201.72	–	–		Phenol hydroxyl	[32]
–	1260.69	–	Ar–O	Alky and aryl ester	[26]
–	–	1301.42	C–N stretch	w, aromatic amine	[33]
1414.49	1409.98	1410.51	C–H in-plane bending coupled with C–C stretching in ring	m, s—Secondary alcohol-free	[30, 34]
–	1437.66	–	–C–H deformation vibration	m, R–CH ₃ ; Alkane	[30]
–	1528.85	–	N–H deformation vibrations	W, Secondary amine	[30]
–	1606.56	1599.37	C≡C conjugated with C=O	S, cyclic alkene, unsaturated ketone	[30]
1631.6	–	–	N–H	M, primary amide	[26]
–	–	2854.06	symmetric stretching vibrations of CH ₂	m, lipid	[28, 35]
–	2922.92	2924.52	Asymmetric stretching vibrations of CH ₂	st,s, lipid or alkanes	[28, 30, 35]
–	3248.16	–	N–H stretching vibration	st, br., proteins and terpenoids	[36]
3334.53	–	3279.25	bonded (–OH) hydroxyl stretching	st, br., Phenol	[36]

* m = medium, st = strong, br. = broad, w = weak, s = sharp

Table 3 Phenolic compounds (μg/ml) detected in RO, Rh and L crude extract using HPLC spectral analysis

Authentic samples	Root	Rhizome	Leaves
<i>Phenolic acids</i>			
<i>Hydroxybenzoic acids</i>			
Gallic acid	31.963	ND	ND
Protocatechuic acid	20.533	105.815	354.789
p-hydroxybenzoic acid	2.809	11.372	1482.069
Vanillic acid	ND	ND	52.405
<i>Hydroxycinnamic acids</i>			
Caffeic acid	ND	ND	47.676
Syringic acid	16.782	ND	ND
p-coumaric acid	6.830	ND	300.830
Cinnamic acid	16.983	68.241	326.846
<i>Flavonoids</i>			
Rutin	26.110	236.460	473.032
Catechin	ND	125.204	ND
Total phenolics detected (μg/ml)	122.01	547.092	3037.647

ND: not detected

their characteristic neutral loss -136 Da (ester linkage cleavage).

6.3 Biological activity

6.3.1 Antioxidant activity

The leaves alcoholic extract exhibits the highest antioxidant activity followed by root then rhizome with scavenger % 76, 70, 66 and IC₅₀, 0.013, 0.055 and ND (not done), respectively.

6.3.2 Antiproliferative activity

Cytotoxicity assay was performed using neutral red assay to evaluate the inhibitory effect of three different *Enhalus acoroides* (L.f.) Royle organs (leaves (L), roots (RO), and rhizomes (RH)) against human adenocarcinoma breast cancer cell line MCF-7, MDA-MB-231, and liver cancer cell line HepG-2 at different concentrations (31.25–1000 μg/mL) for 48 h. The results showed that leaves had a concentration-dependent inhibitory effect on the

Table 4 UPLC-ESI-TOF–MS identified compounds of *Enhalus acoroides* (L.f.) Royle leaves

Compound	<i>m/z</i> (<i>M</i> + 1)	Chemical formula	Chemical group
Diosmin	609.1814	C ₂₈ H ₃₂ O ₁₅	Flavonoid-7-O-glycosides
3-(4-Hydroxy-3,5-dimethoxyphenyl)-2-propenoic acid	225.07574	C ₁₁ H ₁₂ O ₅	Hydroxycinnamic acids
1-O-β-D-glucopyranosyl sinapate	387.12857	C ₁₇ H ₂₂ O ₁₀	Hydroxycinnamic acid glycosides
3,5,7-Trihydroxy-4'-methoxyflavone	301.07068	C ₁₆ H ₁₂ O ₆	Flavonols
3,3',4',5'-Tetrahydroxy-7-methoxyflavone	317.06558	C ₁₆ H ₁₂ O ₇	Flavonols
Myricetin	319.04483	C ₁₅ H ₁₀ O ₈	Flavonols
Naringenin	273.07574	C ₁₅ H ₁₂ O ₅	Flavanones
Kaempferol-3-Glucuronide	463.0871	C ₂₁ H ₁₈ O ₁₂	Flavonoid-3-O-glucuronides
Resveratrol	229.08592	C ₁₄ H ₁₂ O ₃	Stilbenes
Cyanidin-3-glucoside	449.1073	C ₂₁ H ₂₁ O ₁₁	Anthocyanidin-3-O-glycosides
Benzaldehyde	107.0842	C ₇ H ₆ O	Benzoyl derivatives
Caffeic acid derivative	293.1813	Unidentified structure	

cell viability of three cell lines: MCF-7, MDA-MB-231, and HepG-2 (Fig. 4). RO and RH, on the other hand, did not cause significant cytotoxicity in all cell lines. The IC₅₀ values (the half maximal inhibitory concentration) were determined for leaves and doxorubicin as a positive control (data not shown). *Enhalus acoroides* (L.f.) Royle (L) exhibited a significant cytotoxic activity on HepG-2 with IC₅₀ value of 883.61 µg/mL, and the inhibition percentages of cells viability reached up to 42.26% at 1000 µg/mL. On the other hand, (L) inhibited the cell proliferation in MCF-7 up to 73.87%. IC₅₀ value of (L) was determined (628.80 µg/ml) after 48 h. Furthermore, on MDA-MB-231 the percentage of cell inhibition was 68.59% at 1000 µg/ml. The results demonstrated that L exhibited the highest cytotoxic activity among the *Enhalus acoroides* (L.f.) Royle organs extracts (RO, RH). The results indicated that L, RO, and RH were safe on normal cell line HSF with no cytotoxicity.

6.4 Mitochondrial membrane potential

Mitochondrial membrane potential was assessed by JC-1 mitochondrial membrane potential assay kit by treatment of MCF-7 cell line by different concentrations of *Enhalus acoroides* L, R, and RH (0, 62.5, 125, 250, 500, and 1000 µg/mL). The result demonstrated that there was a significant drop in mitochondrial membrane potential (ΔψM) in the treated MCF-7 cells with *Enhalus acoroides* L in dose-dependent manner. On the other hand, both R and RH did not show any significant effect in the induction of mitochondrial membrane potential depletion in treated cells. The mitochondrial membrane potential (ΔψM) is an important indicator of mitochondrial-dependent apoptosis which can be quantified by applying the fluorescent dye JC-1. Treatment of the cells with *Enhalus acoroides* L for 48 h induced a remarkable reduction

in mitochondrial membrane potential (depolarization), expressed as the reductions in JC-1 590/530-nm fluorescence ratios (in control versus in cells treated with the extract (Fig. 5)). We formed the ratio between the aggregate and monomer fluorescence (JC-1 ratio), which decreases upon ΔψM depolarization, and calculated the fold change relative to the negative control (untreated).

7 Discussion

In our study, the total soluble phenolic content was measured using Folin–Ciocalteu method [38]. The results showed that leaves (L) possess the highest phenolic content 0.136 mg/ml GAE and flavonoid content 0.33 mg/ml QE. Furthermore, FT-IR analysis was performed to determine the functional groups of the alcoholic extracts of RO, Rh, and L. The results of FT-IR analysis represent a difference in the functional groups between the three *Enhalus acoroides* (L.f.) Royle organs clarify the difference in their biological activity [39]. Additionally, HPLC data support the total phenolic and total flavonoid content in which L shows the highest concentrations of GAE and QE, respectively [40]. Metabolite profiling of the L alcoholic extract was performed using UPLC-ESI-TOF–MS [41]. The analysis revealed the detection of both primary metabolites (vitamins, sugars, amino acids, and peptides) and secondary metabolites (polyphenols, terpenes, and alkaloids). As aforementioned, our study is concerned with phenolic compounds and their action as antiproliferative candidate. Our findings emphasize the effect of the phenolic compounds on the antiproliferative activity [42]. The leaves (L) alcoholic extract shows the highest phenolic compound content and consequently exhibited higher antioxidant and antiproliferative activities in comparison with RO and RH. Iridoids were detected that show no cytotoxicity on normal cell

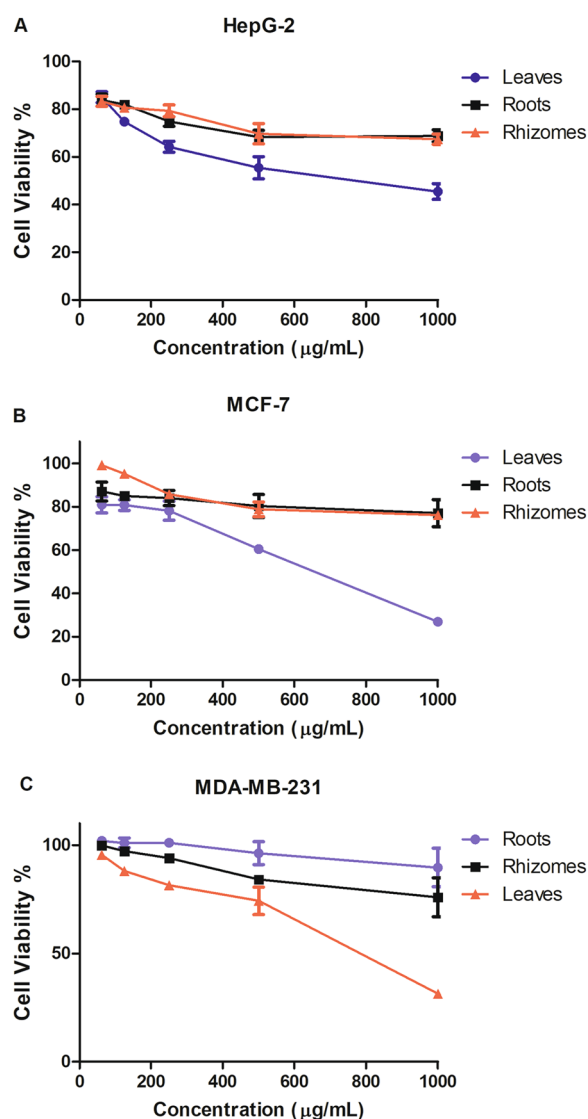


Fig. 4 Cytotoxic effect of *Enhalus acoroides* L, R, and RH on (A) HepG-2 (B) MCF-7, and (C) MDA-MB-231 for 48 h at concentration (31.25–1000 µg/mL). Data are expressed as a mean $\pm$ SD of three identical experiments made in three replicates

line with observed selectivity on different cancer cells. They show anticancer activity against breast cancer cell lines: MCF7 and MDA-MB-231 through a decrease in the mitochondrial function and an increased number of dead cells [43]. Secoiridoids also were detected with its characteristic neutral loss -136 Da (ester linkage cleavage). Medina et al. [44] have stated that phenolic extracts with high content of secoiridoids are more effective against breast cancer cells compared to extract with low and/or null content. According to the literature, this is the first time iridoids have been found in seagrass. The

leaves alcoholic extract exhibits the highest antioxidant activity. Our finding agrees with previous finding of [45] that stated a moderate antioxidant activity of the leaves extract. This result aligns with the HPLC findings that L contains the highest total phenolic and total flavonoid concentration 0.136 mg/ml GAE, 0.333 mg/mL QE, respectively. Furthermore, the scavenger activity of L antioxidant is 70%. Free radicals, ROS production cause oxidative stress which elaborates the development of inflammation processes leading to many degenerative diseases mainly cancer [46]. The antiproliferative assay was performed by neutral red assay. The data show that leaves exhibit significant and higher cytotoxicity activity over other *Enhalus acoroides* (L.f.) Royle organs extract (RO, RH). The results indicated that L, RO, and RH were safe on normal cell line HSF with no cytotoxicity. Therefore, these extracts were able to discriminate between cancerous and normal cell lines. Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed after treatment of MCF-7 with L, R, and RH seagrasses extracts. MCF-7 cell lines showed the highest cell proliferation inhibition among the treated cell lines HepG2 and MDA-MB-231. The results indicated the depletion in mitochondrial membrane potential in leaves treated cells. This disruption of $\Delta\Psi_m$ probably initiated the apoptotic cascade in the treated cells [43]. This result aligns with the capacity of *Enhalus acoroides* L only to induce antiproliferative effect on the treated cells and presence of Iridoids mainly in leaves extract [40]. On contrary, Widiastuti et al. [47] reported the cytotoxicity of *Enhalus acoroides* (L.f.) Royle alcoholic extract in HeLa cervical cancer cells and stated its disability as an anticancer nutraceutical, while Orno and Rantesalu [48] stated its great bioavailability and reported that the leaves show the least cytotoxicity compared to root and rhizome that is coincide with our finding. Thus it is a great demand to deeply fractionate and isolate bioactive compounds from the leaves extract of *Enhalus acoroides* (L.f.) Royle and study its anticancer mechanism of action (our laboratory is conducting additional research).

8 Conclusions

Enhalus acoroides (L.f.) Royle, L, RO, and RH contain varying concentrations of phenolic compounds. Leaves exhibited the highest phenolic and flavonoid content, 136 mg/ml GAE and 0.333 mg/ml QE, respectively, confirmed by HPLC analysis, which reveals the abundance of protocatechuic acid, p-hydroxybenzoic acid, cinnamic acid, and rutin in the three organs, with the highest concentration in leaves (L) and the least in roots. Flavonoids and phenolic acids, in addition to catalpol iridoids and

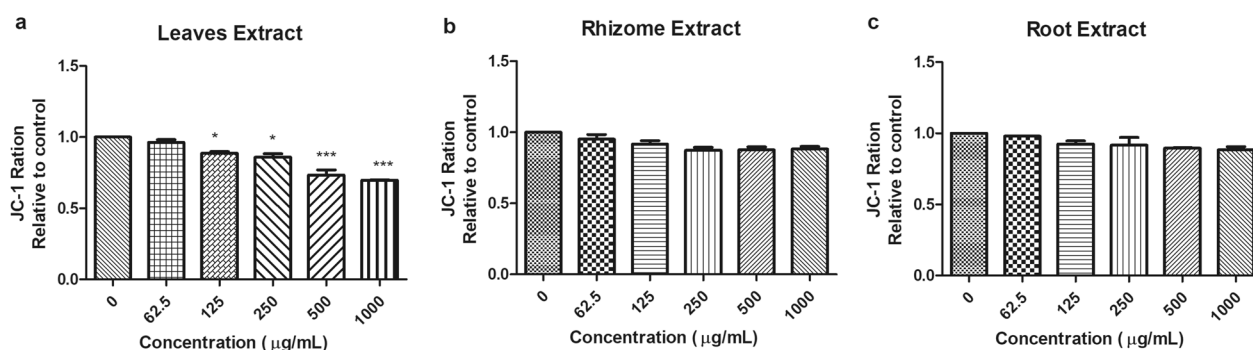


Fig. 5 Effect of *Enhalus acoroides* **a** L, **b** RH, and **c** R on mitochondrial membrane potential of MCF-7. Mitochondrial membrane potential ($\Delta\psi_m$) was measured by JC-1 fluorescence [fluorescence of JC-1 monomers (em 535 nm)/aggregates (em 590 nm)] in MCF-7 cells treated with (0, 62.5, 125, 250, 500, and 1000 $\mu\text{g/mL}$) for 48 h. *Enhalus acoroides* L showed significant decrease in $\Delta\psi_m$ dose-dependently. Data are expressed as mean $\pm$ SD. *** $P < 0.001$, * $P < 0.05$

iridoid glycoside derivatives, were detected in the alcoholic crude extract of leaves using UPLC analysis. The three organs, L, RO, and RH, exhibit antioxidant activity and were safe on the normal cell line HSF with no cytotoxicity. Leaves were the most potent antioxidant and showed a concentration-dependent inhibitory effect on the cell viability of three cell lines: MCF-7, MDA-MB-231, and HepG-2, while RO and RH were insignificant. Additionally, mitochondrial membrane potential was markedly decreased in MCF-7-treated cells with L extract. Thus, phenolic content directly affects the anti-cancer and antioxidant activities. *Enhalus acoroides* (L.f.) Royle leaves are a new nutraceutical candidate. They can be developed as a potential chemotherapeutic agent for the treatment of a variety of human cancers. Nevertheless, more cell-based studies with different cancer cell lines, in addition to in vivo studies of the seagrass on various animal models, are required prior to human studies.

Abbreviations

HPLC	High-performance liquid chromatography
FT-IR	Fourier transform infrared analysis
UPLC-ESI-TOF-MS	Ultra-performance liquid chromatography (UPLC) in combination with time-of-flight (TOF) mass spectrometry (MS)
L	Leaves
RO	Root
RH	Rhizomes
WGNP	Wadi El Gemal National Park
EEAA	Egyptian Environmental Affairs Agency
DPPH	2,2-Diphenyl-1-picrylhydrazyl
IC50	50% Inhibitory concentration
GAE	Gallic acid equivalent
QE	Quercetin equivalent
HepG2	Hepatocellular carcinoma
MCF-7	Human breast cancer cell line
MDA-MB-231	Human breast cancer
HeLa	Cervical cancer cells
$\Delta\psi_m$	Mitochondrial membrane potential
ATCC	American Type Culture Collection
DMEM	Dulbecco's modified Eagle's medium
SD	Standard deviation

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43088-023-00376-7>.

Additional file 1. Figure S1: HPLC chromatogram of *Enhalus acoroides* (L.f.) Royle leaves. **Figure S2:** HPLC chromatogram of *Enhalus acoroides* (L.f.) Royle rhizome. **Figure S3:** HPLC chromatogram of *Enhalus acoroides* (L.f.) Royle root. **Figure S4:** HPLC chromatogram of standard samples for: (1) Gallic acid (RT 4.2 min), (2) Protocatechuic acid (RT 6.7 min) (3) p-hydroxybenzoic acid (RT 10 min) (4) Syringic acid (RT 14.7), (5) Vanillic acid (RT 16.5 min), (6) ferulic acid (RT 20 min), (7) p-coumaric acid (RT 26.3), (7) Cinnamic acid (RT 33.6 min) and flavonoids mainly, (1) Catechin (RT 11.8), (3) Quercetin (RT 36.1 min), (4) Kampeferol at (RT 39.9 min).

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Author contributions

WSAM conducted the phytochemical analysis and analyzed the data, and wrote the draft, SIAM conducted the cytotoxic assays, analyzed the data, and wrote the draft. AEIS helped in collection of Seagrass and revised the final form. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Ethics approval and consent to participate

All procedures were conducted in accordance with the guidelines: <https://portals.iucn.org/library/sites/library/files/documents/2011-057-2nd%20ed.pdf>. Permissions and licenses were obtained by Dr. Amgad El Shaffai (authorized officially to conduct the research and collect samples).

Consent for publication

Not applicable.

Competing interests

There is no conflict of interest.

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