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Comparative study of *Posidonia oceanica* L.: LC/ESI/MS analysis, cytotoxic activity and chemosystematic significance

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Abstract

Posidonia oceanica L., one of nine *Posidonia* species belongs to family Posidoniaceae, is completely restricted to the White Mediterranean Sea. The chemical components of the aqueous methanol extracts of *P. oceanica* leaves and balls were analyzed using LC-ESI-MS technique for the detection and identification of its genuine constituents. Six phenolic acids, six flavonoids, two chalcones, two hydroxyl benzenes, three fatty acid derivatives, one anthocyanin and one sterol were identified or tentatively characterized. Also, the cytotoxic activity of the two extracts were investigated against epidermal carcinoma cell lines of larynx (Hep2), colon (HCT116), liver (HepG2) and breast (MCF7). The leaves extract exhibited obviously higher antiproliferative activity against HepG2 cell line with IC_{50} 17 µg/ml and a moderate activity against Hep2 and HCT116 with IC_{50} 28.3 and 27.8 (µg/ml), respectively while the balls extract showed moderate activity against HepG2, MCF7 and HCT116 with IC_{50} 24.3, 22.6 and 22.5 (µg/ml), respectively. The antiviral activity of the two extracts was also investigated against H5N1 virus, the balls extract showed a moderate % inhibition (45%) and no activity observed for the leaves extract. Furthermore, the chemosystematic significance was characterized to compare between *P. oceanica* and other seagrass species.

1. Introduction

Posidoniaceae Hutch. is a marine family belongs to order Alismatales of monocot. The genus *Posidonia* K.D.Koenig is a monotypic genus of family Posidoniaceae that includes the common Mediterranean *Posidonia oceanica* L. along with nine species widely distributed in the south coast of Australia [1-3]. *P. oceanica* has the ability to grow with a depth reaching 45 m in the clean water and is used as indicator for good sea water quality due to its high sensitivity to marine pollution [4]. *P. oceanica* plays an important role in the cycling of a wide set of trace elements which are not necessarily toxic but many anthropogenic activities increases their natural concentrations causing pollution [5]. While, Neptune balls (Neptune grass) are little balls of seaweed that come from the *P. oceanica* plant, these little balls are usually found with lots of sand inside and clumped together and didn't have much use, but recently it was found that these balls can be converted into high-quality building insulation that is environmentally friendly too [6]. Various studies were carried out on the chemical constituents of *P. oceanica*. It contains primary metabolites such as amino acids [7,8], carbohydrates [9], metallothioneins [10] and fatty acids [11]. The plant also contains numerous classes of secondary metabolites including proanthocyanidins [12], sterols [13], phenolic acids [14-18], chalcones and flavonols [19]. The majority of these studies did not clarify the genuine metabolites for *P. oceanica*. They always detectable after either hydrolysis or saponification of the plant extract [19]. Consequently, the plant might be a source of compounds to be investigated for many activities such as anti-HIV, antioxidant, antibacterial, antitumor and immunostimulant [20].

However, most of secondary metabolites reported from *P. oceanica* were obviously only artifacts [3,19], more investigation should be required for the isolation and structure elucidation of the unknown genuine secondary metabolites of *P. oceanica*. The present study interested to characterize its true natural metabolites using LC-ESI-MS technique for the detection and identification of these genuine constituents, aiming to compare the

chemical constituents between the leaves and the Neptune balls of *P. oceanica* and evaluate their chemosystematic relationships with other seagrass species. Additionally, the cytotoxic activity of the aqueous methanol extracts of leaves and balls against epidermal carcinoma cell lines of larynx (Hep2), colon (HCT116), liver (HepG2) and breast (MCF7) were investigated as well as the antiviral activity against H5N1 virus.

2. Material and Methods

2.1. Plant material

Leaves and Neptune balls of *P. oceanica* were collected from Mersa Matruh, Al-Obayed Beach on May 2016. The plant was authenticated by Dr. Mona M. Marzouk and Dr. Sameh R. Hussein. Voucher specimen was deposited in the herbarium of National Research Centre.

2.2. Extraction

Leaves and Neptune balls of *P. oceanica* were air dried and ground separately; the obtained powder (278.5g and 220 g) were extracted three times at room temperature with MeOH: H_2O, 7:3. The aqueous methanol extracts were evaporated under reduced pressure to obtain residues of (32.5 and 5.5 g), respectively.

2.3. LC-ESI-MS analysis of P. oceanica

LC-ESI-MS analysis system consists of HPLC (Waters Alliance 2695) and mass spectrometer (Waters 3100). The mobile phases were prepared daily by filtering through 0.45 µm membrane disc filter and degassed by sonication before use. The mobile phase for gradient elution consists of two solvents: solvent A (0.1% formic acid (FA) in H_2O) and solvent B (0.1% FA in CH_3CN/MeOH (1:1; *v/v*). The linear gradient profile was as follows: 95% A (5 min), 95-90% A (10 min), 90-50% A (55 min), 50-95% A (65 min), and 95% A (70 min). The injection volume was 10 µL. The flow rate (0.6 ml/min) was split 1:1 before the MS interface. The negative ion mode parameters were as follows: source temperature 150 °C, desolvation temperature 350 °C, cone gas flow 50 L/h, cone voltage 50 eV, capillary voltage 3 kV, and desolvation gas flow 600 L/h. Spectra were recorded in the ESI negative mode between 50-1000 *m/z*. The peaks and spectra were processed using the Maslynx 4.1 software as the method described by El-Wakil *et al*. [21] Known peaks were identified by comparing their retention time and mass spectra with the flavonoid standards (95% purity; UV, NMR) which obtained from our research group (phytochemical and plant systematic department, NRC). Other peaks were tentatively identified by comparing their mass spectra with those in the literatures.

2.4. Cell culture and in vitro anticancer activity

Human tumor cell lines; epidermal carcinoma of larynx (Hep2), colon (HCT116), liver (HepG2) and breast (MCF7) were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.). The cell lines were maintained at the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. The samples were prepared by dissolving Stock solution in dimethylsulfoxide (DMSO) at a concentration 100 mM and stored at -20 °C. The cytotoxic activity of the leaves and balls extracts of *P. oceanica* were carried out using Sulphorhodamine-B (SRB) assay following the method reported by Vichai and Kirtikara [22]. SRB is a bright pink aminoxanthrene dye with two sulphonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mild acidic conditions to provide a sensitive index of cellular protein content. Cells were seeded in 96-well microtiter plates at initial concentration 3×10^3 cell/well in a 150 µl fresh medium for 24 hrs before treatment with the extract to allow attachment of cells to the wall of the plate. Different concentrations of the extracts and pure compounds (0, 5, 12.5, 25 and 50 µg/ml) were added to the cell monolayer in triplicate. Monolayer cells were incubated with the compounds for 48 hrs at 37 °C and in an atmosphere of 5% CO_2. After 48 hrs, cells were fixed, washed and stained with SRB. The excess stain was washed with acetic acid and attached stain was recovered with *tris*-EDTA buffer. Colour intensity was measured at 570 nm with an ELISA reader. The relation between surviving fraction and drug concentration was plotted to obtain the survival curve of each tumor cell line as compared with Doxorubcin; the control anticancer drug.

2.5. Antiviral activity against H5N1 virus

2.5.1. Cells and virus

Madin-Darby Canine Kidney (MDCK) cells were maintained in the Center of Scientific Excellence for Influenza Viruses at the National Research Center. The cells were propagated till confluence in multiwell plates. The highly pathogenic avian influenza (HPAI) virus A/Chicken/Egypt/M7217B/2013 (H5N1) used in this study was isolated from the infected chickens in Egypt in 2013 and characterized at immunologic and molecular levels.

2.5.2. Preparation of extracts for bioassay

Stock solutions of the tested extracts and compounds were dissolved as 0.1 g in 1 ml of 10% DMSO in deionized water. The prepared extract solutions were used for both cytotoxicity and antiviral bioassays.

2.5.3. MTT cytotoxicity assay (TC50)

Samples were 10-fold serially diluted with Dulbecco's Modified Eagle's Medium (DMEM). Stock solutions of the test compounds were prepared in 10 % DMSO in ddH2O. The cytotoxic activity of the extracts were tested in Madin Darby Canine kidney (MDCK) cells by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method described by Salem *et al.* [23] with minor modification. The cells were seeded in 96 well-plates (100 µl/well at a density of 3×105 cells/ml) and incubated for 24 hrs at 37 ^{o}C in 5% CO_2. After 24 hrs, cells were treated with various concentrations of the tested compounds in triplicates. After further 24 hrs, the supernatant was discarded and cell monolayers were washed 3 times with sterile phosphate buffer saline (PBS). MTT solution (20 µl of 5 mg/ml stock solution) was added to each well and incubated at 37 °C for 4 hrs followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 µl of acidified isopropanol (0.04 M HCl in absolute isopropanol= 0.073 ml HCL in 50 ml isopropanol). Absorbance of formazan solutions were measured at λmax 540 nm with 620 nm as a reference wavelength using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined with the following equation: [% Cytotoxicity= (Absorbance of cell without treatment–Absorbance of cell with treatment)/Absorbance of cell without treatment × 100]. The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (LD50).

2.5.4. Plaque reduction assay

Anti-H5N1 activity of the leaves and balls of *P. oceanica* crude extracts were investigated by plaque reduction assay with confluent 24 hrs old monolayer of MDCK cells. Assay was carried out according to the method described by Hayden *et al.* [24] in a six-well plate where MDCK cells (10^5cells/ml) were cultivated for 24 hrs at 37 ^{o}C. A/CHICKEN/7217B/1/2013 (H5N1) virus was diluted to give 10^5 PFU/ well, mixed with the safe concentration of the tested compounds, and incubated for 30 minutes at 37 ^{o}C before being added to the cells. Growth medium was removed from the cell culture plates and virus-Cpd or virus-extract and Virus-oseltamivir mixtures were inoculated (100µl/well). After 1 hr contact time for virus adsorption, 3 ml of DMEM supplemented with 2% agarose containing the virus-Cpd or virus-extract and Virus-oseltamivir mixtures was added onto the cell monolayer, plates were left to solidify and incubated at 37 ^{o}C till formation of viral plaques (3 to 4 days). Formalin (10%) was added for two hours then plates were stained with 0.1% crystal violet in distilled water. Control wells were included where untreated virus was incubated with MDCK cells and finally plaques were counted and percentage reduction in plaques formation in comparison to control wells was recorded as following: % inhibition= viral count (untreated) - viral count (treated)/viral count (untreated)× 100 [23].

3. Results and discussion

3.1. Identification of chemical compounds in P. oceanica using LC-ESI-MS technique

The identification of chemical constituents using LC-ESI-MS analysis detected twenty one compounds in *P. oceanica;* eighteen in the leaves chromatogram and ten in the balls one. Seven common compounds (peaks **1**, **2**, **3**, **9-11** and **21**) were detected in the two parts of the plant (Table 1, Fig. 1). Compound **1** (*Rt*= 4. 26 min) showed a molecular ion peak $[M-H]^-$ at *m/z* 125 and a fragment of *m/z* 97 indicating the presence of a benzene ring and three hydyoxyl groups presented in both leaves and balls chromatograms. This compound was identified as a trihydroxybenzene derivative and suggested to be either 1,3,5-trihydroxybenzene (phloroglucinol) or 1,2,3-trihydroxybenzene (pyrogallol) which previously reported in the leaves of *P. oceanica* [16]. Compounds **2**, **3** and **9** showed the same molecular ion peak $[M-H]^-$ at *m/z* 137. Compounds **3** and **9** were identified as 2-hydroxybenzoic acid (salicylic acid) and 4-hydroxybenzoic acid (*p*-hydroxybenzoic acid), respectively; in comparison to the retention times and mass fragmentations of the authentic references, while the tentative identification of compound **2** indicated the possibility to be 3,4-dihydroxybenzaldehyde (protocatechuic aldehyde) which was previously detected in *P. oceanica* [15, 25]. Compounds **4** and **17** showed molecular ion peaks $[M-H]^-$ at *m/z* 147 and 463 which identified as cinnamic acid and quercetin 3-*O*-glucoside, respectively. The identification of these compounds was also proved by comparing their mass fragmentations and retention times with those of the authentic references. Compounds **3** (salicylic acid) and **17** (quercetin 3-*O*-glucoside) were identified for the first time from *P. oceanica*.

Compound **5** (*Rt*= 14.19 min) showed a molecular ion peak $[M-H]^-$ at *m/z* 401 and fragment at *m/z* 241, it was tentatively identified as campestanol which is a natural phytosterol detected before in the plant [13]. Compound

6 (*Rt*= 18.37 min) presented a molecular ion peak at *m/z* 579 and provided three fragments at *m/z* 447 [M-H-132]$^-$ (loss of pentose), *m/z* 433 [M-H-146]$^-$ (loss of rhamnose) and *m/z* 301 [M-H-278]$^-$ (loss of rhamnose + pentose). The Appearance of two fragments at *m/z* 447 and 433 indicating the glycosylation of two OH groups at different positions of quercetin aglycone [26]. Thus compound **6** was tentatively identified as quercetin-*O*-pentoside-*O*-rhamnoside. Compound **7** (*Rt* 19.8) showed a molecular ion peak [M-H]$^-$ at *m/z* 289 and a fragment at *m/z* 137 suggested to the tentative identification of galloyl benzoic acid. Compound **8** represent a type A procyanidin dimer (*Rt* 20.7), detected by a molecular ion [M–H]$^-$ at *m/z* 577 and fragments at *m/z* 451, 407 and 189 in the leaves of *P. oceanica* [27].

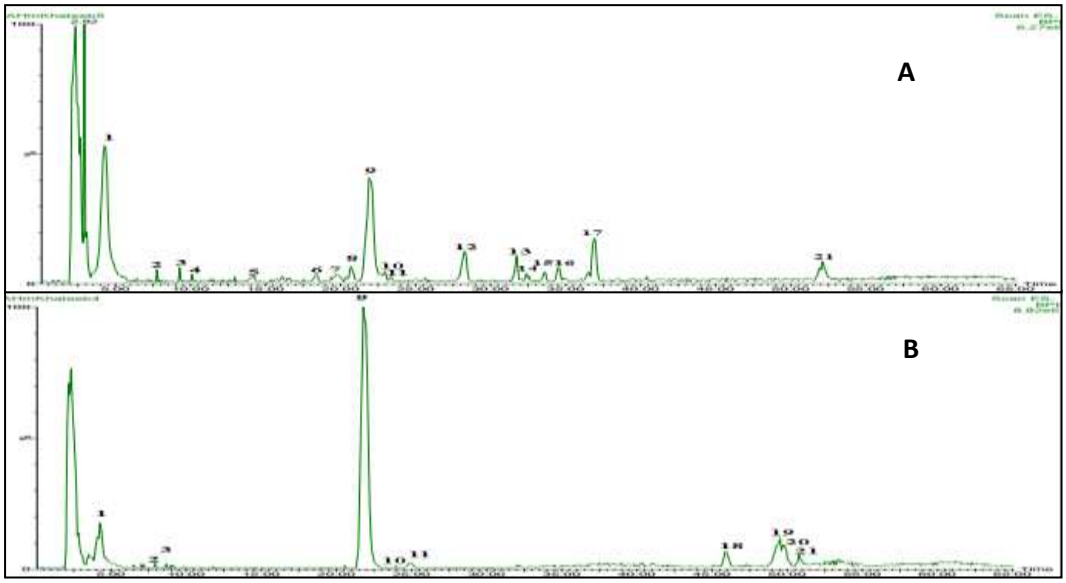

Figure 1: LC-ESI-MS chromatograms of chemical compounds of *P. oceanica*, A: leaves extract; B: Neptune balls extract.

Table 1: Tentative identification of chemical compounds in *P. oceanica* (leaves and balls) using LC-ESI-MS technique

Compds	*Rt* (min)	M	[M –H]$^-$	*m/z* fragments	A	B	Identification
1	4.26	126	125	97, 80, 62	+	+	Trihydroxy benzene
2	7.77	138	137	108, 91,79, 61	+	+	Protocatechuic aldehyde*
3	9.26	138	137	119, 92,62	+	+	Salicylic acid *[@]
4	10.10	148	147	136, 91,80	+	-	Cinnamic acid*
5	14.19	402	401	241	+	-	Campestanol †
6	18.37	580	579	447, 433, 301	+	-	Quercetin-*O*-pentoside -*O*-rhamnoside[@]
7	19.8	290	289	137, 108, 93, 62	+	-	Galloyl benzoic acid[@]
8	20.7	578	577	451, 407, 289, 245, 125	+	-	Procyanidin dimer†
9	21.79	138	137	108, 93, 62	+	+	*p*-Hydroxybenzoic acid*
10	22.04	168	167	153, 123,108, 91	+	+	Vanillic acid *
11	22.9	290	289	245, 221, 122	+	+	Catechin*
12	28.3	450	449	287, 269,179	+	-	Eriodictyol hexoside†[@]
13	31.8	578	577	285	+	-	Kaempferol-*O*-rhamnosyl rhamnoside[@]
14	32.4	288	287	269	+	-	Eriodictyol *[@]
15	33.6	642	641	273, 167	+	-	Phloretin sinapoylhexoside†[@]
16	34.5	612	611	273, 167	+	-	Phloretin feruloylhexoside†[@]
17	36.91	464	463	301	+	-	Quercetin 3-*O*-glucoside*[@]
18	45.9	330	329	329,229	-	+	Fatty acid derivatives†
19	49.4	330	329	311, 229,211, 171, 137	-	+	Linoleic acid derivatives†
20	49.8	330	329	311, 229,211, 171, 137	-	+	Linoleic acid derivatives†
21	52.1	474	473	326,147,97,97,62	+	+	Chicoric acid†

*Compounds identified by comparing their retention times and mass spectrum with the authentic, †Compounds tentatively identified based on the mass spectral data cited in the literature. [@] Compounds detected for the first time from *P. oceanica*. A: leaves extract; B: balls extract.

Compound **10** (*Rt* 22.04) showed a molecular ion peak [M-H]$^-$ at *m/z* 167 and gave fragment at *m/z* 153 and identified as vanillic acid [5,15, 28]. Compound **11** (*Rt* 22.9) showed a molecular ion peak [M–H]$^-$ at *m/z* 289 and identified as catechin by comparing its retention time and mass spectrum with the authentic sample. Catechin was identified before in the hydrophilic extract from *P. oceanica* [29]. Compound **12** (*Rt* 28.31)

showed a molecular ion peak $[M–H]^-$ at *m/z* 449 and a fragment at *m/z* 287 suggested to be identified as dihydroluteolin hexoside (eriodictyol hexoside) [30]. Compound **13** (*Rt* 31.8) showed a molecular ion peak $[M–H]^-$ at *m/z* 577 and gave a fragment at *m/z* 285, after loss of two rhamnose moieties. No fragment was observed at *m/z* 431, indicated that the two rhamnose units were connected to the same hydroxyl group of the aglycone. Thus compound **13** was identified as kaempferol -*O*-rhamnosyl rhamnoside. Compound **14** (*Rt* 32.4) showed a molecular ion peak $[M–H]^-$ at *m/z* 287 and identified as eriodictyol [30]. Compounds **15** and **16** are characterized as chalcones, these class of compounds were previously detected in the leaves of *P. oceanica* as phloretin and phloretin glucoside [15]. Compounds **15** (*Rt* 33.6) with a molecular ion $[M–H]^-$ at *m/z* 641 and **16** (*Rt* 34.5) with a molecular ion $[M–H]^-$ *m/z* 611, both yielding fragment at *m/z* 273 and identified as phloretin sinapoylhexoside and phloretin feruloylhexoside, respectively.

Compounds **18**, **19** and **20** showed the same molecular ion peak $[M–H]^-$ at *m/z* 329 which pointed to be the oxidation products of unsaturated fatty acids (oxylipins) [31]. Compounds **19** (*Rt* 45.9) and **20** (*Rt* 49.4) were suggested to be two derivatives of lineolic acids which characterized by the presence of a molecular ion at *m/z* 171 [OOC $(CH_2)_7$CH-OH]. Neutral loss of 100 amu (329-229; 311-211), corresponding to the loss of an end group HO-CH=CH$(CH_2)_3CH_3$ from the oxylipin molecule, thus both compounds were tentatively identified as lineolic acid derivatives. Compound **21** (*Rt* 52.1) showed a molecular ion peak $[M–H]^-$ at *m/z* 473 and was identified as chicoric acid which is one of the major compounds previously reported from the acetone leaves extract [20, 30].

3.2. Cytotoxic activity

The leaves extract of *P. oceanica* exhibited obviously higher antiproliferative activity against HepG2 cell line with IC50 17 µg/ml and moderate activity with IC50 28.3 and 27.8 (µg/ml) against Hep2 and HCT116, respectively, compared with the regular regimen for cancer treatment in which doxorubicin was used as a protocol even in a relatively low dose while MCF7 cell line showed no activity. Also, the balls extract revealed moderate activity against (HepG2, MCF7 and HCT116) cell lines with IC50 (24.3, 22.6 and 22.5 µg/ml) and no activity against Hep2 cell line (Figure 2). This activity may be due to the presence of *p*-hydroxybenzoic acid which is a major compound in *P. oceanica* and known as anti-inflammatory drug (NSAIDs). Our results are in harmony with Barletta *et al.* [29] who reported that hydrophilic extract from *P. oceanica* was able to strongly decrease gene and protein expression of gelatinases MMP-2 and MMP-9 and to directly inhibit in a dose-dependent manner gelatinolytic activity *in vitro*, the matrix metalloproteinases (MMP) activity represents one of the main mechanisms responsible for the process of invasion and metastasis of tumor cells [32, 33].

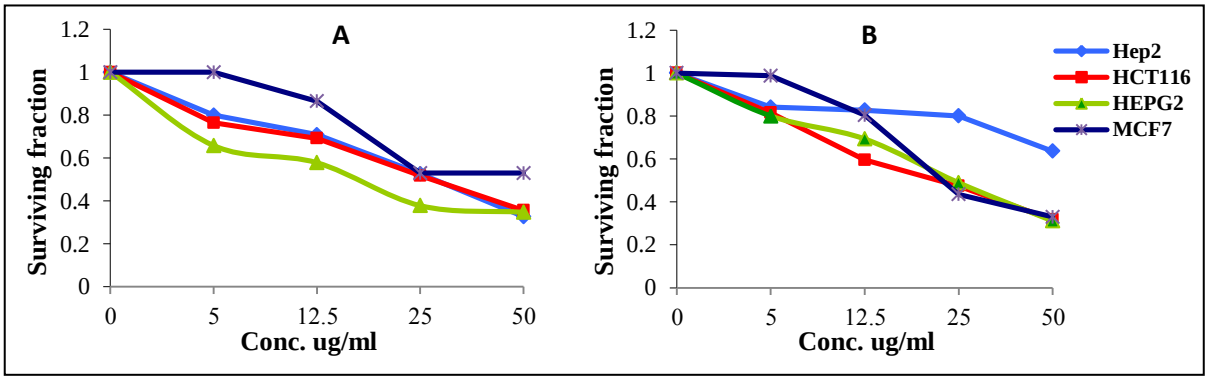

Figure 2: Cytotoxic activity of *P. oceanica* on different carcinoma lines. A: leaves extract; B: balls extract

3.3. Antiviral activity against H5N1 virus

Egypt has the highest incidence of human H5N1 virus infection, temporally associated with a high incidence of H5N1 virus infection in its poultry population. [34] The application of antiviral drugs during the early phase of a pandemic could be of help to control it [35]. Detection of new, potent and cheap anti-influenza agents from plant and marine extracts could be used for the treatment and prevention of influenza, particularly for H5N1 strain [36] as reported in this study. The cytotoxicity of the leaves and balls extracts of *P. oceanica* were determined using MTT assay. The TC50 was found to be 253.0 and 229 µg/µl, respectively, the concentration of 100µg/µl of the leaves extract showed weak inhibition against H5N1 virus about 4% while the Neptune balls extract showed a moderate % inhibition 45%. These results could be due to the high concentrations of *p*-hydroxybenzoic acid which acts previously as antiviral agent, where the ester derivatives of hydroxybenzoic

acid are reported to be widely used for treating infections caused by hepatitis B virus, human papilloma, herpes simplex virus, condyloma acuminate, cervicitis and cervical erosions in human and animals [37].

3.4. Chemosystematic significance

Seagrasses (marine higher plants) are a group of about 60 species belonging to 16 genera distributed all over the world. Only five families of higher plants; Hydrocharitaceae, Cymodoceaceae, Posidoniaceae, Ruppiaceae and Zosteraceae contain exclusively marine species growing in Egypt. The Hydrocharitaceae, that mainly comprise genera restricted to fresh water habitats, also include two marine genera (*Halophila* and *Thalassia*). The Cymodoceaceae represents a solely marine family and encompasses the highest variety of genera (*Cymodocea*, *Halodule* and *Thalassodendron*). Likewise, Posidoniaceae has a monogeneric genus (*Posidonia*), and the Ruppiaceae, also consisting of the one genus (*Ruppia*) as well as Zosteraceae containing the genus *Zostera* [38]. Among the various secondary metabolites, flavonoids have most widely and most effectively used in chemosystematics [39]. They were also frequent metabolites found in all seagrass species thus indicating the possibility that they could be useful as chemosystematic markers. Several structures of flavonoids have been studied in seagrasses, including flavones, flavonols [40] and chalcones [15]. The Hydrocharitaceae family is characterized by varieties of flavone derivatives. Flavone aglycones (luteolin and apigenin) and flavone glucuronides (luteolin 3´-*O*-glucuronide and luteolin 4´-*O*-glucuronide) were obtained from *Enhalus acoroides* [41], while the genus *Thalassia* is characterized by flavone 7-sulfato glycosides. Isoscutellarein 7-sulfatoxyloside has been reported from the Egyptian *Thalassia hemprichii* [42], while 7-sulfato glucoside of apigenin, luteolin and chrysoeriol were reported from the same species which collected from the coast of China [43,44]. Moreover, flavone 6´´-acyl glucosides were characterized for the genus *Halophila* [3,45,46]. Furthermore, all flavonoids isolated from *Zostera* species (Zosteraceae) are flavone 7-*O*-sulfate derivatives [12]. Also, the flavonoids reported for the genus *Thalassodendron* (Cymodoceaceae) are flavonol glycosides and dihydrochalcones [47, 48] To the best of our knowledge there are no reports on the flavonoid constituents from other genera of Cymodoceaceae family (*Cymodocea* and *Halodule*). The family Ruppiaceae is also characterized by flavonols and its glycoside derivatives [49]. Previous studies reported the flavonoids from Posidonaceae are only flavonols [3,19,50] but in the present study, the detected flavonoids were represented as flavonols, dihydroflavones and acylated chalcones. According to the Angiosperm Phylogeny group systems, the plants in the three families Cymodoceaceae, Posidoniaceae and Ruppiaceae form a monophyletic group. Some genera of these families are characterized by their ability to synthesis flavonols and their glycosides and also chalcones.

Conclusions

Total twenty one compounds were identified in *P. oceanica* (leaves and balls extracts) using LC-ESI-MS analysis, among them eight compounds were detected for the first time. The leaves extract exhibited a significance anticancer activity against HepG2 cell line with IC_{50} 17 µg/ml while the ball extract showed a moderate inhibition (45%) against H5N1 virus. Furthermore, the variability of the flavonoid pattern within different families of seagrasses suggested that flavone glucuronoids, flavone acyl glucosides, sulphated flavone, flavone sulphatoglycosides, chalcones and dihydrochalcones could be used as chemosystematic markers to discriminate among different genera of seagrasses. Also, more efforts should be required to have a complete view on the chemosystematics study of family Cymodoceaceae (genera *Cymodocea* and *Halodule*).

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