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by

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Abstract

Posidonia oceanica (L.) Delile is an endemic Mediterranean sea-grass distributed in the infralittoral zones, where it forms meadows playing a recognized ecological role in the coastal marine habitat. Although its use as a traditional herbal remedy is poorly documented, recent literature reports interesting pharmacological activities as antidiabetic, antioxidant and vasoprotective. Differently from previous literature, this study presents a hydrophilic extraction method that recovers metabolites that may be tested in biological buffers. We showed for the first time in the highly invasive HT1080 human fibrosarcoma cell line that our 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*. Moreover, we have revealed that our extract strongly inhibited HT1080 cell migration and invasion. Biochemical analysis of the hydrophilic extract showed that catechins were the major constituents with minor contribution of gallic acid, ferulic acid and chlorogenic plus a fraction of uncharacterized phenols. However, if each individual compound was tested independently, none by itself was able to induce a direct inhibition of gelatinases as strong as that observed in total extract, opening up new routes to the identification of novel compounds. These results indicate that our hydrophilic extract from *P. oceanica*might be a source of new pharmacological natural products for treatment or prevention of several diseases related to an altered MMP-2 and MMP-9 expression.

Keywords: cell migration and invasion, gelatinase, hydrophilic extract, MMP-2, MMP-9, Polyphenols, *Posidonia oceanica*.

Introduction

Degradation of the extracellular matrix by specific proteases is involved in numerous physiological processes such as cell proliferation, cell adhesion and migration, angiogenesis, bone development, wound healing.[1,2] Among the different proteases responsible for the degradation of the extracellular matrix *in vivo*, an important role is played by the matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases.[3,4] In particular, MMP-2, also known as gelatinase A, and MMP-9, also known as gelatinase B, degrade type IV collagen, one of the extracellular matrix component of basement membrane, or denatured collagen (gelatin), and therefore these MMPs play a pivotal role in cell invasion and migration.

An increased expression and activity of MMPs has been reported in various pathological processes such as inflammation and vascular diseases.[5,6] Moreover, MMP activity represents one of the main mechanisms responsible for the process of invasion and metastasis of tumor cells.[7,8]

In the last few years, several studies have shown that polyphenolic compounds of dietary origin are able to play an inhibitory role on the process of activation of MMPs.[9,10] Polyphenols are one of the most common classes of secondary metabolites in terrestrial and marine plants. Since these compounds have low toxicity, in recent years growing interest has been focused on evaluating their role as chemopreventive agents in cancer. Indeed, polyphenols are able to inhibit gene and protein expression as well as activity of gelatinases in various malignancies.[9-14]

The angiosperm *Posidonia oceanica* (PO) is a sea-grass belonging to the Posidoniaceae family. Despite its name, it is endemic in the Mediterranean sea and its dense underwater meadows cover tens of thousands square kilometres. It is considered as fundamental for the Mediterranean environment and an important ecological indicator.[15-17] Studies addressing PO as a bioremediator agent have been drawn since long time.[18]

Although PO is widely distributed, its role as a traditional herbal remedy is poorly documented, except for the benefit of the decoction of the leaves of PO against diabetes mellitus and hypertension used by the villagers who live near the west coast of Anatolia.[19] The folk medicine reports that Egyptians attributed to the seagrass curative properties, especially for sore throats and skin problems, and PO has also been reported in the popular pharmacopoeia by an old botanical handbook of Cazzuola.[20,21]

PO has emerged as an important reservoir of bioactive compounds with important antidiabetic, antioxidant, vasoprotective, antibacterial and antifungine actions.[19,22,23] PO has been shown to contain partially known phenolic mixtures composed of flavonoids and condensed tannins such as proanthocyanidins as well as new compounds (e.g. a novel sesquiterpene).[24-28] A comparison of results from different extraction methods reported in literature is hardly feasible because of the different strategies used and because PO leaves were not collected from the same seasonal periods and ecological conditions (e.g., fresh and polluted waters). The main phenols found in PO are 4-

hydroxybenzoic acid, 4-coumaric acid, cinnamic acid, caffeic acid, ferulic acid, myricetin, quercetin, isorhamnetin, kaempferol, gentisic acid, chicoric acid and vanillin.[25,26,29]

Several polyphenols found in PO might be able to inhibit activity and expression of specific MMPs involved in cell invasion and migration.[30-33] Nevertheless, to the best of our knowledge, a possible activity of *Posidonia oceanica* compounds on cells with such aggressive phenotype has never been reported. Therefore, in the present study we aimed at investigating whether PO extracts might play a role in cell migration and cell invasiveness through inhibition of the MMP-2 and MMP-9. We optimized a water-ethanol extraction system that recovers hydrophilic compounds and we analyzed the effect of this hydrophilic extract on cell migration/invasion and on expression of MMP-2 and MMP-9 in the highly invasive HT1080 human fibrosarcoma cell line which constitutively expresses MMP-2 and MMP-9.

Results

Hydrophilic extraction recovers water-soluble compounds

Since we aimed at collecting substantially hydrophilic compounds from PO leaves in order to perform experiments in biological buffers, we optimized an extraction method based on 70% ethanol followed by hexan-based removal of hydrophobic compounds. This procedure allowed us a consistent extraction (0.13 g dry extract from 1 gr of dry leaves, a yield of 7,7%, see **Table 1**), and an easy and complete solubilization of the dried material in 20% ethanol.

Table 1. Biochemical characterization of POE. Values represent the mean and the standard deviation of at least 4 independent extractions. Abbreviations: TC, total carbohydrate; TP, total polyphenols; RS, radical scavenging activity

Biochemical characterization and antioxidant activity

	Method	Fresh POE mg/mL	1 week POE mg/mL	Reference compound
Dry extract	Weighting	43.0 ± 0.5	43.0 ± 0.5	–
TC	Phenol-sulfuric acid	33.4 ± 10.8	31.0 ± 9.7	D-glucose
TP	Folin-Ciocalteau	7.4 ± 0.4	7.3 ± 0.5	Gallic acid
Antioxidant	FerroZine™	8.03 ± 0.34	8.20 ± 0.41	Ascorbic acid
RS	DPPH	6.01 ± 0.55	5.80 ± 0.62	Ascorbic acid

As shown in **Table 1**, *Posidonia oceanica* extract (POE) was found to contain carbohydrates (33.4 ± 10.8 mg glucose/mL) and polyphenols (7.40 ± 0.40 mg gallic acid/mL). Since phenols and polyphenols are known to act as scavenger for free radicals and as antioxidant in general, we tested these POE activities with DPPH and FRAP assays, as reported in **Table 1**. POE showed a radical scavenging and antioxidant activity of 6.01 ± 0.55 mg/mL and to 8.03 ± 0.34 mg/mL ascorbic acid equivalents, respectively.

Natural products are known to have a limited stability during time. We tested our POE at 1 week after solubilisation and storage at 4°C. We verified that its composition and activities were very similar to that of fresh batches, demonstrating its stability (**Table 1**). However, in order to ensure the reproducibility of the experiments, all experiments were done with fresh POE.

Phenolic composition

We investigated the phenolic fraction of POE using UPLC and a battery of reference compounds (**Fig. 1**). We were able to identify and quantify about 88% of POE phenols. The principal compound (over 83% of the total of identified phenols) was (+) catechin and the remaining 5% fraction was a mixture of gallic acid, ferulic acid, (−) catechin, epicatechin and chlorogenic acid. Using the area under the chromatographic peaks we estimated the concentration of each known compound, in particular we measured (+) catechin at a concentration of 420 µg/mL. **Table 2** reports the relative abundance and the estimated concentration of the known phenolic constituents in POE. The

remaining 12% fraction was composed by several minor peaks, indicating the presence of additional compounds that, although detectable as phenols, results as unknown/uncharacterized.

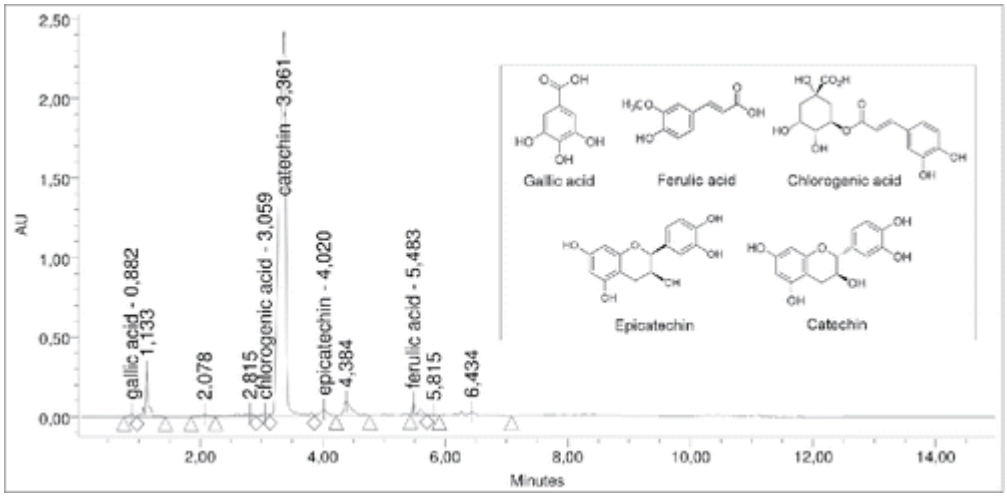

Figure 1. UPLC chromatogram of POE. The names and retention times of phenolic standards are indicated. In the box, the structures of the main constituents of POE are depicted.

Table 2. Characterization of the polyphenolic component of POE by UPLC.

Phenol name	Retention Time (min)	Area (%)	Concentration (µg/mL)
Gallic acid	0.882	0.374	1.94 ± 0.78
NA	1.133	4.303	NA
NA	2.078	0.410	NA
NA	2.815	1.352	NA
Chlorogenic acid	3.059	0.639	8.46 ± 0.03
(+) Catechin	3.361	84.762	418.44 ± 9.00
Epicatechin	4.022	1.383	33.31 ± 0.60
NA	4.384	3.634	NA
Ferulic acid	5.483	1.729	10.68 ± 1.19
NA	5.815	0.043	NA
NA	6.434	1.371	NA

Effect of POE on cell viability

Cell viability of HT1080 cells exposed to POE was tested using the MTT assay. As shown in **Fig. 2**, during exposure of HT1080 cells to 1:2000 dilution (corresponding to ~13 µg dry extract/mL and 0.21 µg/mL or ~4.5 µM catechin) and to 1:1000 dilution of POE (corresponding to ~26 µg dry extract/mL and 0.42 µg/mL or ~9 µM catechin) cell viability was the same as untreated control cells. In addition, even higher concentrations of POE had a very slight effect on cell viability: at 1:62.5 dilution, corresponding to 416.87 µg/mL of dry extract, we observed 87.2 ±5.1% of cell viability with respect to control untreated cells.

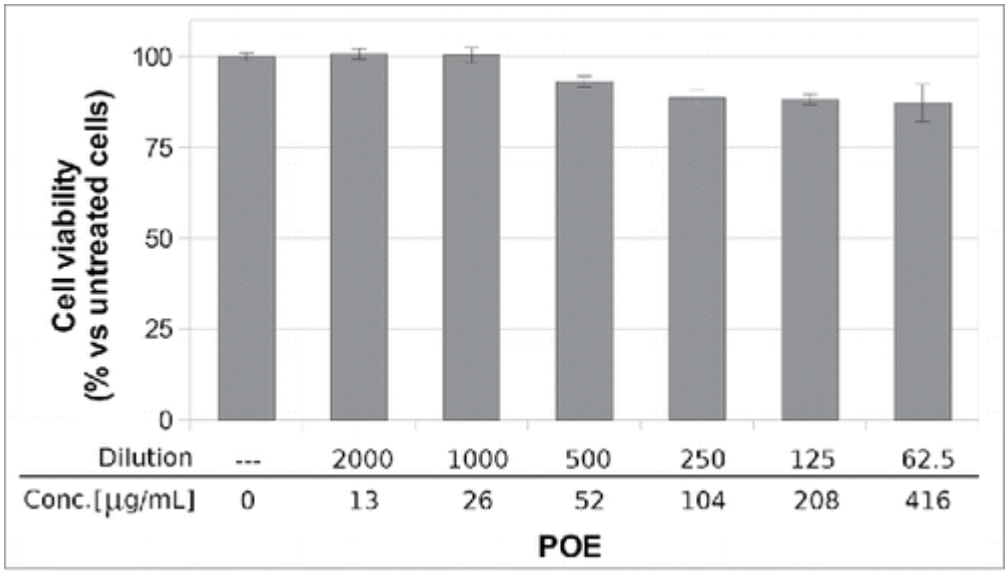

Figure 2. Effect of POE on HT1080 cell viability. Values are expressed as percent with respect to control untreated cells. The labels of bars report the used POE dilution and the corresponding concentration in term of µg dry extract / mL. Error bars represent standard deviation of 3 different experiments. POE at 1:1000 dilution was chosen as safe concentration for further experiments.

POE reduces cell migration and invasiveness

When cell migration was evaluated by the scratch wound healing assay (**Fig. 3**), POE exposure determined a dramatic reduction of cellular motility, that in turn determined a very limited closure of the wound area (see a representative time lapse movie in supplementary materials). In particular, control untreated cultures HT1080 cells completely closed the wound as early as 6 h from the initial scratching (20.7 ± 9.4% of wound area) and a complete closure (9,6 ± 1,9% of wound area) after 12 h, while in the presence of POE (1:1000 dilution, previously shown to be non-toxic to cells by MTT assay) about 70% of wound area was still open (72.3 ± 2,3%) at 12 h.

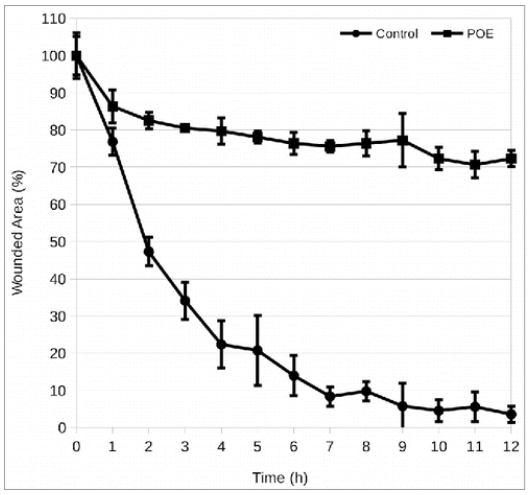

Figure 3. POE delays wound healing by HT1080 cells (scratch test). Time course analysis of the percent of wounded area in untreated (circles) and treated (squares) cells. Points are

expressed in terms of mean ± standard deviation of 3 consecutive frames (spanning 1 minute).

Figure 4 shows the degree of invasiveness of HT1080 cells trough the reconstituted basement membrane Matrigel®. During control culture conditions, 182.5 ± 6.4 cells/filter were able to invade Matrigel®. When cells were treated with POE (1:1000 dilution) a marked and significant reduction of invasiveness was observed (53.75 ± 2.9 cells/filter, about 70% reduction, $P < 0.05$, t-test) with respect to untreated cells.

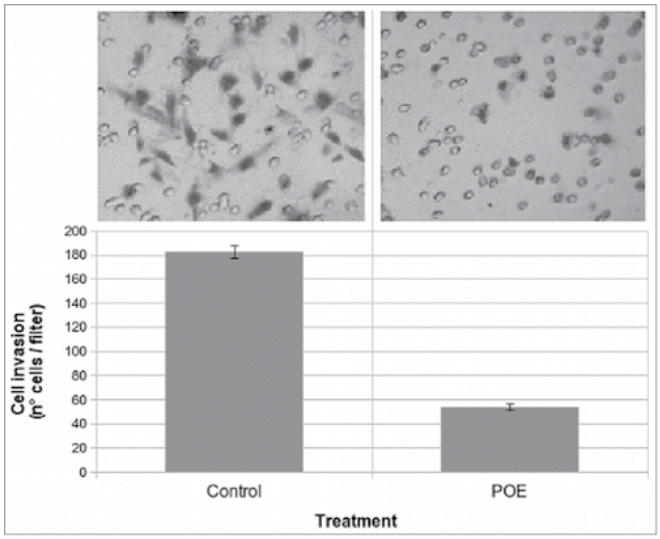

Figure 4. POE reduces Matrigel® invasion by HT1080 cells. Top panels show microscopic images of Matrigel® with cells stained with hematoxylin and eosin. Bottom panels report the corresponding number of cells per filter.

Modulation of gelatinase activity and expression

Gelatine zymography of conditioned medium from control HT1080 cell line showed the presence of gelatinolytic activity of an apparent molecular weight of 92 kDa, 72 kDa and 64 kDa (**Fig. 5A**). Western-blotting (**Fig. 5B**) of the same conditioned medium evidenced that zymographic bands comprise MMP-9 (MMP-9, 94 KDa), pro-active MMP-2 (pro-MMP-2, 72 KDa) and active MMP-2 (64 KDa) respectively. Treatment of cells with POE showed a 3 to 5 fold reduction of the amount of MMPs released in the medium (**Fig. 5A**), also confirmed by Western blot of MMP-9 and MMP-2 (**Fig. 5B**). Analysis of MMP-9 and MMP-2 gene expression by real time PCR revealed that inhibition of MMP secretion by exposure to POE was likely induced by a reduction of the MMP gene expression (**Fig. 5C**). The expression of TIMP-1, TIMP-2 and MMP-14, the physiological inhibitors and activator of gelatinases, respectively, assayed by RT-PCR, was not affected by POE treatment (*P*-value > 0.5, 2-tails t-test, data not shown).

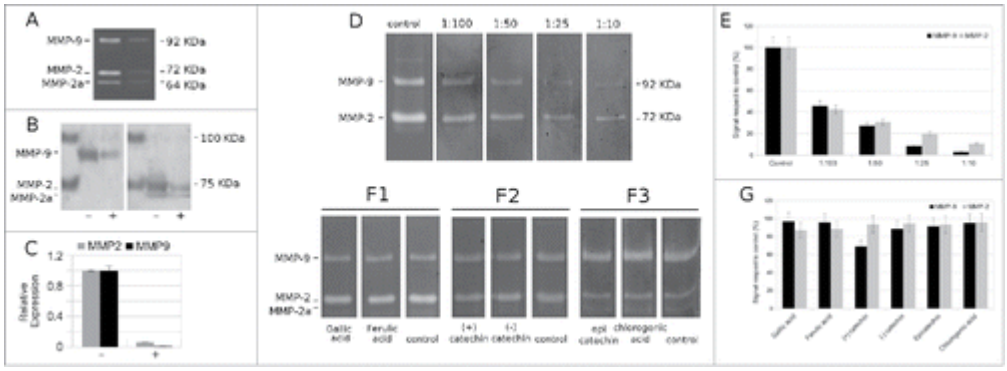

Figure 5. Gelatinase inhibition by POE. (A) Gelatin zymography: incubation of HT1080 with POE (+) reduces the amount of MMP-9 and MMP-2 released in the medium with respect to control (−). (B) Western blotting: identification of MMP-9 (left) or MMP-2 (right) in conditioned medium showed in A by specific antibodies. (C) Real time PCR: incubation of HT1080 with POE (+) reduces the expression levels of MMP-9 and MMP-2 with respect to control (−). (D) Gelatin zymography: *in vitro* dose dependent inhibition of MMP-2 and MMP-9 by POE. (E) Densitometric quantification of signals form bands in panel D. (F) *in vitro* effect of single, known phenols determined to be present in POE by UPLC at concentrations equivalent to those at 1:10 POE. (G) Densitometric quantification of signals form bands in panel F. In E and G the values are expressed in terms of inhibition percent with respect to controls and error bars represent the standard deviation of at least 3 independent experiments.

In a second set of zymographic experiments we showed that POE has a direct effect on MMP-2 and MMP-9 activities. In fact, incubation for 16 h of zymographic gel-lanes (prepared from untreated HT1080 control cells) in reaction buffer supplemented with POE, showed a dose-dependent inhibition of gelatinases (**Fig. 5D and E**), that was not abolished by the addition in large excess of the divalent cations Ca^{2+} and Zn^{2+} (data not shown).

Catechin alone can not explain POE direct inhibition of MMP activity

In order to dissect the relative contribution of each identified phenolic compounds of POE, we incubated the zymographic gel slabs with pure (+) catechin, (−) catechin, epicatechin, ferulic acid, gallic acid and chlorogenic acid (together constituting the 88% of POE polyphenols) at concentrations equivalents to that achieved with 1:10 extract, i.e. the dilution that determined a nearly complete (>90%) direct *in vitro* MMP inhibition (**Fig. 5D**, last strip). As shown in **Fig. 5F1-3** (and quantified in **Fig. 5G**), apart from (+) catechin, that showed a reduction in the overall intensity of both MMP bands of about 30%, all other compounds evidenced a very small reduction of MMP activity (in most cases lower than 10%). These results let us suppose that the fraction of phenols we were not able to identify (comprising about 12% of the total phenolic composition) should confer most of the *in vitro* inhibitory activity of POE.

Discussion

Cell invasion and migration play a pivotal role in several physiological and pathological conditions, particularly these processes that are essential for malignant cancer cells to invade the tissue micro-environment by degrading extracellular matrix components and to disseminate far from their primitive site, a necessary step for the metastatic process. Matrix metalloproteases MMP-2 and MMP-9, by degrading type IV collagen and other extracellular matrix adhesive proteins, have been shown to play a crucial role in tumor cell migration and invasion. In this study we demonstrated for the first time that POE is able to inhibit MMP-2 and MMP-9 expression and to drastically reduce migration and invasion of the highly invasive human fibrosarcoma HT1080 cell line, indicating that PO might be a suitable source of phytochemicals against tumor cell dissemination.

We introduced an extraction method that combines a water/ethanol extraction and a degumming step with *n*-hexane. Most literature on PO describes methods mainly aimed at recovering the hydrophobic polyphenolic components in the organic phase (e.g. ethyl-ether), therefore a direct comparison of our results with these studies is hardly feasible. First, our method allowed recovering water-soluble secondary metabolites and compounds compatible with biological buffers for *in vitro* and *in vivo* studies. Moreover, our POE retains antioxidant activities as those observed in hydrophilic and hydrophobic extracts isolated from PO by previous studies, suggesting that our extraction method makes no depletion of the main bioactive properties of PO. It is widely accepted that antioxidant and radical scavenger compounds might play a role in regulation of MMPs since reactive oxygen species induce activation and release of MMPs, especially MMP-2 and MMP-9[34] and they also modulate the redox state of the cell and influence the NF-κB pathway, that in turn modulates MMP transcription. Therefore, in this study POE might affect cell migration and invasion through its antioxidant properties. Finally, we revealed that our hydrophilic POE mostly contains (+) catechin (>80%) and minor amounts of ferulic, gallic and chlorogenic acids. All these biochemical characteristics were not previously reported by any literature on phenolic composition of PO leaves.[35] Catechins, are not only well known antioxidants, but they are also able to inhibit MMPs, as documented in a wealth of literature related to epigallocatechin gallate and its relatives.[36,37] Through inhibition of MMP-2 and MMP-9, catechins and its epimer epicatechin as well as their gallic acid conjugates are strongly implicated in cell migration and invasion.[38]

Although ferulic acid, which was in minor concentrations in our POE, seems to possess diverse pharmacological functions some of which still remaining unknown, most of its beneficial effects were ascribed to its antioxidant activities which might modulate MMP expression/activity. In fact, ferulic acid is able to modulate protein expression of MMP-2 and MMP-9 in liver alcoholic fibrosis and in end-stage cirrhosis.[39]

Gallic acid, the other minor compound in POE, inhibits gene expression of gelatinases MMP-2 and MMP-9 in human leukemia cells by reducing the binding of c-Jun/ATF-2 and c-Jun/c-Fos with

promoter region of MMP-2 and MMP-9 genes and it also inhibits cell migration and invasion of a human squamous cell carcinoma cell line by suppressing MMP-2 and MMP-9 expression through inhibition of NF-κB.[40,41]

Similarly, chlorogenic acid, another minor compound of POE, exhibits inhibitory effects on cell migration and MMP-2 secretion in a glioblastoma cell line and it is also able to inhibit other matrix metalloproteinases such as MMP-1, MMP-3, and MMP-13 which play a role in cell migration and invasion.[42,43]

Although anti-invasive properties of the above mentioned polyphenols were revealed in pure substances, their concomitant presence in the same extract (POE in this case) is reasonably leading to even more powerful effect (a principle of synergy well known to the pharmacognosy discipline[44]). In fact, POE showed a powerful inhibition of MMP-2 and MMP-9 both at protein and gene levels.

Further, our data clearly depict an additional role of POE in cell migration and invasion that is possibly correlated to a direct effect of POE on MMP-2 and MMP-9 activities. In fact, when zymography of conditioned medium of HT1080 was incubated in the presence of POE, we observed a dose dependent inhibition of gelatinolysis that was not related to chelating properties of polyphenols, since a large excess of Zn^{2+} or Ca^{2+} was not able to recover the inhibition by POE, in agreement with previous results.[45,46] It is important to underline that none of the identified POE phenols was by itself able to induce a direct inhibition of MMP-2 and MMP-9 gelatinolytic activity as strong as that observed in POE and their cumulative effect was insufficient. This lead us to speculate that POE could contain some not yet identified component with strong inhibitory activity on MMPs. Since this bioactive component or mixture is not supposed to have either a lipid, a complex carbohydrate or a protein origin, due to our extraction method, it is reasonable to suppose its phenolic origin. However, it is beyond the scope of this study to identify this compound and to elucidate its mechanism of inhibition on both MMPs and cell migration/invasion.

In conclusion, in this study we characterized POE at biochemical and compositional levels and proved its important role in modulating cell migration and invasion. We showed that gelatinases, which promote tumor invasion and metastasis through degradation type IV collagen, are affected by POE at a dual level: on the one hand, POE was very effective in inhibiting gene and protein expression of MMP-2 and MMP-9; on the other hand, POE directly inhibited the activity of synthesized and secreted gelatinases, thereby reducing the capability of degrading extracellular matrix components. Therefore, *Posidonia oceanica* can be considered as an interesting reservoir for new natural compounds useful for treatment of pathologies related to altered expression of MMP-2 and MMP-9.

Materials and Methods

Materials

α,α-Diphenyl-β-picrylhydrazyl (DPPH), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″ isulfonic acid sodium salt (Ferrozine®), Folin–Ciocalteu's phenol reagent, gallic acid, ascorbic acid, D-glucose, gelatin and Coomassie brilliant blue R-250, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan, Cell Growth Determination Kit MTT based (Cat. CGD1-1KT), Dulbecco's Modified Eagle's Medium (DMEM), heat inactivated Fetal Bovine Serum (FBS) and UPLC phenol standards were purchased from Sigma-Aldrich. Cell culture plastics were from Nunc (Thermo Fisher Scientific). Transwell filter units were from Costar. 30% Acrylamide/Bis 37.5:1 solution, ammonium persulfate (APS), 1,2-Bis (dimethylamino) ethane (TEMED), and Tris/Glycine buffers were purchased from Bio-Rad. UPLC solvents and equipment were from Waters. Matrigel® was purchased from Collaborative Research. Monoclonal antibodies against MMP-2 (clone 42-5D11) and MMP-9 (clone 56-2A4), were purchased from Calbiochem-Novabiochem International. Blotting membranes, Immobilon polyvinylidene difluoride (PVDF), were from Millipore. Goat anti-mouse IRDye 680LT antibody (Cat# 926-68020) was purchased from Li-Cor Biosciences. The RT-PCR primers for matrix metalloproteases (MMP-2: Hs00234422_m1 and MMP-9: Hs00234579_m1), tissue inhibitors of MMPs (TIMP-1: Hs00171558_m1 and TIMP-2: Hs00234278_m1), membrane-type 1 MMP (MMP-14/MT1-MMP: Hs00237119_m1) and for glyceraldehyde-3-phosphate dehydrogenase (Hs00266705_g1), as well as TaqMan universal PCR master mix, were purchased from Applied Biosystems. RT-PCR ABI 7500 PCR instrument was from Applied Biosystems. Photometric measurements in multiwell plates were recorded on an iMARK microplate reader (Bio-Rad). Western blotting signals were acquired using an infrared imaging system (Odyssey; Li-Cor Biosciences).When not otherwise specified, all chemicals and solvents, such as ethanol, methanol and *n*-hexane were of the highest analytical grade and were purchased from Sigma-Aldrich.

Sample collection

Leaves of *Posidonia oceanica* (L.) Delile (see algaeBASE species id 18577 and references therein) were collected from scuba divers in July 2013 in Le Ghiaie meadow (Elba Island, Tyrrhenian Sea, Italy, 42°49′1″ N, 10°18′15″ E) at -10 m. Even though the sampling zone is a Biological Protection Reserve, at the depth of 10 m the plant was lusher and more protected. Soon after their collection, the leaves were removed from the rhizomes and kept at low temperature (10°C) and dark during the transport to the laboratory.

In the laboratory only adult leaves[47] were selected and washed repeatedly in fresh water. From the leaves, fragments (1–5 cm in length) free from visible epiphytes were cut off with non metallic tools. The fragments were washed with distilled water and dried at 60°C in darkness (to preserve

polyphenol composition, according to[24]) for about 24 h and stored in plastic food bags until processing.

Extraction method

Extraction of phenolic compounds involved the central part of the leaves. Dried leaves were minced and suspended in 10 mL 70% ethanol per gram of leave. Extraction was performed overnight at room temperature, under continuous agitation and at 65°C for further 3 h. The PO ethanol extract was separated from debris by filtration and mixed with *n*-hexane (ratio 1:1), then vigorously mixed in a separatory funnel. The organic phase containing hydrophobic compounds was discarded and 1 mL batches of the cleaned PO extract, mainly containing hydrophilic compounds, were dried by Univapo^{TM} vacuum-spin concentration. Dry extracts were then stored at room temperature in the dark. For assays, batches of dry extracts were suspended until complete solubilisation in 0.5 mL 20% ethanol in sterile water and directly used. Hydrophilic Extract from PO were hereinafter called POE.

Ultra performance liquid chromatography

The lyophilized extract dissolved in ethanol and the standards (previously dissolved in methanol) were filtered with 0.45 µm microfilters before analysis. The analysis of polyphenols present in the extract was carried out by using an ACQUITY^{TM} Ultra Performance Liquid Chromatography (UPLC) system linked to a PDA 2996 photodiode array detector. Empower software (Waters) was used to control the instruments and for data acquisition and processing. The analyses were performed at 30°C using a reversed phase column (BEH C_{18}, 1.7 µm, 2.1 × 100 mm, Waters).[48] The mobile phase consisted of 7.5 mM acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 250 µL/min. Gradient elution was employed, starting with 5% B for 0.8 min, then 5% – 20% B over 5.2 min, isocratic 20% B for 0.5 min, 20% – 30% B for 1 min, isocratic 30% B for 0.2 min, 30% – 50% B over 2.3 min, 50% – 100% B over 1 min, isocratic 100% B for 1 min, and finally 100% – 5% B over 1.0 min. At the end of this sequence, the column was equilibrated under the initial conditions for 2 min. The pressure ranged from 6000 to 8000 psi during the chromatographic run. The effluent was introduced into an LC detector (scanning range: 210 – 400 nm, resolution: 1.2 nm). The injection volume was 5 µL.

Carbohydrate content

The total carbohydrate content (TCC) of POE was determined according to the phenol-sulfuric acid method optimized from with minor modifications.[49] Briefly, scalar aliquots of POE were added to 96-well microplate and diluted with water (final volume 70 µl), then 150 µL of concentrated sulfuric acid was added to each well. After 5 min of incubation at RT under continuous shaking, 30 µL of 5% phenol solution was added to each well and heated for 5 min at 90°C. After cooling to room

temperature for 20 min, the absorbance at 490 nm was recorded with a microplate reader. Carbohydrate content was determined by linear regression using D-glucose as a reference in the range 0–50 µg.

Total polyphenol content

The total polyphenol content (TPC) of POE was determined according to the colorimetric Folin-Ciocalteau method.[50] Scalar volumes of POE (final volume 20 µL) were added to 100 µL of Folin–Ciocalteu (Folin–Ciocalteu's phenol reagent diluted 1:10 in H_2O). After incubation for 5 min at RT, 80 µL of 7,5% sodium carbonate solution was added and incubated for further 2 h. The absorbance at 595 nm was recorded with a microplate reader. Polyphenol content was determined by linear regression with gallic acid as a reference in the range 0–10 µg.

Radical scavenging activity

The radical-scavenging activity (RSA) of POE was determined adapting the method from Fukumoto and Mazza.[51] In a 96-well microplate, scalar aliquots of POE were diluted with 95% methanol (final volume 100 µL) and mixed with 100 µL of freshly prepared DPPH solution (0.15 mg/mL methanol). After 30 min incubation in the dark at room temperature, the absorbance was read at 490 nm with a microplate reader. Radical scavenging activity was determined by linear regression with ascorbic acid reference in the range 0–4 µg.

Antioxidant activity

The antioxidant activity (TAA) of POE was estimated using the FRAP (ferric-reducing/antioxidant power) method adapted from Pulido et al.[52] Scalar aliquots of POE were diluted with water (final volume 50 µL) and 200 µL of Ferrozine™ reagent (10 mM Ferrozine™ in 40 mM HCl : 20 mM ferric chloride : 0.03 M acetate buffer pH 3.6 ratio 1:1:10) were added to each aliquot. After 4 min incubation at 37°C, the absorbance was measured at 595 nm at room temperature with a microplate reader. Antioxidant activity was determined by linear regression with ascorbic acid reference in the range 0–4 µg.

Cell lines and culture conditions

The HT1080 human fibrosarcoma cell line (ATCC CCL-121) was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of heat inactivated fetal bovine serum (FBS). Cell cultures were incubated at 37°C in a 5% CO_2 -humidified atmosphere and sub-confluent cultures were propagated by trypsinization (trypsin 0.025% – EDTA 0.5 mM).

Cell viability

Cell viability was assessed by the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) metabolic activity assay according to the cell growth determination kit from Sigma-Aldrich. Briefly, HT1080 cells were seeded in 96-well culture dishes in the presence of complete culture medium at a density of 1×10^4 cells/well. After 24 h of incubation at 37°C in a 5% CO_2-humidified atmosphere, cell monolayers were washed with serum-free medium supplemented with 25 µg/mL heat-treated bovine serum albumin (BSA) and exposed to dried Posidonia extract resuspended in serum-free medium at 6 1:2 serial dilutions from 1:62.25 to 1:2000. Cells incubated in serum-free medium served as control untreated group. After 24 h of incubation at 37°C in a 5% CO_2-humidified atmosphere, culture medium was removed, cell monolayers were washed with phosphate buffered saline (PBS) and then tested for cell viability. All experiments were performed in triplicate and absorbance values at 595 nm were averaged. Cell viability was expressed as percent relative to untreated control.

Invasion assay

The invasiveness of HT1080 cells through reconstituted basement membrane represented by Matrigel® was evaluated in Transwell filter units with pores of 8 µm in diameter. Aliquots (100 µl) of a Matrigel solution in serum-free culture medium (2 mg protein per mL) were layered on the top of each filters and dried overnight. Then, cells detached from stock cultures with EGTA (0.5 mM in PBS) were suspended at a density of 3×10^5 cells/mL in serum-free medium supplemented with 25 µg/mL heat-treated BSA and POE 1:1000 dilution. 0.5 mL of cell suspension was layered on the upper surface of filters that were then immersed in wells filled with 1 mL serum-free medium supplemented with BSA and POE 1:1000 dilution. Control untreated cells were treated as above except for the absence of POE. Transwell filter units were then incubated at 37°C in a 5% CO_2 - humidified atmosphere for 18 h. Non-invading cells were removed from the upper surface of the filter by wiping with a cotton swab, and the filters were then fixed in a methanol:acetic acid mixture (70:30 v/v) for 1 h at room temperature. The filters were stained with hematoxylin and eosin, and the invading cells were quantified by examining the whole lower surface of the filter under a light microscope (200-fold magnification). Each assay was performed in duplicate.

Cell migration assay

The scratch wound healing assay of tissue-culture cell monolayers was used in order to analyze the effects of POE on cell migration. Briefly, HT1080 cells were seeded in 9 cm^2 slide flasks at high density (5×10^5 cells/flask) and allowed to form monolayers overnight in the presence of medium supplemented with serum. Cell monolayers were then wounded with a sterile rounded glass tip and washed several times with PBS to discard detached cells and cell debris. In order to maintain proper pH without requirement of CO_2, cell monolayers were then exposed to serum-free medium supplemented with BSA and 20 mM HEPES and POE 1:1000 dilution. Cells treated with serum-free

adhesion medium supplemented with BSA and 20 mM HEPES without POE served as control untreated cells. The wounded cell-free area was then observed under phase contrast microscopy for 48–72 h at 37°C. Three frames from the same optical field were captured every minute by time-lapse recording and wound size was then analyzed with the TScratch software (ETH CSElab, Zurich, Swiss).

Gelatin zymography

Conditioned medium from HT1080 cell cultures was prepared by incubating cells for 12 h in serum-free medium or in serum-free medium in absence or supplemented with POE 1:1000 dilution. In brief, cells detached from stock cultures with EGTA were seeded at a density of 2.5×10^4 cells/cm^2 in 6-well culture plates in the presence of complete culture medium supplemented with 10% FBS. After 18 h of incubation at 37°C in a 5% CO_2 -humidified atmosphere, culture medium was removed, cell monolayers were washed twice with PBS and incubated in serum-free medium supplemented with 25 µg/mL heat-treated BSA or in serum-free medium supplemented with BSA and POE 1:1000 dilution. After 24 h of incubation, conditioned medium was collected and centrifuged at 12000 rpm for 1 min at 4°C in order to pellet non adherent cells. Then, same volumes of conditioned media from control or treated HT1080 cells were assayed for gelatinase activity by gelatin zymography. Briefly, 25 µl aliquots of conditioned medium were electrophoresed under non-reducing conditions in 8% polyacrylamide gels containing 1 mg/mL gelatin. After the electrophoretic separation, gels were washed twice in 2.5% Triton X-100 for 1 h to remove SDS, rinsed briefly, and incubated at 37°C for 24 h in reaction buffer (50 mM Tris-HCl pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$, 1 1 µM mu;M $ZnCl_2$).

When the effect of POE on gelatinase activity was assayed *in vitro*, the medium of untreated cells was electrophoresed as above, then strips of gels corresponding to lanes were cut, treated with Triton X-100 and incubated separately in reaction buffer containing scalar dilutions of POE or pure phenols at appropriate concentrations.

In order to exclude inhibition by a chelating effects, the reaction buffer was optionally supplemented with concentration of Ca^{2+} and Zn^{2+} ranging form 5 mM to 100 and from 1 µM to 20 M respectively.

Gels or gel strips were stained with 0.05% Coomassie Brillant Blue R-250 dissolved in 40% methanol and 10% acetic acid and destained with the same Coomassie-free solution. Zones of enzymatic activity appeared as clear bands against a blue background. Images were acquired with a digital scanner.

Western blotting

In order to identify individual protease activities, conditioned medium form treated or untreated cells was analyzed for immuno-reactive MMP-2 and MMP-9. In brief, 25 µl aliquots form the same conditioned medium used for zymography (see above for details) were electrophoresed under non reducing conditions on 8% SDS-polyacrylamide gel and transferred by electroblotting onto

Immobilon PVDF membranes. Red Ponceau staining was used to check for equal loading and protein transfer. After blocking with 2% fat-free dry milk in 20 mM Tris-HCl (pH 7.4), 0.9% NaCl, 0.1% Tween-20 (TBS-Tween), the membrane was incubated with 2 µg/mL anti human matrix metalloproteinase MMP-2 monoclonal antibody (clone 42–5D11) or with anti human matrix metalloproteinase MMP-9 monoclonal antibody (clone 56–2A4). After washing with TBS, the membranes were incubated with goat anti-mouse and revealed by an Odissey infrared imaging system.

Real time PCR

Total cytoplasmic RNA was isolated as described elsewhere[53] from sub-confluent HT1080 cells cultured for 24 h in control serum free-medium or in serum-free medium supplemented with 1:1000 dilution of POE. RNA concentration after extraction was measured using a NanoDrop (Thermo Scientific) and first-strand cDNA was synthesized from 1 µg of total RNA. Relative expression of MMP-2, MMP-9, TIMP1, TIMP2, MMP14/MT1-MMP and GAPDH gene, which represents the housekeeping gene, were quantified using TaqMan real-time polymerase chain reaction using the primer sets described above. Each reaction was performed in triplicate using the following protocol: 95°C for 10 min for one cycle, 50 cycles of 95°C for 15 s and 58°C for 1 min. Negative controls for amplification consisted in non-template control and no-RT enzyme control. The relative quantification (RQ) of gene expression was obtained by the comparative CT method, 2-ΔΔCt, as follows: first, the mean of the triplicate threshold cycle (CT) values for the target gene was normalized to the mean of the triplicate CT values for the internal control GAPDH gene in the same samples (ΔCT = mean CT_{Target} – mean CT_{GAPDH}); second it was normalized with the control – i.e., cells unexposed to POE – (ΔΔCT = ΔCT – $\Delta CT_{Control}$) and the fold change in gene expression was obtained by calculating 2-ΔΔCT.[54] Reported data are representative of 3 independent experiments with similar results.

Statistical analysis

All the results were expressed in terms of mean ± standard deviation (SD) on the basis of at least 3 independent extractions for the main biochemical activities. Replicates for other experiments are detailed in appropriate sections. Linear regression analysis of reference compounds were performed with Microsoft Office Excel (Microsoft Corporation).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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