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Myrtle Seeds (*Myrtus communis* L.) as a Rich Source of the Bioactive Ellagitannins Oenothein B and Eugeniflorin D_2

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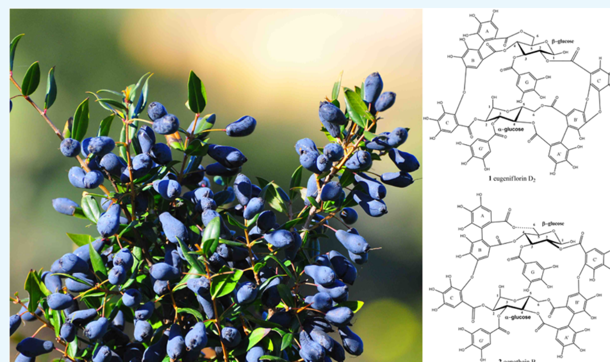
ABSTRACT: The increasing popularity of "Mirto" liqueur, produced from *Myrtus communis* berries, has led to the planting of domesticated cultivars, expanding myrtle berry production. To promote the use of cultivated berries, the content in the nutraceutical compounds ellagitannins has been investigated both in spontaneous and cultivated fruits. Oenothein B and eugeniflorin D_2, characterized by ^1H and ^{13}C NMR, were isolated and quantified using ultrahigh-performance liquid chromatography−diode array detector−tandem mass spectrometry (UPLC−DAD−MS/MS). The antifungal and anti-inflammatory activities of oenothein B were assayed in vitro. Large amounts of oenothein B and eugeniflorin D_2 were detected in seeds (12 ± 2.4 and 5.8 ± 1.2 mg/g). The oenothein B concentration in liqueurs was 194 ± 22 mg/L. This macrocyclic ellagitannin dimer showed anti-*Candida* (minimal inhibitory concentration <8−64 μg/mL) and anti-inflammatory properties. Cultivated myrtle berries are a source of nutraceutical compounds. The high concentration of oenothein B in liqueur suggests a possible contribution to the organoleptic and biological properties of the beverage.



1. INTRODUCTION

Myrtus communis L. is an evergreen shrub or small tree growing spontaneously in the Mediterranean basin, Madeira, and West Asia.[1] In Sardinia (Italy), myrtle berries are largely employed in the food industry, principally by the liqueur industry to produce the popular "Mirto di Sardegna", recognized as a geographical indication of the island of Sardinia, according to EC Reg. no. 110/2008. Annual production of Mirto di Sardegna is ca. 2 million bottles. With the scope of protecting production, given its economic importance in the Sardinian agricultural sector, manufacturing specifications have been established since 1998 on the basis of an in-depth study of the chemical composition of the berry and the production process for the liqueur.[2] The berries used to produce the liqueur must be gathered without the use of pesticides or fertilizers and picked in Sardinia, and no flavoring agents, colorants, and preservatives should be added.

The popularity of the liqueur suggested a need for agronomical research to domesticate spontaneous plants, and the result was the planting of about 200 ha of myrtle plants in Sardinia.[3] Unfortunately, liqueur producers prefer fruit from wild plants, which are available in sufficient quantities; therefore, the market demand for products made with nonspontaneous plants is poor. For this reason, there is a strong interest in seeking applications in fields other than the liqueur industry. Better knowledge of the nutraceutical properties of myrtle berries might represent an incentive for their future exploitation in the food, pharmaceutical, and cosmetic industries.

M. communis L. berries are rich in phenolic compounds, including phenolic acids (gallic acid, ellagic acid), flavonols (myricetin glycosides with small quantities of quercetin and kaempferol glycosides), flavan-3-ols (epigallocatechin, epigallocatechin 3-*O*-gallate (EGCG), and epicatechin 3-*O*-gallate), and anthocyanins.[2,4,5] The distinctive tannic and astringent taste of myrtle berry hydroalcoholic extract cannot be explained by the limited presence of proanthocyanidins, whose content ranges from 93 to 503 mg/L in liqueur.[2]

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Table 1. Berry Composition[a]

sample (no. of specimens)	berries (g)	pericarp (g)	seeds (g)	% seeds
		Year 2008−2009		
cultivated (12)	10.4 ± 0.51	8.5 ± 0.24	2.0 ± 0.23	19.0 ± 0.53
wild (6)	10.4 ± 0.23	8.5 ± 0.35	2.1 ± 0.56	24.9 ± 1.55
		Year 2010−2011		
cultivated (11)	10.4 ± 0.34	8.5 ± 0.53	1.9 ± 0.75	22.6 ± 1.12
wild (11)	10.3 ± 0.65	8.0 ± 0.24	2.3 ± 0.43	28.9 ± 0.84
		Year 2013−2014		
cultivated (16)	10.0 ± 0.46	8.0 ± 0.49	2.0 ± 0.42	24.5 ± 2.34
wild (12)	10.0 ± 0.53	7.9 ± 0.34	2.0 ± 0.15	25.8 ± 1.45

[a]Data are mean ± standard deviation from three independent experiments.

Although the phytochemical profile of myrtle berries has previously been described, the ellagitannin (ET) content in berries and liqueur has been little investigated. Ellagitannins (ETs) are a complex class of bioactive compounds, with distribution restricted to dicotyledonous angiosperms.[6] From a chemical point of view, ETs are a complex class of hydrolysable tannins, characterized by the presence of one or more hexahydroxydiphenoyl units, generally esterified to a glucopyranose group. Their presence has been detected in edible fruits of plants belonging to the orders Fagales, Rosales, and Myrtales, such as walnut, strawberry, raspberry, pomegranate, and myrtle (*Myrtus communis* L.).[6,7] Consumption of food containing ETs has been associated with improved health and reduced chronic pathologies such as inflammatory, cardiovascular, and neurodegenerative diseases and cancer,[8−10] and, thus they are classified as potential nutraceutical ingredients.[11] The biological activities of ETs are related to two principal properties: their antioxidant potential,[12] involved in the prevention and delayed development of carcinogenesis, and their ability to participate in the complexation of macromolecules, thus defending against inflammation and other gastrointestinal disorders such as infections caused by microorganisms.[11,13] Due to the important properties of ETs for human health described above and to promote the use of myrtle berries, we investigated their presence in fruits collected from both cultivated and wild plants. We focused in particular on the chemical and biological properties of myrtle seeds.

The aim of the first part of this study was (i) to isolate and structurally characterize the main ETs present in myrtle berries and (ii) to quantify their presence in *M. communis* L. picked from different locations in Sardinia. We compared the composition of domesticated cultivars and wild plants to evaluate the possibility of promoting the nutraceutical properties of cultivated fruits.

The aim of the second part of the study was the investigation of (iii) the antifungal and (iv) the anti-inflammatory properties of oenothein B, the most abundant ET found and isolated from *M. communis* L. berries.

2. RESULTS AND DISCUSSION

2.1. Berry Composition. Myrtle berries are round fruits, composed of a fleshy pericarp and a snail-shaped seed. We investigated the composition of 38 samples obtained from both cultivated (23) and wild (15) plants to estimate the relative mass of seeds and pericarp tissues. Seeds represented on average 22.3% of the fruit, while the rest of the weight was represented by fleshy tissues, as shown in Table 1. A slight difference, albeit not significant ($p > 0.05$), was observed in berries obtained from cultivated and wild plants.

2.2. Ellagic Acid and Ellagitannins from Myrtle Seeds. Myrtle fruit is rich in secondary metabolites. The seeds in particular have previously been reported to contain a larger amount of phenolic compounds in comparison with other tissues in the pericarp.[4,14,15] Ellagic acid is released after hydrolysis of ellagitannins, and its presence in myrtle berries is well documented.[14,16] We investigated its content in seeds and found that the average amount of EA was 0.49 ± 0.14 mg/g, both for wild and cultivated samples (Table 2). No significant differences ($p > 0.05$) were observed between cultivated and wild plants.

Table 2. Ellagic Acid and Ellagitannins in Myrtle Berry Seeds[a]

samples (no. of specimens)	ellagic acid	eugeniflorin D_2	oenothein B
	Year 2008−2009		
cultivated (12)	0.48 ± 0.09	5.35 ± 2.10	13.61 ± 2.20
wild (6)	0.55 ± 0.15	5.14 ± 1.80	11.03 ± 1.13
	Year 2010−2011		
cultivated (11)	0.33 ± 0.09	4.91 ± 0.76	10.84 ± 2.50
wild (11)	0.40 ± 0.15	5.32 ± 1.23	10.55 ± 3.07
	Year 2013−2014		
cultivated (16)	0.65 ± 0.17	7.75 ± 1.65	15.15 ± 2.25
wild (12)	0.56 ± 0.21	6.54 ± 0.91	13.60 ± 1.23

[a]Data are expressed as mg/g dry weight and are mean ± standard deviation from three independent experiments.

Two main dimeric ellagitannins, eugeniflorin D_2 (**1**) and oenothein B (**2**), with a specific macrocyclic structure were isolated from seed extracts and chemically characterized. Eugeniflorin D_2 was first isolated from *Eugenia uniflora*, a plant belonging to the Myrtaceae family shown to have digestive properties.[21] Oenothein B was found in *Oenothera erythrosepala*[18] and subsequently detected in other species belonging to the Myrtales order. Interestingly, eugeniflorin D_2 can be converted into oenothein B when kept in aqueous solution at 80 °C for 1 day.[17] The presence of eugeniflorin D_2 and oenothein B in myrtle berries was investigated. Chemical analysis revealed that the compounds are accumulated in all parts of the fruit, but the amount was particularly high in seeds (Table 2). Here, eugeniflorin D_2 and oenothein B were found in average quantities of 5.8 ± 1.2 and 12 ± 2.4 mg/g, respectively, while in pulp and peel extract the concentrations were 1.7 ± 0.1 and 1.8 ± 0.1 mg/g, respectively (data not shown). Thus, in *M. communis* L. berries, seeds represent the main reservoir for both ellagitannins.

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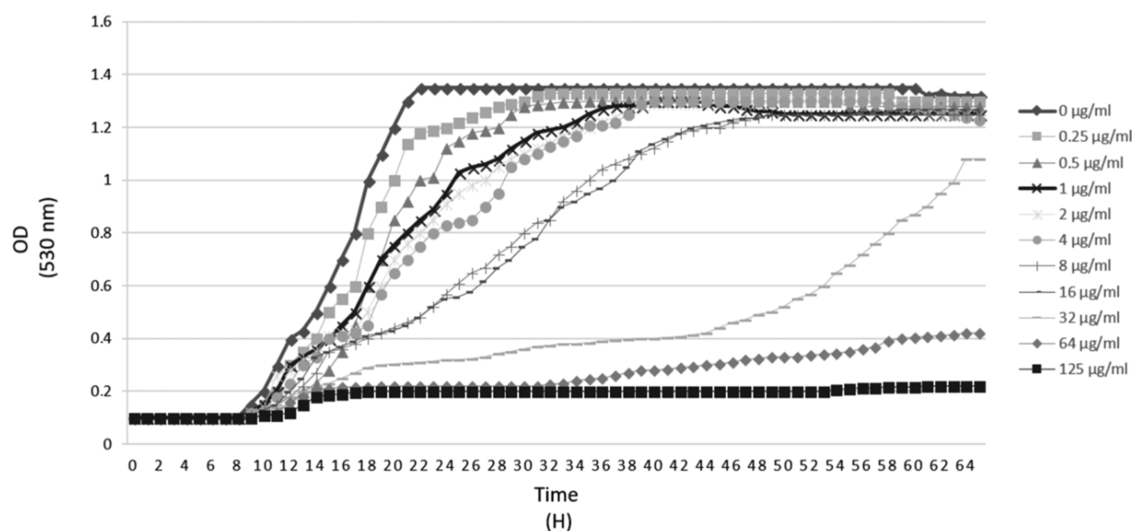

Figure 1. Oenothein B inhibits the growth of *C. glabrata* (strain RTT199_3) in a dose-dependent manner. *C. glabrata* was treated with increasing concentrations of oenothein B for 65 h. The cells were allowed to grow in dark conditions, at 37 °C, and with continuous shaking. The growth curves were recorded using a spectrophotometer (530 nm). The graph shows the geometric means of three independent experiments.

No significant differences were observed in wild and cultivated samples ($p > 0.05$). In myrtle, eugeniflorin D_2 and oenothein B have previously been detected in leaves;[19] however, to the best of our knowledge, there are no reports relating to berries in the literature.

2.3. Oenothein B Content in Mirto di Sardegna Liqueur. We also investigated the content of the eugeniflorin D_2 and oenothein B in Mirto di Sardegna liqueur. No traces of eugeniflorin D_2 were detected, while the average amount of oenothein B in liqueur samples obtained from the three main Sardinian producers was 194 ± 22 mg/L. According to our analysis of berry composition, the estimated amount of fresh berries used (up to a maximum of approx. 180 g/L) is even higher than the legal minimum (150 g/L) prescribed by the production protocol. Our analysis therefore contributed toward highlighting the quality of Sardinian Mirto liqueur.

The presence of oenothein B in liqueurs at a concentration of 124 μM, which is well above the taste threshold concentrations for the astringent sensation of similar taste-active ETs in aqueous solution (pH = 4.5), in the range of 0.2−6.3 μM,[20] is thought to contribute toward explaining the slightly tannic, astringent taste of the liqueur. Moreover, recent studies have proven that astringency is a trigeminal perception in humans and have reported the existence of a trigeminal G protein-coupled receptor specific for galloylated molecules.[20] This study described the dose−response curves for the activation of isolated mouse trigeminal ganglion cells by several phenolic plant astringents, indicating that galloyl moieties are necessary for strongly activating substances. Oenothein B has two valoneoyl and galloyl moieties each in its structure (Figure 4).

Finally, it has been shown that ETs can activate human bitter taste receptors,[21] with all ETs investigated in this library of polyphenols found to be capable of interacting with the bitter receptor TAS2R7, with the half-maximal activation agonist concentration (EC50) being in the range of 2.43−7.26 μM. In conclusion, oenothein B has all of the structural requirements to be taste-active and we suggest that it could explain the liqueur's particular oral sensation. Further work is needed to precisely evaluate the contribution of oenothein B to astringency and bitterness in the specific matrix.

Oenothein B has been shown to have anticancer, anti-inflammatory, antioxidant, and antimicrobial properties.[22] Due to the documented biological properties, it is reasonable to believe that oenothein B might contribute to the beneficial effect of berry-derived products and liqueur on human health. Seed extracts have a positive effect on the treatment of oesophagitis-induced reflux and colitis in rats, exhibiting anti-inflammatory properties.[23] Myrtle liqueur also has vasodilatory properties,[24] and extracts obtained from myrtle berries have been shown to exert antibacterial activity against food-borne pathogens and to have an ulcer-protective effect.[25,26]

2.4. Oenothein B Shows Antifungal Activity against Fluconazole-Sensitive and -Resistant *Candida* Strains. Oenothein B was subjected to further studies to assess its biological properties and the possible beneficial effects deriving from the consumption of foods containing this metabolite. Oenothein B was able to inhibit the growth of all of the *Candida* strains tested, in a concentration-dependent manner (Figure 1), with MIC_{50} values ranging from <8 to 64 μg/mL (Table 3). The inhibitory properties of the molecule against fluconazole-resistant strains of *Candida albicans*, *Candida tropicalis*, and *Candida glabrata* are worth noting.

As previously reported, oenothein B is active against the pathogen *Paracoccidioides brasiliense*, which is the causal agent of the most prevalent human systemic mycosis in Latin America.[27] Through analysis of the transcription profile of

Table 3. Anti-*Candida* Activity of Oenothein B from Myrtle Seeds[a]

strain (no.)	MIC range (μg/mL)	
	oenothein B	fluconazole
C. albicans (3)	<8−64	0.125 to >64
C. glabrata (2)	8−64	0.125 to >64
C. parapsilosis (1)	32	0.125
C. tropicalis (1)	<8	>64

[a]Data are geometric mean of the MIC_{50} values measured for each strain in three independent experiments.

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fungi treated with oenothein B, it has been found that the compound exerts its antifungal activity by inhibiting the transcription of genes like 1,3-β-glucan synthase (PbFKS1), GLN1, and KRE6, which are involved in cell wall biosynthesis.[27] The human gut is commonly colonized by *Candida* spp., but alteration of the normal gastrointestinal flora (dysbiosis), often associated with the use of antibiotics, physical and psychological stress, hormone intake, and dietary changes, can increase levels of *Candida* colonization, which is associated with several diseases of the gastrointestinal tract, inflammation, and delays in the healing of inflammatory lesions. Moreover, the gut is considered to be a source for yeast dissemination and systemic candidiasis development, which is associated with high rates of morbidity and mortality in immunocompromised patients.[28] Considering that agents able to reduce the levels of barrier alteration and inhibit the growth of *Candida* species inhabiting the gastrointestinal tract are important for control of the dissemination and development of candidiasis, consumption of new products obtained from myrtle berries and containing oenothein B could exert a beneficial effect on controlling proliferation of *Candida* spp. in the human gut during episodes of dysbiosis.

2.5. Oenothein B Inhibits IL-8 Release in TNFα- or IL-1β-Treated AGS Cells through Inhibition of NF-κB Signaling. The stomach is one of the first sites of action of bioactive molecules consumed with the diet, and ETs have already shown anti-inflammatory activity at the gastric level, both in vitro and in vivo.[9,10] *Helicobacter pylori* is the main etiological agent of gastritis. This bacterium can establish a persistent infection in the gastric mucosa, and the local production of chemokines represents an important step in the recruitment and activation of inflammatory cells. The chemokine IL-8 plays a pivotal role during *H. pylori* infection by promoting neutrophil infiltration, and gastric epithelial cells are among the main producers of this chemokine in gastric mucosa.[29] IL-8 mucosal or serum levels have been positively associated with gastric inflammation, even in the absence of *H. pylori*.[29,30] Furthermore, *H. pylori* strains, associated with peptic ulceration, induce a higher level of IL-8 in vitro[31] and increased IL-8 production has been correlated with more severe grades of gastritis. This chemokine is regulated at the transcriptional level by the transcription factors NF-κB and AP-1, and its secretion by gastric epithelial cells is induced by *H. pylori* or cytokines such as TNFα and IL-1β.[32] To test the inhibitory activity of oenothein B on IL-8 secretion, human gastric epithelial AGS cells were treated with TNFα (10 ng/mL) or IL-1β (10 ng/mL) in the presence of increasing concentrations of the ellagitannin (0.25−10 μM) for 6 h.

In both conditions, the pure compound inhibited IL-8 release in a concentration-dependent fashion (Figure 2, panels A, B), with low IC_{50} (0.54 and 1.29 μM for IL-8 release induced by TNFα or IL-1β, respectively). Since IL-8 release is NF-κB-dependent, we investigated whether oenothein B could impair the NF-κB pathway in AGS cells. Oenothein B inhibited the NF-κB-driven transcription induced by TNFα or IL-1β in a concentration-dependent manner, with IC_{50} 1.34 and 2.01 μM, respectively (Figure 3, panels A, B). To confirm the effect of the pure compound on the NF-κB pathway, cells were treated with TNFα or IL-1β in the presence of increasing concentrations of oenothein B for 1 h, and the amount of p65 in the nuclear fraction was measured with enzyme-linked immunosorbent assay (ELISA) and normalized using the protein content. The compound inhibited nuclear translocation induced by both cytokines with a similar profile (IC_{50} 1.18 and 2.72 μM, following induction with TNFα or IL-1β, respectively), thus confirming that inhibition of IL-8 release was due, at least in part, to impairment of the NF-κB pathway (Figure 3, panels C, D).

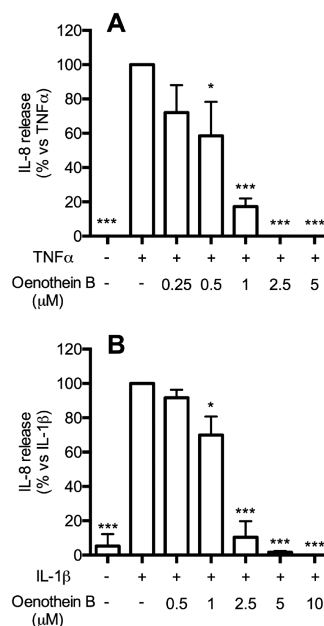

Figure 2. Oenothein B inhibits IL-8 release induced by TNF-α or IL-1β in AGS cells. To test the effect on IL-8 secretion, AGS cells were treated with 10 ng/mL TNF-α (panel A) or IL-1β (panel B) in the presence of increasing concentrations of oenothein B for 6 h. The amount of released IL-8 was measured using the ELISA assay. The graphs show the means ± standard deviation (sd) of at least three experiments. Treatment with the reference compound (20 μM EGCG) reduced the secretion by 70 and 36% for TNF-α and IL-1β, respectively. Statistical analysis: one-way analysis of variance (ANOVA), followed by Bonferroni as the post hoc test. *$p < 0.05$, ***$p < 0.0001$ versus stimulus alone.

None of the concentrations used in the assays (0.25−25 μM) showed signs of cytotoxicity in the 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay after 6 h of treatment.

The results obtained in our cell model clearly show that oenothein B significantly attenuates the release of IL-8 by gastric epithelial cells acting on the NF-κB pathway. Previous in vitro studies have shown that ETs are fairly stable in the physiological conditions of the stomach: gastric pH and digestive enzymes are not able to hydrolyze or modify ETs and they are not absorbed in this area,[33] suggesting possible action directly in situ. Moreover, oenothein B showed an anti-*H. pylori* effect on four different strains, thus increasing the interest in this pure ET as a potential antigastritis compound.[34]

This is the first time that oenothein B has been shown to have anti-inflammatory properties at the gastric level, supporting the beneficial effects attributed to the consumption of food enriched with ETs in the gastrointestinal tract.

3. CONCLUSIONS

ET-rich foods are very promising ingredients in the human diet. Here, we have provided further evidence on the additional health effects of native myrtle berry ETs. Myrtle berries, and the seeds in particular, have been found to contain high amounts of the valuable ETs eugeniflorin D_2 and oenothein B,

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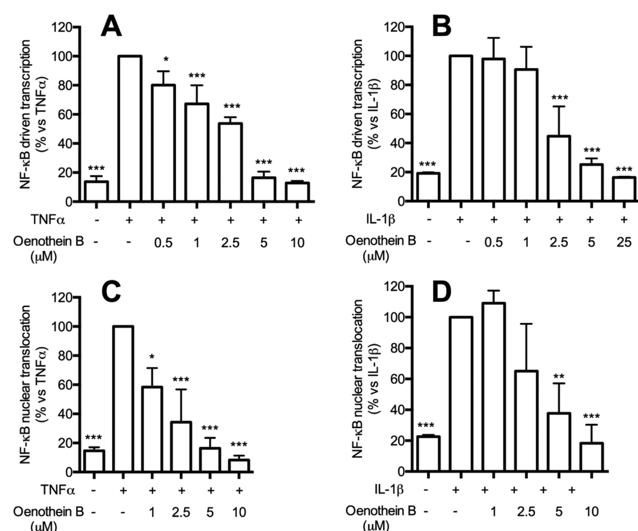

Figure 3. Oenothein B inhibits NF-κB-driven transcription and nuclear translocation induced by TNF-α or IL-1β in AGS cells. AGS cells were transiently transfected with the NF-κB-LUC plasmid and then treated with increasing concentrations of oenothein B in the presence of TNF-α (panel A) or IL-1β (panel B). Treatment with the reference compound (20 μM EGCG) reduced transcription by 85 and 64% for TNF-α and IL-1β, respectively. The amount of p65 in the nuclear fraction following treatment with oenothein B in the presence of the proinflammatory stimuli for 1 h was also assessed (panels C and D). Treatment with the reference compound (20 μM EGCG) reduced translocation by 90 and 64% for TNF-α and IL-1β, respectively. The graphs show the means ± sd of at least three experiments. Statistical analysis: one-way analysis of variance (ANOVA), followed by Bonferroni as the post hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$ versus stimulus alone.

and no differences were detected between wild and cultivated fruits. As the main ellagitannin detected, oenothein B was subjected to further studies to assess its biological properties. Here, we showed that it is able to inhibit the growth of several sensitive and fluconazole-resistant *Candida* species inhabiting the gastric tract, encouraging further investigations that could lead to the discovery of broader antifungal action and thus to characterization of a new antifungal compound. In addition, oenothein B was not only toxic for human cell lines but also showed growth inhibition properties against *H. pylori*[35] and anti-inflammatory activity in human gastric epithelial cells, suggesting that it may represent a possible support in the antigastritis therapy.

Taken together, our findings underline the value of *M. communis* L. seeds, without discriminating between samples obtained from cultivated or wild sources. They are a rich source of a valuable metabolite whose properties could be exploited by the food and pharmaceutical industries for the development of products useful for human health, taking advantage of the benefits derived from ellagitannin-enriched food consumption on the intestinal tract.

4. EXPERIMENTAL SECTION

4.1. General Section. *4.1.1. Solvents.* Methanol and acetone were of HPLC grade, acetonitrile was of LC−MS grade, and all were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid 98−100% was also purchased from Sigma-Aldrich. Milli-Q water was used for chromatography.

4.1.2. Apparatus. For chemical analysis, a ultrahigh-performance liquid chromatography−diode array detector−tandem mass spectrometry (UPLC−DAD−MS/MS, Waters Xevo TQ) apparatus equipped with an ACQUITY UPLC HSST3 column (1.8 μm, 2.1 × 100 mm^2) was used. Compound isolation was performed on a Waters HPLC equipped with a 2695 sample manager, 2996 PDA (Milford, MA), and a Supelco Discovery HS C18 column (10 mm × 250 mm, 10 μm). NMR analysis were performed using a Bruker Avance 400 MHz NMR spectrometer. Mass spectrometry was performed using a LTQ Orbitrap XL Hybrid Ion Trap-Orbitrap mass spectrometer (Thermo Fisher, Waltham, MA) equipped with an electrospray ion source (ESI).

4.2. Plant Material. Berries from cultivated *Myrtus communis* L. plants belonging to the Angela, Maria Rita, Rosella, Maria Elisa, Erika, Uta, Comune, Piccola, LAC11, and Michela Bianco cultivars were obtained from several farmers, while those from wild plants were collected at several locations on the island of Sardinia in the winters of 2008−2009, 2010−2011, and 2013−2014, as reported in Table 1. For each sample, a stock of berries was frozen soon after picking and kept at −20 °C till use, while another stock was weighed to register the whole mass and then crushed gently to manually separate the seeds from the pericarp to determine the relative mass. The analysis was performed in triplicate. One-way analysis of variance (ANOVA) followed by Bonferroni as a post hoc test was applied. Differences in p-values of <0.05 were considered to be statistically significant.

4.3. Mirto di Sardegna Liqueur. The samples of Mirto di Sardegna liqueur, collected from three producers, were prepared in compliance with the product specifications reported in the Decree of June 8, 2016, published in Italian Official Journal no. 139 of June 16, 2016. In short, the production requires an infusion of ripe, freshly collected berries, in stainless steel tanks, for at least 15 days, with an hydroalcoholic solution (≥40% vol). The infusion is then extracted, and demineralized water can be added to the berries for a second extraction. The berries are then pressed to recover the liquid. The two infusions and the press fraction are then pooled and filtered for the preparation of the liqueur. The liqueur is prepared by mixing the infusion with alcohol and a syrup made of water and sugar, and optionally honey (less than 15% of the sugar). Addition of antioxidants, colorants, preservatives, and other spirits is not allowed. Bottling can only occur in glass containers, and the final alcohol content is adjusted to a value between 28 and 36% vol. The minimum quantity of berries is 150 g/L in the final liqueur.

4.4. Compound Isolation. Aliquots of berries obtained from the *M. communis* L. cultivars listed above were mixed in equal amounts, and seeds were collected from a total of 1.2 kg of fresh berries. Seeds (ca. 270 g) were manually separated from the pericarp, washed under tap water, and then ground under liquid nitrogen using a laboratory mill to obtain a fine powder. The powder was defatted with hexane and then subjected to extraction with a solution of 70% acetone in water (1:10 w/v). The solid seed biomass was separated from the raw extract by centrifugation for 15 min at 4000g and 5 °C, and the extraction solution was evaporated in a rotary evaporator. The extract was suspended in 100 mL of methanol before injection in semipreparative high-performance liquid chromatography (HPLC).

Compound isolation was performed using a Waters HPLC equipped with a 2695 sample manager, 2996 PDA (Milford,

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MA), and a Supelco Discovery HS C18 column (10 mm × 250 mm, 10 μm). Mobile phases were composed of Milli-Q water (A) and acetonitrile containing 0.1% formic acid (B). The column temperature was set to 50 °C, the injection volume was 100 μL, and the flow rate was 1 mL/min. After an initial 6 min for equilibration, the gradient conditions were as follows: 0−1 min, 0% B; 1−5 min, 0−10% B; 5−10 min, 10−25% B; 10−20 min, 25−100% B; and 20−22 min 100% B.

Fractions containing the target compounds (retention range 2.8−3.6 min for compound **1** and 4−5.1 min for compound **2**) were collected with an automatic collector based on UV detection and the HPLC−UV chromatogram recorded at 270 nm. Fractions containing the single compounds were combined, the solvent was removed by rotary evaporation, and the residue was suspended in water and frozen at −20 °C. The samples were then lyophilized and prepared for nuclear magnetic resonance (NMR) measurements and mass spectrometry analysis.

4.5. NMR and Mass Spectrometry Experiments. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra for the purified ETs were recorded in acetone-d_6 and D_2O (only data for D_2O are reported here) at 298 K using a 5 mm BBI probe with 90° proton pulse length of 8.7 μs at a transmission power of 0 db and equipped with pulsed gradient field utility. The chemical shift scale (δ) was calibrated on the residual proton signal of deuterated acetone at δ_H 2.040 and δ_C 29.80 and on the residual proton signals of deuterated water at δ_H 4.78. The following NMR experiments were carried out: ^1H NMR; decoupled ^{13}C NMR; $^1H-^1H$ correlation spectroscopy; $^1H-^{13}C$ heteronuclear single quantum coherence; and $^1H-^{13}C$ heteronuclear multiple bond correlation.

For the mass spectrometry measurement, negative ion analysis was performed in data-dependent analysis mode. The ESI conditions in negative mode are as follows: spray voltage, −3.5 kV; heated capillary temperature, 320 °C; capillary voltage, −35 V; and tube lens, −110 V. In the LTQ component of the instrument, nitrogen was used as both the sheath gas (35 U) and auxiliary gas (5 U), and helium was used as the damping gas. All measurements were made using the automatic gain control of LTQ to adjust the number of ions entering the trap. Mass calibration was performed with every sequence run just prior to starting the batch, using the flow injection of the manufacturer's calibration standard mixture, allowing for mass accuracy lower than 5 ppm in external calibration mode. Full-scan accurate mass spectra were obtained at a mass resolution of 30 000 FWHM (m/z 400) in profile mode, while the resolving power for MS^2 scans was 7500. Product ions were generated in the LTQ trap at a collision energy of 35 eV using an isolation width of 1 Da.

4.5.1. Compound 1. According to NMR and HRMS data, compound **1** was identified as eugeniflorin D_2 (Figure 4): ^1H NMR (400 MHz, deuterium oxide) δ 7.16 (s, galloyl-I G, 2H), 7.15 (d, J = 2 Hz, dehydrovaloneoyl, 1H), 7.09 (s, galloyl-II′, 2H), 6.80 (s, valoneoyl, 1H), 6.77 (s, valoneoyl, 1H), 6.58 (s, valoneoyl, 1H), 6.49 (s, valoneoyl, 1H), 6.04 (s, valoneoyl, 1H), 6.05 (d, J = 2.4 Hz, H1-αglucose, 1H), 5.73 (t, J = 9.8 Hz, H3-αglucose, 1H), 5.57 (dd, J = 2.4, 9.8 Hz, H2-αglucose, 1H), 5.49 (brs, dehydrovaloneoyl, 1H), 5.28 (t, J = 9.8 Hz, H2-βglucose), 5.25 (dd, J = 13.5, 6.0 Hz, H6-αglucose, 1H), 5.22 (t, J = 9.8 Hz, H3-βglucose, 1H), 4.96 (t, J = 9.8 Hz, H4-βglucose, 1H), 4.93 (dd, J = 14.1, 5.8 Hz, H6- βglucose, 1H), 4.78 (d, 8.0, H1-βglucose, 1H), 4.66 (t, J = 8.0 Hz, H4-βglucose, 1H), 4.57 (dd, J = 6.5, 9.8, H5-αglucose, 1H), 4.47 (t, J = 9.8, H4-αglucose, 1H), 4.25 (dd, J = 9.8, 6.4 Hz, H5-βglucose, 1H), 3.97 (d, J = 13.5 Hz, H6-αglucose, 1H), 3.79 (d, J = 14.1 Hz, H6-βglucose, 1H). Through ^1H NMR signal integration, the purity of eugeniflorin D_2 was estimated to be around 90%.

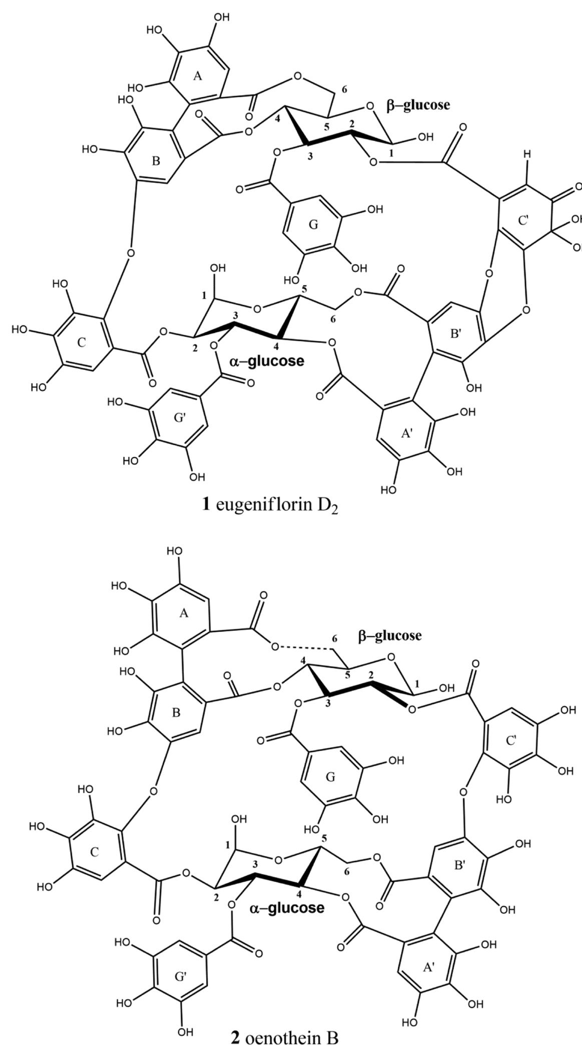

Figure 4. Structures of eugeniflorin D_2 and oenothein B.

HRMS data for compound **1**: ESI(−)MS: $[M - H]^-$ 1583.1383 ± 0.008, calculated for $C_{68}H_{47}O_{45}^-$: 1583.1395; $[M - 2H - H_2O]^{2-}$ 782.0604 ± 0.004, calculated for $C_{68}H_{44}O_{44}^{2-}$: 782.0608.

4.5.2. Compound 2. According to NMR and HRMS data, compound **2** was identified as oenothein B (Figure 4).

NMR data of compound **2**: ^1H NMR (400 MHz, deuterium oxide) δ 7.16 (s, ring G, 2H), 6.86 (s, ring G′, 2H), 6.78 (s, ring C′, 1H), 6.69 (s, ring B, 1H), 6.58 (s, ring A′, 1H), 6.51 (s, ring C, 1H), 6.49 (s, ring B′, 1H), 6.44 (s, ring A, 1H), 5.82 (t, J = 10.3 Hz, H3-αglucose, 1H), 5.60 (d, J = 3.0 Hz, H1-αglucose, 1H), 5.55 (t, J = 9.0 Hz, H4α-glucose, 1H), 5.47 (t, J = 9.0 Hz, H3-βglucose), 5.30 (dd, J = 13.5, 6.0 Hz, H6-αglucose, 1H), 5.22 (t, J = 8.7 Hz, H2-βglucose, 1H), 5.10 (t, J = 10.1 Hz, H4-βglucose, 1H), 4.85 (dd, J = 14.1, 5.8 Hz, H6-βglucose, 1H), 4.74 (m, H2-αglucose, 1H), 4.70 (d, J = 8.0 Hz, H1-βglucose, 1H), 4.66 (dd, J = 9.8, 5.2 Hz, H5-αglucose, 1H), 4.19 (dd, J = 9.8, 5.2 Hz, H5-βglucose, 1H), 3.98 (d, J = 13.5 Hz, H6-βglucose, 1H), 3.95 (d, J = 14.1 Hz, H6-α-glucose, 1H). Through ^1H NMR signal integration the purity

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Table 4. UPLC−ESI−MS/MS Conditions for Quantification and Identification of Metabolites

metabolite	rt (min)	ESI mode	cone voltage	MRM transitions quantifier ion	MRM transitions qualifier ion	supplier
acid ellagic	4.03	+	32	302.91 → 257.2 (26)	302.91 → 201.1 (32)	Sigma-Aldrich
eugeniflorin D_2	1.63	−	34	1582.8 → 935.2 (40)	1582.8 → 831.0 (37)	isolation
onothein B	2.54	−	38	1576.2 → 765.2 (42)	503.41 → 937.2 (44)	isolation

of oenothein B was estimated to be around 93%. ^{13}C NMR (100 MHz, D_2O) δ 170.58 (s), 170.10 (s), 169.72 (s), 169.40 (s), 167.82 (s), 166.98 (s), 147.15 (s), 145.70 (s), 145.27 (s), 144.95 (s), 144.54 (s), 144.18 (s), 144.03 (s), 143.17 (s), 142.59 (s), 141.89 (s), 140.51 (s), 139.51 (s), 139.41 (s), 139.30 (s), 139.08 (s), 138.53 (s), 138.48 (s), 136.76 (s), 136.39 (s), 135.87 (s), 135.31 (s), 126.00 (s), 125.66 (s), 125.58 (s), 123.36 (s), 121.88 (s), 120.53 (s), 119.98 (s), 117.50 (s), 117.09 (s), 115.74 (s), 115.63 (s), 114.14 (s), 113.94 (s), 111.19 (d, 2C), 110.83 (d), 109.81 (d), 109.70 (d, 2C), 107.76 (d), 106.33 (d), 95.55 (d), 90.23 (d), 75.54 (d), 75.03 (d), 73.12 (d), 71.79 (d), 71.38 (d), 71.23 (d), 70.24 (d), 68.34 (d), 64.46 (t), 63.97 (t).

HRMS data for compound **2**: $[M - H]^-$ 1567.1398 ± 0.008, calculated for $C_{68}H_{47}O_{44}^-$: 1567.1446; $[M - 2H]^{2-}$ 783.0668 ± 0.004, calculated for $C_{68}H_{46}O_{44}^{2-}$: 783.0686.

4.6. Sample Preparation and Chemical Analysis.

Pulp, seeds, and liqueur were subjected to chemical analysis. Samples of pulp and seeds obtained from 10 g of fresh berries for each sample were extracted separately with 20 mL of a solution of acetone and water (70:30 v/v). Traces of the solvent were removed using a rotary evaporator, and the extract was suspended in a 20 mL solution of methanol and water (50/50) before chemical analysis. Samples of three commercial Mirto di Sardegna liqueurs were mixed with an equal volume of a 50% methanol solution and stored at −20 °C before chemical analysis via ultrahigh-performance liquid chromatography−diode array detection−tandem mass spectrometry (UPLC−DAD−MS/MS).

Extracts were analyzed according to the method described by Vrhovsek et al.[36] for the presence of ellagic acid and oenothein B using a UPLC−DAD−MS/MS (Waters Xevo TQ) apparatus equipped with an ACQUITY UPLC HSST3 column (1.8 μm, 2.1 × 100 mm^2) and separation was carried out at 40 °C. The mobile phase was composed of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile). Acetonitrile of LC−MS grade and pure standard of ellagic acid (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO), while oenothein B was isolated as reported above. Milli-Q water was used for chromatography.

The flow rate was set to 0.4 mL/min, and the gradient profile was the following: 0 min 5% B, from 0 to 3 min linear gradient to 20% B, 80% A; from 3 to 5 min, linear gradient to 95% B; isocratic elution from 5 to 7 min and from 7 to 10 min and re-equilibration to the initial conditions of 5% B. The injection volume was 2 μL for both sample and standard solutions. Each sample was analyzed in triplicate. After each injection, the needle was rinsed with 600 μL of a weak washing solution (water/methanol, 90:10) and 200 μL of a strong washing solution (methanol/water, 90:10). Samples were kept at 6 °C during the analysis. The DAD spectrum was recorded between 200 and 600 nm. Mass spectrometry detection was performed on a Waters Xevo TQMS (Milford, MA) instrument equipped with an electrospray (ESI) source. Capillary voltage was 3.5 kV in positive mode and −2.5 kV in negative mode; the source was kept at 150 °C; the desolvation temperature was 500 °C; the cone gas flow was 50 L/h; and the desolvation gas flow was 800 L/h.

Each metabolite was directly infused in the MS system in negative mode with 50:50 v/v of two mobile phases A and B to optimize the detection process. The two most abundant fragments were selected for each metabolite, one as a quantifier (most abundant) and the other as a qualifier ion using the multiple reaction monitoring (MRM) method for selective quantification. The MRM conditions were optimized automatically by Waters Intellistart software. MRM transitions for ellagic acid, eugeniflorin D_2, and oenothein B are reported in Table 4.

For the quantification, linear calibration curves were obtained by the injection of 10 dilutions of the stock solution in a concentration range of 0.178−670 mg/L for oenothein B and 0.130−208 mg/L for eugeniflorin D_2. The calibration curve of ellagic acid was obtained by the injection of eight dilutions of the stock solution in a concentration range of 0.056−180 mg/L. All standard solutions were prepared in water. Data processing, calibration curve calculation, and quantification were carried out by Waters TargetLynx software. All curves showed good linearity over the concentration range. The limit of detection (LOD) for onothein B was 0.059 mg/L, and the limit of quantification (LOQ) was 0.244 mg/L. For eugeniflorin D_2, the LOD was calculated to be 0.064 mg/L and the LOQ was 0.190 mg/L, while for ellagic acid, the LOD was 0.028 mg/L and the LOQ was 0.095 mg/L.

4.7. Antifungal Susceptibility Assay.

For antifungal susceptibility testing, clinical strains isolated from human gut *C. albicans* MFB005FS3, *C. albicans* MFB008MM1, *C. albicans* YMS 102_2, *C. parapsilosis* MFB005FS5, *C. glabrata* MFB004, *C. glabrata* MFB005FS4, *C. glabrata* RTT99_3, and *C. tropicalis* RTT037-3[37] were tested for their susceptibility to oenothein B.

Yeasts were grown on Sabouraud agar medium (Sigma-Aldrich, St. Louis, MO) for 48 h at 30 °C and resuspended in distilled water at a concentration of (1−5) × 10^5 CFU/mL before testing.

Yeasts were tested for their susceptibility to oenothein B (five dilution series, ranging from 125 to 8 μg/mL) following the European Committee for Antimicrobial Susceptibility Testing protocol.[38] Briefly, cells were grown in RPMI-1640 medium supplied with 2.0% glucose, counted, and inoculated at a concentration of (1−5) × 10^5 CFU/mL. Minimal inhibitory concentration (MIC) values were determined using a spectrophotometer (at 530 nm) after 48 and 120 h of incubation as the lowest concentration of the drug that resulted in a ≥50% inhibition of growth relative to the growth control.

Growth curves for oenothein-treated and untreated *Candida* spp. were recorded using a spectrophotometer (at 530 nm). Inoculated plates were kept in the dark at 37 °C, with gentle agitation, and the cell density values were measured every 30 min for over 65 h.

4.8. Anti-inflammatory Assay.

Human adenocarcinoma epithelial gastric cells (AGS, CRL-1739, LGC Standard S.r.l.,

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Milan, Italy) were grown at 37 °C in DMEM F12 (Gibco-Invitrogen) supplemented with 100 U penicillin/mL, 100 mg of streptomycin/mL, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum (FCS) (Euroclone S.p.A, Pero, Italy) in a humidified atmosphere containing 5% CO_2. During treatments with oenothein B or vehicle alone (<0.1% dimethyl sulfoxide), cells were placed in a medium deprived of FCS.

To test IL-8 release, AGS cells were grown in 24-well plates (30 000 cells/well) for 48 h; then, cells were treated with the proinflammatory stimuli TNFα or IL-1β (10 ng/mL) in the presence of oenothein B. IL-8 release reached its maximum at 6 h, and this time was selected for experiments to test the effect of the individual compound (0.25−10 μM). After 6 h, the medium was collected and stored at −20 °C until IL-8 quantification with an enzyme-linked immunosorbent assay (ELISA) kit (Peprotech, Rocky Hill, NJ), as previously described.[17] These assays were performed in duplicate, and at least three experiments were performed.

To evaluate the effect on NF-κB-driven transcription, cells were plated in 24-well plates (30 000 cells/well). After 48 h, cells were transiently transfected using the calcium-phosphate method with the reporter plasmids NF-κB-LUC (50 ng/well), which contain the luciferase gene, under the control of three κB-responsive elements. The plasmid was a gift from Dr. N. Marx (Department of Internal medicine, Cardiology, University of Ulm, Germany). After 16 h, cells were stimulated with TNFα or IL-1β (10 ng/mL) and pure ellagitannin was tested at 0.5−25 μM. After 6 h, cells were lysed and luciferase assay was performed using BritelitePlus reagent (PerkinElmer Inc., Massachusetts), according to the manufacturer's instructions; the signal was read with VictorX3 (PerkinElmer, Walthman MA). Data were expressed considering 100% of luciferase activity related to cytokine-induced NF-κB-driven transcription. These assays were performed in triplicate within each experiment, and at least three experiments were performed.

To assess the effects of oenothein B on NF-κB (p65) nuclear translocation, AGS cells were plated at a density of 1.5×10^6 cells/mL in 100 mm plates. After 48 h, cells were treated for 1 h with TNFα or IL-1β (10 ng/mL) and oenothein B (1−10 μM). Nuclear extracts were prepared using the nuclear extraction kit from the Cayman Chemical Company (Michigan), and stored at −80 °C until assay. The same amount of total nuclear proteins (10 μg/well), measured according to the Bradford (Bio-Rad) assay, was used to assess NF-κB nuclear translocation using the NF-κB (p65) transcription factor assay kit (Cayman) followed by spectroscopy at 450 nm, 0.1 s (VictorX3, PerkinElmer, Walthman MA). Data were expressed considering 100% of absorbance related to cytokine-induced NF-κB nuclear translocation. These assays were performed in duplicate within each experiment. At least three experiments were performed. Epigallocatechin 3-*O*-gallate (EGCG) 20 μM was used as the reference compound for each inflammation-related test.

4.9. Cytotoxicity. The cytotoxicity of oenothein B was evaluated with the 3,4,5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT) assay, as previously described.[39]

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Notes

The authors declare no competing financial interest.

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