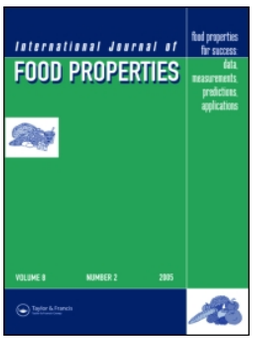

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Screening and comparative study of *in vitro* antioxidant and antimicrobial activities of ethanolic extracts of selected Vietnamese plants

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ABSTRACT

This study aimed to screen the *in vitro* antioxidant and antimicrobial activities of ethanolic extracts from 20 plants and three herbal commercial products empirically used for aquaculture improvement in Vietnam. The results of 2,2-diphenyl-1-pycrylhydrazyl (DPPH) radical scavenging assays showed that *Phyllanthus amarus* extract was the strongest antioxidant, followed by four extracts in the subsequent order: *Piper betle* > *Psidium guajava* > *Euphorbia hirta* > *Mimosa pudica*. These five plant extracts were very active in a DPPH radical scavenging assay with concentrations needed to scavenge half of the DPPH (IC_{50}) below 30 µg/mL. Seven plant extracts showed an IC_{50} ranging from 31.9 to 59.7 µg/mL, while eleven extracts showed an IC_{50} above 70 µg/mL. A positive association was found between phenolic content (expressed as gallic acid equivalents) and antioxidant activity of the plant extracts. Concerning *in vitro* antimicrobial activities, *P. amarus* extract showed the highest activity against two different strains of *Aeromonas hydrophila* as demonstrated by its low minimal inhibitory concentration (MIC) of 156 and 625 µg/mL, respectively; whereas, *P. betle* displayed a moderate activity against *Edwardsiella ictaluri* with a MIC value of 625 µg/mL. Tannins were observed as significant factors contributing to antioxidant and antimicrobial properties of the plant extracts tested.

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Phyllanthus amarus; *Aeromonas hydrophila*; *Edwardsiella ictaluri*; tannins; Vietnam

Introduction

Medicinal therapies using plant extracts expanded popularly in the late 1990 s.[1] Beside the Chinese herbs, during the last decades, Vietnamese medicinal plants have also received interest as novel sources of alternative medication. It is estimated that approximately 2500 species of the Southeast Asian tropical exotic herbals have been used in folk medicine for their biological/therapeutic properties,[2,3] such as diuretic,[4] antioxidant,[5] cytotoxicity,[6,7] and antimicrobial.[8] Moreover, a variety of herbs and plants have been receiving enormous interest as alternatives to synthetic additives or preservatives in the food industry in general and in aquaculture products in particular.[9]

Aquaculture in Vietnam has been growing remarkably in recent years, producing 3.84 million tons aquaculture products in 2017.[10] Mekong Delta is the main fish production area in the

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Southern part of Vietnam with a contribution of 70% to the national aquaculture production. Striped catfish (*Pangasianodon hypophthalmus*) is the dominating cultured species for export with an annual production of 1.25 million tons.[11] However, *Aeromonas hydrophila* and *Edwardsiella ictaluri* are pathogenic bacteria causing major diseases in the striped catfish industry, including "bacillary necrosis of pangasius" (BNP)[12] or motile aeromonad septicemia (MAS),[13] which is often related to stressed or immunocompromised hosts.[14]

Chemicals including antimicrobials have been extensively applied to control pathogens and water quality management.[15,16] The imprudent use of antimicrobials to control bacterial disease in fish farming may lead to development of antibiotic resistant bacteria, residues of antimicrobials in fish products, environmental impacts, etc. The access to export markets is certainly warned by the risk of noncompliance to international food safety regulations and quality standards. The final outcome could be a decline of profits made by the aquaculture sector, which is one of the most important activity in Vietnam (especially in the Mekong Delta).

Nowadays, more environment friendly prophylactic and protective solutions are claimed and natural bio-active products are examined for enhancing the immune system and health status of cultivated animals.[17,18] However, in spite of a great variety of wild plants allocated in the various eco-regions of Vietnam and the concern of aquaculture farmers in using alternatives to antibiotics, the use of natural products in aquaculture is not yet popular in the country.

In this study, 20 plants, which are locally available, inexpensive and have been empirically used by fish farmers in the Mekong Delta regions, have been selected for a literature review about their antioxidant and antimicrobial activities (in particular against *A. hydrophila* and *E. ictaluri*). Ethanolic extracts of these 20 selected plants have been tested *in vitro* testing for these activities (Table 1). Besides these 20 plants, three commercial products have been added (Table 2), coming from Vietnamese companies, which are supposed to be efficient in treating white feces syndrome, enteritis and hepatic disease in shrimp, as well as in regenerating liver tissue in fish.

Materials and methods

Chemicals and media

DPPH (2,2-diphenyl-1-pycrylhydrazyl), (±)-α-tocopherol, Folin-Ciocalteu's reagent, gallic acid, resazurin and gentamicin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Brain Heart Infusion media (BHI), isosensitest media, buffer pepton water (BPW) and plate count agar (PCA) were obtained from Oxoid (Basingstoke, UK). All solvents and reagents used in the analysis were of analytical grade.

Plant extracts preparation

Twenty fresh plants (Table 1) were collected from various areas in Mekong Delta, Vietnam. The plants were authenticated by the Department of Biology, College of Natural Science, Can Tho University and compared to literature. All collected parts of plants were then washed to remove mud and dust; the rotten and damaged parts were also discarded. Samples were air dried in shade for some days and then put in an oven at about 60°C until well-dried. After that, they were ground into a fine powder with a blender and stored in sealed containers in a dry and cool place. The dried-powder (100 g) was soaked in ethanol 96% (800 mL) for at least 24 hours at room temperature with frequent agitation. The solvent-containing extracts were then decanted and filtered. The ground samples were further extracted 4 times with ethanol 96%. The filtrates from each extraction were combined and the solvent was evaporated under reduced pressure using a rotary evaporator to give crude ethanolic extracts.

Three commercial products, named A, B, C were purchased from purchasers willing to stay anonymous. However, they gave us as a personal communication the plant composition of their

Table 1. List of 20 selected plants used for the screening of their antioxidant and antimicrobial activities.

Identification number*	Scientific name	Common name	Ecology	Distributed in Vietnam	Level of threats in the wild	Status of species	Part used	Reference
CTU1731	*Ageratum conyzoides*	Billygoat-weed, chick weed	Grow and develop on different soils; from lowland to highland 1800 m	Widely grown in Vietnam	Low	Invasive	The whole plant	[19–21]
CTU1922	*Allium sativum*	Garlic	Spice herb	Widely grown in Vietnam	No	Native	Bulb	[19–21]
CTU1716	*Alternanthera sessilis*	Sessile joyweed and dwarf copperleaf	Uncultivated growth in the garden, paths at edge of ricefield, edge of canal	Widely grown in Vietnam (at height 1000 m)	Low	Native	Aerial parts (leaves + twigs)	[19–21]
CTU1720	*Annona reticulata*	Custard apple; bullock's heart; raamphal plant	Aquatic growth, edge of canal (alum tolerant tree)	Widely grown in Mekong Delta	Low	Native	Leaves	[20–22]
CTU1608	*Azadirachta indica*	Neem	Grow in the forest, leaves harvested as vegetable	Forest: Ninh Thuận, An Giang, Kiên Giang; cultivated in the South of Vietnam	Low	Native	Leaves, flower and stem bark	[19–21]
CTU17161	*Cayratia trifolia*	Fox grape	Scattered growth in secondary forest, shrub, garden	Widely grown in Vietnam	Low	Native	The whole plant	[20,21,23]
CTU1823	*Centella asiatica*	Asiatic pennywort, Indian pennywort	Grow in clusters at wild land, paths at edge of ricefield, roadside	Widely grown in Vietnam	Low	Native	The whole plant	[20,21,23]
CTU1836	*Eclipta prostrata/ Eclipta alba*	False daisy	Grow in paths at edge of ricefield, roadside, from lowland to highland 1800 m	Widely grown in Vietnam	Low	Native	The whole plant	[19–21]
CTU1874	*Euphorbia hirta*	Asthma-plant	Photophilic herb, grow in wild land, paths at edge of ricefield	Widely grown in Vietnam	Low	Native	The whole plant	[20,21,23]
CTU17174	*Houttuynia cordata*	Fish mint	Grow in clusters at wild land, paths at edge of ricefield, edge of canal	Uncultivated and widely grown in Vietnam	Low	Native	Leaves + stem	[21,22,24]
CTU17113	*Mimosa pudica*	Sensitive plant, sleepy plant, shy plant	Grow in roadside, wild land, sea wall	Widely grown in Vietnam (exotic species)	Medium	Introduced*	The whole plant	[21,22,24]
CTU1871	*Momordica charantia* L.	Bitter melon, bitter squash	Uncultivated growth in secondary forest or cultivated in farms	Widely grown in Vietnam (as vegetable)	Low	Native	Leaves, stem	[21,22,24]
CTU1896	*Ocimum basilicum*	Basil	Photophilic herb	Widely grown in Vietnam (as spice herb)	No	Native	Aerial parts (leaves + twigs)	[21,23,24]
CTU1899	*Perilla frutescen*	Perilla mint	Photophilic herb	Widely grown in Vietnam (as spice herb)	No	Native	Leaves	[21,23,24]
CTU1778	*Phyllanthus amarus*	Chamber bitter, gripe-weed, shatterstone, stone-breaker or leaf-flower	Uncultivated grow in paths of grass, paths at edge of ricefield or mountain field, roadside	Widely grown in Vietnam	Low	Native	Aerial parts (leaves + twigs)	[21,23,24]
CTU1623	*Piper betle*	Betel	Planted in home gardens	Widely grown in Vietnam	No	Native	Leaves	[21,22,24]

(*Continued*)

Table 1. (Continued).

Identification number*	Scientific name	Common name	Ecology	Distributed in Vietnam	Level of threats in the wild	Status of species	Part used	Reference
CTU17137	*Portulaca oleracea*	Purslane	Scattered growth or in clusters at wild land, edges of ricefield	Widely grown in Vietnam	Low	Native	The whole plant	[19,21,24]
CTU17125	*Psidium guajava*	Apple guava	Planted in farms	Widely grown in Vietnam	Low	Introduced*	Leaves	[21,23,24]
CTU1644	*Wedelia chinensis*	-	Uncultivated grown in paths of grass	Widely grown in the Central and North of Vietnam	Low	Native	Leaves	[19,21,24]
CTU1898	*Zingiber officinale* Rosc.	Ginger	Uncultivated grown in forest	Widely grown in Vietnam	Low invasion	Native	Rhizome	[19,21,24]

*Species identification was done in/by Can Tho University (CTU), Vietnam.
Introduced*: this introduced species was perennially planted in Mekong Delta, Vietnam.

Table 2. List of 3 commercial products used for the screening of their antioxidant and antimicrobial activities.

Product name	Composition*	Form
A	*Blumea balsamifera*	Solid
B	*Cynara cardunculus* var. *scolymus* *Phyllanthus emblica* *Terminalia arjuna*	Liquid
C	*Phyllanthus urinaria* *Eclipta prostrata*	Solid

*Personal communication from the purchasers who asked to remain anonymous

product (Table 2). For both commercial powder products, a 1 g dry sample was continuously shaken in 30 mL ethanol (96%) for 24 hours. After filtration, the filtrates obtained were evaporated to dryness as explained above. The liquid commercial product was lyophilized. All ethanolic extracts were finally lyophilized until dryness to remove any trace of water and stored in a dry place at room temperature before use.

Total phenolic compounds determination

The amount of total phenolic compounds (TPC) was determined using Folin-Ciocalteu reagent.[25] This assay was performed in triplicate for all plant extracts, dissolved in methanol. A standard curve was established using gallic acid as standard, in the range of 0–10 µg/mL (in methanol). The amount of total phenolic compounds in plant extracts was calculated as gallic acid equivalents (GAE) in mg of GAE per 100 mg (%) of freeze-dried plant material.

Antioxidant capacity

Antioxidant capacity of plant extracts was measured through DPPH (2,2′-diphenyl-1-picrylhydrazyl) radical scavenging assay.[26] The assay was performed in triplicate for each plant extract sample and α-tocopherol was used as a positive control. Plant extracts dissolved in methanol were tested for their DPPH scavenging activity in concentrations ranging from 1 to 125 µg/mL. Plant extract concentrations were plotted against percentages of remaining DPPH after 30 minutes reaction. A sigmoidal curve was then fitted, allowing the determination of the $IC_{50,}$ i.e. the plant extract concentration (in µg/mL) needed to scavenge 50% of the DPPH initially introduced.

In vitro investigation of antimicrobial activities of plant extracts

Resazurin method was performed as described by Sarker et al.[27] with some adaptations for minimum inhibitory concentration (MIC) determination of plant extracts against *A. hydrophila* and *E. ictaluri*. Stock solutions of the plant extracts were prepared at a concentration of 10 mg/mL in sterilized normal saline containing 25% dimethyl sulfoxide (DMSO) (when testing *A. hydrophila)* or 12.5% DMSO (when testing *E. ictaluri)*. The resazurin and the antibiotic gentamicin solutions were prepared in sterile distilled water at the concentration of 10 mg/mL and 200 µg/mL, respectively. Two strains of *Aeromonas hydrophila* (1 and 2) were isolated from red tilapia (*Oreochromis* sp.), whereas, *Edwarselia ictaluri* was isolated from striped catfish (*P. hypophthalmus*) in Mekong Delta, Vietnam. These strains were subcultured in BHI broth, incubated for 24 h at 37°C (*A. hydrophila*) and for 48 h at 30°C (*E. ictaluri*), and then pelleted in 20 mL of sterile normal saline solution. The approximate number of bacteria were estimated by the optical density at 600 nm, reaching a value of 4×10^8 and 1×10^8 cfu/mL for *A. hydrophila* and *E. ictaluri*, respectively according to the turbidity of McFarland Standards (e.g., Dalynn Biologicals, cat no. TM50-TM60). Further dilutions were performed to use final concentrations of 2×10^4 in double strength isosensitest broth and 0.5×10^4

cfu/mL in BHI broth for *A. hydrophila* and *E. ictaluri*, respectively. One mg of resazurin dissolved in water was then added into 10 mL of the liquid inoculum.

The MIC determination was performed in a 96-well plate, using concentrations of plant extracts ranging from 5 µg/mL to 2500 µg/mL and 100 µL of working inoculum solution per well. The plates were prepared in triplicate and gentamicin was used as positive control in each plate. After incubation at 37°C for 18 h (*A. hydrophila*) or 48 h (*E. ictaluri*), plates were visually observed to determine the lowest concentration at which color changed from purple to pink, which was taken as the MIC value.[28]

Tannins quantification and removal from plant extracts

The extracts of *P. amarus* and *E. hirta* were prepared at 10 mg/mL in methanol. Tannins were quantified according to the European pharmacopoeia method and the results were expressed in pyrogallol equivalents (g/100 g extract).[29] Tannins were excluded from methanolic plant extracts by using a polyamide column (Macherey – Nagel, Germany), as described by Houghton and Raman.[30] Ten grams of polyamide were soaked in 100 mL of water during one night. A column was packed with the gel. Ten milligrams of plant extract dissolved in 1 mL of methanol were loaded onto the column. A volume of 200 mL of methanol was gradually poured into the column for the elution. An empty dried flask was prepared to collect the eluates, which were evaporated to dryness. Tannins were retained on the column while the dried residue contained the extract free from tannins.

Statistical analysis

Data of total phenolic content and IC_{50} values were expressed as mean ± standard deviation by Microsoft Excel software. Analysis of variance (one-way ANOVA) was performed by using SPSS 16.0 (SPSS Inc, Chicago, II, USA).

Results and discussion

Total phenolic content and antioxidant activity of 23 plant extracts

The content of phenolic compounds determined in the 23 plant extracts samples ranged between 0.5 and 18.8 mg gallic acid equivalent/100 mg plant extract (Table 3). Among the first five plant extracts mentioned in Table 3, considered as having a "high" antioxidant activity (see below), *P. amarus* showed the significantly ($p < .05$) highest total phenolic content. Interestingly, the commercial products contained lower levels of phenolic compounds than these 5 plant extracts.

The antioxidant activity measured using the DPPH assay was expressed as an IC_{50}, corresponding to the concentration of plant extract needed to scavenge half of the DPPH. According to Thiangthum et al.[26], the antioxidant activity of samples can be classified as high ($IC_{50} < 30$ µg/mL), intermediate ($30< IC_{50} < 50$ µg/mL), low ($50< IC_{50} < 70$ µg/mL) or absent ($IC_{50} > 70$ µg/mL). In our assay, the IC_{50} of the (±)-α-tocopherol, the reference antioxidant, was 12 µg/mL. Eleven out of the 23 extracts displayed an antioxidant activity, with concentrations able to inhibit half of the maximum response (IC_{50}) ranging from 5.83 to 49.5 µg/mL (Table 3). *P. amarus* extract showed the apparent strongest radical scavenging effect ($IC_{50} = 5.83$ µg/mL) but no significant difference ($p > .05$) was observed with the antioxidant activities of *P. betle* and *P. guajava* extracts. The remaining 2 samples showing a high antioxidant activity (i.e. $IC_{50} < 30$ µg/mL) were *E. hirta* and *M. pudica*. A group of six samples, including *Z. officinale*, commercial product B, *E. prostrata*, commercial product A, *A. reticulata, H. cordata*, showed an intermediate antioxidant capacity (i.e 30 µg/mL $< IC_{50} < 50$ µg/mL).

For the 23 selected plant extracts, the antioxidant activity was related with their phenolic content with a correlation coefficient $R^2 = 0.9137$ (Figure 1). The antioxidant properties of plant extracts might be influenced by the number of specific chemical groups from phenolic compounds, such as

Table 3. Total phenolic content and results from the DPPH radical scavenging assay (IC_{50}) of 23 plant extracts (the plants are shown by decreasing antioxidant capacity, i.e. increasing IC_{50}), and comparison with data from the literature.

	This study		Literature studies		
Scientific name	Phenolic content (%)*	IC_{50} (µg/mL)	Phenolic content (%)*	IC_{50} (µg/mL)	Reference
Phyllanthus amarus	$18.8^{j} \pm 0.75$	$5.83^{a} \pm 0.50$	17	11	[26,31]
Piper betle	$16.4^{i} \pm 0.76$	$8.32^{a} \pm 0.90$	15.4	11	[32]
Psidium guajava	$14.5^{h} \pm 0.79$	$8.55^{a} \pm 0.53$	15.6	1.56	[33]
Euphorbia hirta	$10.3^{g} \pm 0.91$	$17.8^{b} \pm 1.28$	29.1	2.81	[34]
Mimosa pudica	$7.46^{f} \pm 0.29$	$18.0^{b} \pm 0.96$	4.3	127	[35]
Zingiber officinale	$6.92^{f} \pm 0.31$	$31.9^{c} \pm 2.48$	1	46.5	[36]
Commercial product B	$0.53^{a} \pm 0.04$	$33.7^{cd} \pm 0.34$	-	-	-
Eclipta prostrata	$4.57^{d} \pm 0.25$	$36.6^{cd} \pm 0.86$	13.2	42	[37]
Commercial product A	$5.65^{e} \pm 0.20$	$38.1^{d} \pm 2.55$	-	-	-
Annona reticulata	$5.01^{de} \pm 0.24$	$45.1^{e} \pm 4.58$	13.6	51	[38]
Houttuynia cordata	$5.45^{e} \pm 0.28$	$49.5^{e} \pm 5.83$	12.6	73	[39]
Cayratia trifolia	$3.31^{c} \pm 0.22$	$59.7^{f} \pm 5.11$	7.3	74	[40,41]
Perilla frutescens	$3.61^{c} \pm 0.21$	$73.7^{g} \pm 2.09$	11.6	7.97	[42]
Azadirachta indica	$4.33^{d} \pm 0.39$	$79.2^{h} \pm 3.93$	10.1	60.6	[43]
Commercial product C	$2.04^{b} \pm 0.17$	$85.9^{i} \pm 1.95$	-	-	-
Ageratum conyzoides	$2.05^{b} \pm 0.07$	$118^{j} \pm 1.92$	0.85	214	[44]
Portulaca oleracea	ND	> 125	0.43	2950	[45]
Allium sativum	ND	> 125	0.005	600	[46]
Ocimum basilicum	$2.25^{b} \pm 0.26$	> 125	0.07	350	[47]
Centella asiatica	$2.27^{b} \pm 0.09$	> 125	2.4	45	[48]
Wedelia chinensis	$1.66^{b} \pm 0.02$	> 125	-	45	[49]
Momordica charantia	ND	> 125	1.02	307	[50]
Alternanthera sessilis	$1.73^{b} \pm 0.11$	> 125	3.7	946	[51,52]

*mg gallic acid equivalent/100 mg dry plant extract; ND = Not determined; "-": no information available.
High antioxidant activity: IC_{50} < 30 µg/mL, Intermediate antioxidant activity: 30< IC_{50} < 50 µg/mL, No activity: IC_{50} > 70 µg/mL.
Values are mean ± SD (n = 3). Mean values within a column with the same letter are not significantly different ($p > 0.05$).

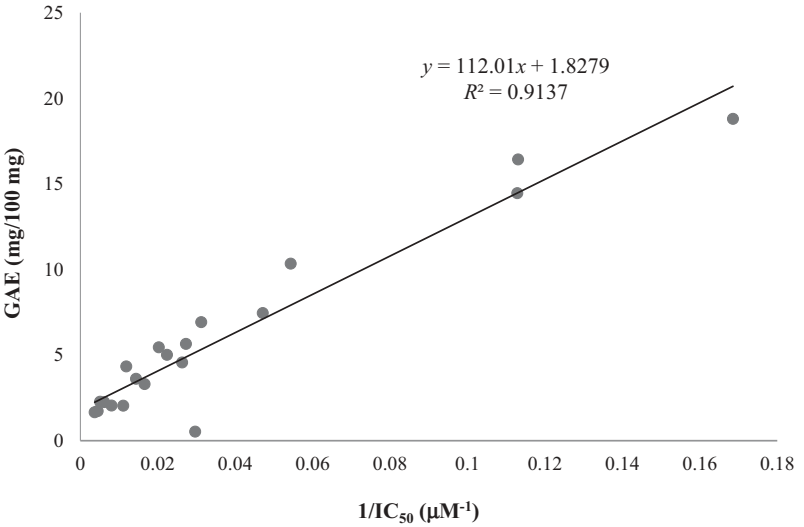

Figure 1. Correlation between phenolic compounds contents (expressed as mg GAE/100 mg extract) and antioxidant activity measured in the DPPH assay (expressed as $1/IC_{50}$, μM^{-1}) of the 23 selected extracts.

hydroxyl or methoxy groups, keto or free carboxylic groups, as well as by other antioxidant secondary metabolites, such as vitamins, volatile oils and carotenoids.[53] In the present study, the highest antioxidant activity was found for the *P. amarus* extract. This result agrees with the previous study of Thiangthum et al.[26] reporting that *P. amarus* extracts showed high antioxidant activity as

shown by low IC_{50} values in a DPPH assay, ranging from 10 to 16 μg/mL. Regi Raphael et al.[54] also reported that the extract of *P. amarus* was found as a remarkable antioxidant throughout its inhibition capacity in scavenging free radicals *in vitro*, while other studies confirmed that the high content of phenolic compounds in this plant was positively correlated with its radical scavenging potential.[55]

Many investigations were conducted concerning the chemical components of this *Phyllanthus* species and bioactive constituents. According to Igwe et al.[56], extracts from leaves of *P. amarus* (which were the part of the plant used in this study) contained high level of saponins and tannins: 24 and 17%, respectively. In the study of Sharma et al.[57], the leaves of *P. amarus* were shown to contain a high quantity of lignans (phyllanthin (0.7%) and hypophyllanthin (0.3%)) in comparison with other parts of the plant. These active compounds might be responsible for the major part of the antioxidant activity.

The good relationship found in this study between total phenolic contents and DPPH measurements is also in agreement with a study of Dudonne et al.[58] who showed a high positive correlation between the free radical scavenging and total phenolic content of 30 plant extracts. For both total phenolic content and IC_{50} found in the DPPH assay, Table 3 compares the results of this study and data found in the literature. For most of the tested plants, the results are roughly similar except for two plants giving opposite results. In this study, the ethanolic extract from the whole *Mimosa pudica* displayed a high antioxidant activity (IC_{50} of 21 μg/mL) while Das et al.[35] reported an IC_{50} of 127 μg/mL for leaves methanolic extract of the same plant, which would mean no antioxidant capacity (Table 3). On the contrary, for *Perilla frutescens*, the ethanolic extract from its leaves showed a low antioxidant capacity in this study (IC_{50} of 70 μg/mL), but a high one in Lin et al.[42] (IC_{50} of 8 μg/mL).

Surprisingly, the antioxidant capacity determined in two (A and B) out of the three tested commercial products was shown to be intermediate only and was lower than the one of the 5 plant extracts mentioned here above, while the third commercial product (C) was qualified as non active. Plants used as the main ingredients in each product are herbs that have been acknowledged since ancient times for their valuable and therapeutic efficiency. Extracts of *Blumea balsamifera* (contained in the commercial product A), *Cynara cardunculus* var. *scolymus, Phyllanthus emblica, Terminalia arjuna* (contained in the commercial product B), or *Phyllanthus urinaria* and *Eclipta prostrata* (contained in the commercial product C) revealed different activities in the DPPH assay with IC_{50} values of 72 μg/mL,[59] 23 μg/mL,[60] 11 μg/mL,[61] 8 μg/mL,[62] 17 μg/mL,[63] and 42 μg/mL,[37] respectively. However, it is well known that many factors of manufacture (such as the materials, the extraction procedure of plant extracts, the ingredients, etc.) can affect the product's activity. Moreover, Bruno and Munro[64] determined that the formulation of commercial products frequently include constituents other than the active ingredient(s), which can be stabilizers, carriers or diluent agents.[65]

Antimicrobial activities of plant extracts against A. hydrophila and E. ictaluri

The *in vitro* antimicrobial activities of the 23 samples against two *A. hydrophila* strains isolated from red tilapia (*Oreochromis* sp.) in Vietnam are presented in Table 4, which also presents the antibacterial activities of the selected plants reported in the literature. According to Kuete[82], antimicrobial activities can be classified as weak or absent if MIC are above 1250 or 2500 μg/mL, respectively. For MIC between 156 and 625 μg/mL, the antimicrobial activity was qualified as moderate. Four plant extracts (*P. amarus, P. betle, P. guajava* and *E. hirta*) and one commercial product (A) showed moderate antimicrobial activities against the first strain of *A. hydrophila* (1) (Table 4), with MICs values ranging between 156 and 625 μg/mL, while the rest of the samples showed weak or no antibacterial activity (MIC ≥ 1250 or 2500 μg/mL, respectively). The growth of the first strain of *A. hydrophila* (1) was also inhibited by *P. amarus, P. betle* and commercial product A, but at higher concentrations of plant extracts (MIC = 625 μg/mL). *P. guajava* and *E. hirta*, which

Table 4. *In vitro* antibacterial activity of 23 plant extracts against two strains of *A. hydrophila* (1 and 2) and *E. ictaluri*, and other antibacterial activities from literature studies.

	MIC (µg/mL)			
Scientific name	*A. hydrophila* 1	*A. hydrophila* 2	*E. ictaluri*	MIC against other bacteria, from literature
Phyllanthus amarus	156	625	>2500	*Staphylococcus aureus* (100 µg/mL), *Streptococcus pneumoniae* (400 mg/mL), *Shigella* spp. (25 µg/mL), *E. coli* (50 µg/mL)[66]
Piper betle	156	625	625	*S. aureus* (1000 µg/mL), *Propionibacterium acnes* (4000 µg/mL)[67]
Psidium guajava	312	1250	>2500	*S. mutans* (250 µg/mL), *S. mitis* (250 µg/mL), *S. oralis* (250 µg/mL)[68]
Commercial product A	312	625	625	-
Euphorbia hirta	625	1250	>2500	*S. aureus* (25 mg/mL), *Candida albicans* (12.5 mg/mL)[69]
Mimosa pudica	1250	2500	>2500	*Escherichia coli (250* mg/mL), *S. aureus* (250 mg/mL), *Bacillus subtilis* (200 mg/mL)[70],
Eclipta prostrata	1250	1250	>2500	*E. coli* (12.5 mg/mL), *S. aureus* (3.125 mg/mL), *B. subtilis* (6.25 mg/mL), *B. cereus* (1.56 mg/mL)[71]
Commercial product B	>2500	>2500	>2500	-
Zingiber officinale	2500	2500	>2500	*Pseudomonas aeruginosa* (40 mg/mL), *E. coli* (40 mg/mL), *S. aureus* (20 mg/mL)[72]
Annona reticulata.	2500	2500	2500	*E. coli* (30 µg/mL), *S. aureus* (40 µg/mL), *B. subtilis* (10 µg/mL)[73]
Houttuynia cordata	2500	2500	>2500	*Bacillus dysenteriae* (0.08 mg/mL)[74]
Cayratia trifolia	2500		>2500	-
Perilla frutescens	>2500		>2500	-
Azadirachta indica	>2500		>2500	*E. coli* (0.781 mg.mL), *K. pneumonia* (1.562 mg/mL), *E. faecalis* (3.125 mg/mL), *S. aureus* (1.562 mg/mL), *P. aeruginosa* (1.562 mg/mL)[43]
Commercial product C	2500		>2500	-
Ageratum conyzoides	>2500		>2500	*E. coli* (100 µg/mL), *S. aureus* (200 µg/mL)[75]
Portulaca oleracea	>2500		>2500	*S. aureus* (12.5 mg/mL), *Streptococcus pyogenes* (12.5 mg/mL), *P. aeruginosa* (50 mg/mL), *E. coli* (50 mg/mL)[76]
Allium sativum	2500		>2500	*E. coli* (150 µg/mL), *Klebsiella pneumonia* (150 µg/mL), *B. subtilis* (100 µg/mL)[77]
Ocimum basilicum	>2500		>2500	Bacillus cereus (62.5 µg/mL), *B. subtilis* (125 µg/mL), *S. aureus* (62.5 mg/mL), *E. coli* (125 µg/mL), *Salmonella typhi* (500 µg/mL)[78]
Centella asiatica	2500		>2500	*S. aureus* (8 mg/mL)[79]
Wedelia chinensis	>2500		>2500	*B. cereus* (3.13 mg/mL), *B. subtilis* (6.25 mg/mL), *S. aureus* (6.25 mg/mL), *E. coli* (25 mg/mL)[80]
Momordica charantia	>2500		>2500	*Enterococcus faecalis* (1.25 mg/mL), *E. coli* (5 mg/mL), *K. pneumonia* (5 mg/mL)[81]
Alternanthera sessilis	>2500		>2500	-
Gentamicin	6.25	6.25	12.5	

Significant activity: MIC < 100 µg/mL
Moderate activity: 100 < MIC ≤ 625 µg/mL
Weak activity: MIC ≥ 625 µg/mL

inhibited the growth of the first strain of *A. hydrophila* (1) at a concentration of 312 and 625 µg/mL respectively, needed a concentration of 1250 µg/mL to inhibit the second strain (2) (Table 4). The results showed that both strains of *A. hydrophila* display the same pattern of sensitivity toward tested plant extracts, but the first strain was more sensitive than the second one. The MIC of the gentamycin antibiotic, used as positive control, was 6.25 µg/mL for both strains (Table 4).

The antibacterial activities of the 23 samples against *Edwardsiella ictaluri* strain are summarized in Table 4. Results show that most plant extracts do not possess antimicrobial activities (MIC ≥ 2500 µg/mL). *Piper betle* and commercial product A showed a moderate activity, with MIC values of 625 µg/mL. However, the strain of *E. ictaluri* used in this work was not very sensitive to gentamicin (MIC = 12.5 µg/mL). In another study, where 64 different strains of *E. ictaluri* were tested for their sensitivity to antibiotics, gentamicin showed MIC of 2 µg/mL for 50% of the tested strains and 1 µg/mL (25%) or 4 µg/mL (25%) for the remaining strains.[83]

Limited information is available about previous studies concerning the antimicrobial capacity of *P. amarus* and *P. betle* against *Aeromonas* spp. and *Edwardsiella* spp. For example, a methanolic extract of *P. amarus* showed an antibacterial activity against *A. hydrophila* with a MIC of 128 µg/mL according to De Britto et al.[84], while, for the same bacteria, a MIC of 25 µg/mL was found by Caruso et al.[85] for the ethanolic extract from leaves of *Piper betle*. The capacity of *P. amarus* to inhibit bacterial growth showed a concentration-dependent antibacterial activity particularly against gram-negative microbes,[86,87] while Kaveti et al.[88] showed that ethanolic extracts of *P. betle* leaves were active against several strains of gram positive and negative bacteria.

The findings of this study regarding a significant antibacterial activity of two plant extracts (*P. amarus* and *P. betle*) against two strains of the pathogenic bacteria *A. hydrophila* could be useful for the initial selection of natural alternative to antibiotics as well as to prevent bacterial growth in fish products during storage.

Impact of tannin removal on the antimicrobial and antioxidant activities of P. amarus and E. hirta ethanolic extracts

The medicinal usefulness of the *P. amarus* and *E. hirta* have been the subject of numerous chemical and microbiology studies. Guha et al.[89] demonstrated that the extract of *P. amarus* is active in inhibiting lipid peroxidation and in scavenging hydroxyl and superoxide radicals. Moreover, Sheikhlar et al.[83] investigated the antibacterial activity of several extracts of *E. hirta, Citrus lemon* and *Trigonella foenum-graecum*. Results showed that the methanolic extract of *E. hirta* exhibited the strongest antimicrobial activity with the lowest MIC (70 µg/mL) against *A. hydrophila* and their potential to be a beneficial dietary supplement for enhancing the resistance of *Clarius gariepinus* to *A. hydrophila* contamination. That is why *P. amarus* and *E. hirta* were chosen as the representative plants for further investigations. Previous screenings of bioactive compounds in *P. amarus* and *E. hirta* revealed that tannins may be responsible for their antioxidant and antimicrobial properties.[90,91]

Tannins constitute a group of secondary metabolites which are widely distributed among vegetal species.[92] They are found in approximately 80% of wooden and 15% of herbal dicotyledonous species. Tannins are known to be responsible for general antimicrobial and antioxidant activities.[93,94] Up to now, investigations on the effects of tannin removals from crude plant extracts are limited. Additionally, the prediction of which class of compounds (alkaloids, anthraquinones, flavonoids, saponins and tannins) possess antioxidant and antimicrobial activities is a challenge.-[1,95,96] Hence, the comparison of the activities of two selected extracts before and after tannins removal on a polyamide column was performed to clarify whether tannins are the main contributors to their antioxidant and antimicrobial activities.

The results showed that, after tannin removal, both *P. amarus* and *E. hirta* extracts were less active against both strains of *A. hydrophila* (Table 5), while their inhibiting activity against *E. ictaluri* remained as weak as before tannin removal (Table 5). As expected, for both plant extracts, the total

Table 5. Antimicrobial activity of *Phyllanthus amarus* and *Euphorbia hirta* before and after tannins removal.

	MIC (µg/mL)			
	Before removing tannins		After removing tannins	
		Aeromonas hydrophila 1		
Phyllanthus amarus	156	Moderate	625	Moderate
Euphorbia hirta	625	Moderate	2500	Weak
		Aeromonas hydrophila 2		
Phyllanthus amarus	625	Moderate	>1250	Weak
Euphorbia hirta	1250	Weak	2500	Weak
		Edwardsiella ictaluri		
Phyllanthus amarus	>2500	Weak	>2500	Weak
Euphorbia hirta	>2500	Weak	>2500	Weak

Table 6. Antioxidant activities of *Phyllanthus amarus* and *Euphorbia hirta* before and after tannins removal.

	Before removing tannins		After removing tannins	
Name	DPPH assay IC_{50} (µg/mL)	Phenolic content (%*)	DPPH assay IC_{50} (µg/mL)	Phenolic content (%*)
Phyllanthus amarus	5.83 ± 0.50	18.8 ± 0.75	83.6 ± 5.38	2.68 ± 0.20
Euphorbia hirta	17.8 ± 1.28	10.3 ± 0.91	38.3 ± 2.40	5.02 ± 0.54

Values are mean ± SD (n = 3); * Expressed as mg GAE per 100 mg plant extract

phenolic content decreased after tannin removal while their IC_{50} in the DPPH assay increased, meaning less antioxidant activity (Table 6). About half of the initial phenolic content remains for *E. hirta* extracts and only the seventh for *P. amarus*, showing that a high amount of tannins was present in these extracts. This apparent higher proportion of tannins in *P. amarus* was confirmed after quantification of tannins in both extracts according to the European pharmacopoeia method, which gave 5.6% for *P. amarus* and 1.79% for *E. hirta* (expressed in pyrogallol). Hydrolysable tannins appear to be the main polyphenol constituents found in *P. amarus*, with geraniin being the most abundant.[97–99] This could explain the fact that *P. amarus* displayed high antioxidant and antimicrobial activities. This is in agreement with Catteau et al.[100], reporting that tannins were at least in part responsible for the activities of several plant methanolic extract effects, but other compounds may also explain a part of the activity.

Patel et al.[101] reported that high content of tannins could be isolated from *P. amarus* which are associated with some health importance and antimicrobial activity. According to Bensky et al.[90], hydrolysable tannins are among the main secondary metabolites, which have been discovered to prevent viral DNA polymerase and reverse transcriptase in HIV infection, and to act on angiotensin-converting enzymes in diabetes complication. Moreover, antimicrobial activity of *E. hirta* was attributed to tannins and other bioactive components. Yoshida et al.[91] and Yoshida et al.[102,103] reported the isolation of hydrolysable dimeric ellagitannins (euphorbin A, B, C and E) from the leaves of the plant.

Conclusion

In our study, twenty three ethanolic extracts from 20 plant and 3 commercial products obtained in Mekong Delta (Vietnam) were screened for their *in vitro* antioxidant and antimicrobial activities. The results from DPPH free radical scavenging assays revealed that the *P. amarus* extract showed the highest antioxidant activity. Four other plant extracts showed a high antioxidant activity in the following descending order: *P. betle* > *P. guajava* > *E. hirta* > *M. pudica*. There was a positive correlation between total phenolic content and antioxidant activity of the 23 extracts. Beside its high antioxidant activity, *P. amarus* extract also showed antibacterial activity against two different strains

of *A. hydrophila* and one strain of *E. ictaluri*. Tannins were shown to be mainly responsible of these activities, as after tannins removal from *P. amarus* and *E. hirta* extracts, both antioxidant and antimicrobial activities decreased. Both *P. amarus* and *E. hirta* appeared to be promising active plants for further studies. The extracts of these two plants could be valuable natural antibiotic alternatives or natural additives to improve seafood and aquaculture product preservation.

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