Original Article

HPLC Analysis, Antioxidant, Antimicrobialactivities of Alcaloids and Methanolic Extract of *Pancratium maritimum* Growing in Djerba

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Abstract

Pancratium maritimum is a species of bulbous plant that belongs to the Amaryllidaceae family, it is among one of the most important alkaloid-containing plant families. This study aimed to investigate the antioxidant, antibacterial, and antifungal activities of *Pancratium maritimum* extracts and their UHPLC-MS analysis. The total phenolic, tannins, and flavonoid content of Tunisian *Pancratium maritimum* aerial part methanolic extract were determined by colorimetric method. The phytochemicals in *P.maritimum* methanolic were identified using the UHPLC-MS method. The antioxidant activity was assessed using a DPPH assay. The antibacterial and antifungal properties were evaluated against a diverse array of pertinent pathogens using the broth dilution technique.

This study showed the presence of alcaloids in methanolic extract as hordenine, lycorine and galanthamine, a strong antioxidant activity was recorded for *P.maritimum* methanolic extract with a DPPH inhibition of (86.7%). The alkaloid fraction exhibited the most potent antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis* bacteria, with minimum inhibitory concentration (MIC) values ranging from 1.125 to 2 mg/mL. Furthermore, it demonstrated significant anticandidal activity against *Candida glabrata* and *Candida parapsilosis*, with MIC values ranging from 0.031 to 0.125 mg/mL. *P.maritimum* extracts have the potential to be utilized for controlling bacterial biofilms in food and food-related situations.

Keywords: *P.maritimum*, HPLC, Antibacterial, Extracts, Antifungal

Introduction

Plants are a source of a vast range of natural products with varied medicinal qualities and are constantly studied to generate innovative medications [1, 2]. These bioactive chemicals such as polyphenols, flavonoids, terpenes, and alcaloids provide biological activity against a variety of disease-causing substances [3]. In recent years, research into these phytochemicals has been a major focus in the creation of functional foods, nutraceuticals, and cosmeceuticals, with the discovery that the usage of plant extracts gives bioactive characteristics and positive effects [1]. Natural antioxidants are mostly derived from food and medicinal plants, including fruits, vegetables, cereals, flowers, spices, and traditional medical herbs [4]. In addition, enterprises that process agricultural byproducts may be key sources of natural antioxidants [5]. Generally, natural antioxidants, particularly polyphenols, and carotenoids, possess a diverse array of biological effects, including antibacterial, anti-inflammatory, antiviral, preventing aging, and cancer-fighting capabilities [6-9] *Pseudomonas aeruginosa* has become the primary cause of Gram-negative infection, particularly in patients with weakened immunity. Therefore, the search for novel antimicrobials has become a necessity [10]. Isolated extracts derived from medicinal plants have been documented to demonstrate diverse biological properties, including antibacterial, anti-inflammatory, and antioxidant activity [11]. In this context, *Pancratium maritimum* plants have been widely investigated for their therapeutic potential [12, 13]. The genus *Pancratium*, which belongs to the *Amaryllidaceae* family, comprises around 20 species [13]. Often referred to as marine narcissus, *P. maritimum L.* is a type of plant that grows along sandy beaches from the Mediterranean to the Black Sea and certain areas of the Atlantic coast [14]. Despite

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being widely spread, populations of *P. maritimum* have significantly declined as a result of urbanization, tourism development, alteration and destruction of dune systems, and excessive harvesting [14]. The antiviral, antimalarial, purgative, analgesic, anticancer, and cytotoxic properties of P. maritimum are utilized in traditional medicine in many Mediterranean countries [15-20], all these activities are attributed to alkaloid molecules since the Amaryllidaceae family is known for producing beneficial alcaloids. Several investigations focused on alcaloids as the principal bioactive ingredients [18]. Assessing the antibacterial and antifungal properties of *P. maritimum methanolic extract and alcaloids grown in Tunisia* was the aim of our investigation.

Materials and Methods

Plant Material

Aerial parts (Stems and leaves) of *P. maritimum*, were collected from the island of Djerba in the south of Tunisia during the flowering period in July. The identification of the plant was assessed by Prof. Fethia Skhiri, a botanist at the Hight Institute of Biotechnology in Monastir, Tunisia. A voucher specimen was submitted to our laboratory located in the Faculty of Pharmacy, Monastir, Tunisia (MM-H).

Extract Preparation

A total of 50 grams of leaves and stems were cleansed by rinsing them with flowing water to eliminate any dust particles. Afterward, they were air-dried at room temperature and subsequently ground into a fine powder. The resulting powder was then soaked in 500 milliliters of methanol for a period of four days on each occasion. The extract was filtered and evaporated at low pressure [21].

Total Phenolic Content

A modified Folin-Ciocalteu method [22] was used to measure total phenols. To do this, 20 liters of extract solution were combined with 100 liters of the Folin-Ciocalteu reagent that had been diluted ten times. A 7% Na2CO3 solution in 80 µl was added to the mixture after 30 seconds. After 30 minutes at 23 °C in the dark, the blue complex that resulted was then evaluated for absorbance at 760 nm using a spectrophotometer (Spectro UV-VIS, Double Beam PC UVD-2950, Labomed). Gallic acid equivalents per gram of dry weight (mg GAE/g DW) were used to express the total phenol concentration. The gallic acid calibration curve range was 0–1000 g/mL. All of the samples were measured in triplicate.

Total Flavonoid Content

The trichloride aluminum method was employed to assess the flavonoids' total content [23]. A reference quercetin solution or 100 l of samples was combined with 100 l of ALCL3 (2%) and then incubated for 30 min. The Spectro UV-VIS, Double Beam PC UVD-2950, and Labomed instruments were used to measure the absorbance at 510 nm. The measurements were taken for both the sample and a blank mixture without the sample. The estimation was made to determine the amount of flavonoids in plant organs, measured in milligrams of quercetin equivalents per gram of dry weight (mg QE/g DW).

Total Condensed Tannin Contents

Using catechin as a reference, the tannin contents were calculated using a previous method [24] from 1978 with a minor modification. 50 liters of extract and a few drops of concentrated sulfuric acid were added to 100 liters of vanillin (1% in methanol) solution. At 500 nm, the absorbance was measured after 15 minutes of incubation. As (mg EC/g DW), the condensed tannin was expressed.

UHPLC-MS Analysis

The UHPLC-MS analyses of the various plant extracts were performed according to a previous study [25] with some modifications. The Acquity UHPLC H-Class Waters® System (located in Guyancourt, France) was equipped with two separate pumps, a controller, a strip detector, diodes (DAD), and a QDa electrospray quadrupole mass spectrometer. A Uptisphere C18-AQ column with dimensions of 2.1 x 100 mm and a particle size of 2.6 micrometers was employed as the stationary phase. The mobile phase consisted of two components: (A) ultrapure water with 0.1% formic acid from Carlo Erba Reagents®, Val de Reuil, France, and (B) acetonitrile with 0.1% formic acid from Carlo Erba Reagents®, Val de Reuil, France. The flow rate of the column was set at 0.3 mL/min, while the temperature was set to 30 °C. The wavelength range between 200 and 790 nm was selected with an accuracy of 1.2 nm. We carried out ionization with masses ranging from 50 to 1250 Da in both positive and negative modes. Cone voltage was measured at 15 V, and capillary voltage was measured at 0.8 kV. The volume of the injection was adjusted to four microliters. The UHPLC-UV-MS analyses were conducted using the elution gradients as follows: 5% to 80% (B) from 0 to 9 minutes, 100% (B) from 9.5 to 10.5 minutes, and 5% (B) from 11 to 13 minutes. The samples were produced using MeOH of analytical grade at a concentration of 2 mg/mL and were then subjected to centrifugation for 20 minutes. The injections were made with supernatants.

Alkaloid's Extraction

500 mg of methanol extract was suspended in 100 mL water acidified to pH = 2 by HCl 37 %, followed by liquid-liquid partitioning with hexane (4 x 100 mL) and chloroform (CHCl3) (4 x 100 mL). Next, the water phase was basified to pH = 9-10 by NH4OH 35 % before partitioning with CHCl3 (4 x 100 mL) and BuOH (4 x 100 mL) [16].

Estimation of the Antioxidant Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) experiment was used to evaluate the antioxidant activity of *P.maritimum* preparations [26]. The concentrations of the methanolic extracts tested ranged from 5 mg/mL to 1 mg/mL. In a nutshell, 810 l of daily generated DPPH methanolic solution (4 mM/l) were coupled with 90 µL of each diluted extract.

The combination was then kept in the dark at 37 °C for 20 minutes. A decrease in absorbance was recorded at 517 nm. The antioxidant activities of the substances were expressed using Equations (DDPH scavenging activity (%) or AA (%) = [1 − (As − Ab/Ac)) × 100]. Where Ac represents the control absorbance (no extract), As represents the absorbance of the tested extract, and Ab represents the absorbance of the extract sans DPPH radical. Ascorbic acid served as the positive control, and methanol served as the negative control.

Evaluation of the Antimicrobial Activity

Microbial Strains

Different reference bacteria strains and fungi have been used in antibacterial and antifungal activity, including *Pseudomonas aeruginosa* ATCC 27583, *Escherichia coli* ATCC 25922, *Candida glabrata* ATCC 90030, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 90028, *Candida tropicalis* ATCC 3121, and *Candida parapsilosis* ATCC 22019. All the above species were kindly provided by the Laboratory of Microbiology, Hospital of Fattouma Bourguiba, Monastir.

Estimation of Minimum Inhibitory Concentration (MIC) Values

Using 96-well microtiter plates and the microdilution technique, the MIC value of *P. maritimum* extract was ascertained. Every bacterial strain was cultured overnight in MHB plates to establish its active culture. The turbidity of the suspended active bacterial cells was either modified or maintained at 0.5 Mc Farland standards (108 CFU/mL). The extract was mixed with dimethyl sulfoxide (DMSO) at a concentration of 10%. In order to ascertain the Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC), 20 liters of each well that showed no visible growth were reinoculated onto Müller-Hinton agar (MH) plates and incubated at 37 °C for 24 hours. The smallest amount of extract exhibited no evidence of bacterial or fungal development [27].

Results and Discussion

Total Phenols, Flavonoids and Tannins

P.maritimum methanolic leaf and stem extract were subjected to total phenolic, flavonoids, and tannins determination. The results are gathered in **Table 1** below.

In this study, *pancratium maritimum* methanolic extract showed relatively a high concentration of phenolic compounds (164.316 ±3.92 mg), however, traces of tannins are present in this extract (0.4±0.01). The phenolic composition of bulb and leaf extracts was investigated by previous studies [20].

Total phenols and total flavonoid content of different *P. maritimum* extracts were determined previously and showed that ethanolic fruit extract of *P.maritimum* contained the highest amount of phenols (277.8 ± 2.9mg of chlorogenic acid equivalents/g of extract) and flavonoids (52.7 ± 0.3mg of quercetin equivalents/g of extract) [13].

Table 1. Results of the total phenols, flavonoids and tannins.

	MeOH
Total phenols (mg GAE/g DW)	161,31 ±3.9
Total flavonoids (mg QE/g DW)	126.7±3
Total tannins (mg CE/g DW)	0.4±0.01

MeOH: methanolic extract

Chemical Composition of Methanolic Extract by UHPLC-MS

The methanolic extract was investigated for its chemical composition via the UHPLC-MS method. Results are illustrated in **Table 2**. The outcomes revealed the dominance of alcaloids such as zef-betaine, lycorine, galanthamine, and hordenine in *P.maritimum* leaf and stem methanolic extract Phenolic acids in *P.maritimum* extracts were previously identified by several authors [20] as 5-hydroxy-7-methoxy-2-methylchromone. Previous Studies in Italy demonstrated the presence of lycorine as an alkaloid in the bulb extract of *P.maritimum* [28]. Similarly, hordenine and galanthamine were identified in the bulbs of Turkish *P.maritimum* [29].

Table 2. HPLC analysis of *P.maritimum* methanolic extract.

	RT	$[M]^+$	Compound
1	0,734	288,15	Zefbetaine isomer
1	0,964	268,12	Zefbetaine
2	1,218	166,07	Hordenine
3	1,431	288,10	Galanthamine
4	2,112	288,16	Lycorine
5	3,764	318,11	Ungiminorine
6	3,907	318,09	Lycorenine
7	4,005	302,13	9-O-demethylhomolycorine
8	4,448	302,12	Haemanthamine
9	4,595	246,17	Haplopine
10	8,939	353,24	7-hydroxy-6-methoxy-4-[(2-oxochromen-7-yl) oxy]chromen-2-one

RT: retention time

Determination of the Antioxidant Activity

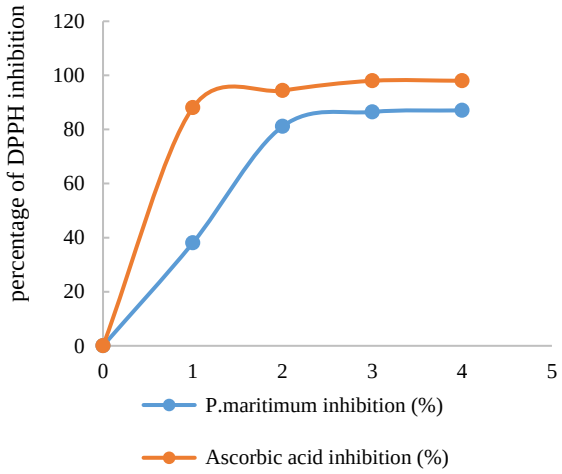

Figure 1. Determination of the antiradical activity of *P.maritimum* methanolic extract

The methanolic extract of *Pancratium maritimum* exhibited good antioxidant activity **(Figure 1)** compared to ascorbic acid. It recorded high DPPH radical scavenging activity (87.04%). One possible source of free radicals in nature is plants. In order to survive, it generates a variety of antioxidative chemicals to inhibit reactive oxygen species (ROS) [30]. The antioxidant capacity of plant extracts relies on the presence of phenols. These bioactive chemicals have the ability to function as reducing agents, scavengers of free radicals, chelators of metals, and deactivators of singlet oxygen. They may also perform multiple functions simultaneously [31]. High DPPH radical scavenging activity was recorded in Pancratium maritimum methanol extract. This activity is probably explained by the enormous number of flavonoids [20], alcaloids [32], and amino acid derivatives (betaine) whose antioxidant activity was evaluated in mice [33]. Many authors investigated the good antioxidant activity of *pancratium maritimum* bulb and leaf extract [20, 34].

Evaluation of the Antimicrobial Activity of Methanolic and Alcaloids Fractions (mg/mL)

Evaluation of the Antimicrobial Activity of P.Maritimum Methanolic Extract

The antibacterial and antifungal activities of *P.maritimum* methanolic extract are summarized in **Table 3**.

Table 3. Antibacterial activity of *P.maritimum* methanolic extract (mg/mL)

Strains	MeOH extract		
	MIC	MBC/ MFC	MBC (MFC) /MIC Ratio
Staphylococcus aureus ATCC6583	20	20	1
Pseudomonas aeruginosa ATCC27583	40	40	1
Enterococcus faecalis ATCC08152	20	20	1
Escherichia coli ATCC25922	10	20	2

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

Table 4. Antifungal activity of *P.maritimum* methanolic extract

Strains	MeOH extract		Fluconazole
	MIC	MBC	MIC
Candida albicans ATCC 942	40	40	1
Candida tropicalis ATCC 3121	40	40	1
Candida parapsilosis ATCC 924	20	40	2
Candida glabrata ATCC 913	10	20	2

MFC: minimum fungicidal concentration

The methanolic extract exhibited moderate antibacterial activity, as demonstrated by MIC values ranging from 10 to 40 mg. An effective activity was recorded against *E. coli* bacteria and *C.glabrata* fungi (MIC=10 mg/mL) respectively **(Table 4)**. *P. maritimum* methanolic extract exhibited bactericidal and fungicidal activity since MBC/MIC is lower than 4 [35]. A previous study reported no antibacterial activity of *P.maritimum* bulb methanol extract however it was effective against *C. krusei* ATCC 6285 and *C.albicans* ATCC 10231 yeasts (inhibition zone diameter mm =20 and 26 mm respectively) [36].

Evaluation of the Antimicrobial Activity of Alkaloid Fractions

Results of the antibacterial and antifungal activities of an alkaloid fraction are summarized in **Tables 5 and 6**.

The antibacterial and antifungal activities of *P.maritimum* extracts were assessed, The methanol extract of *P. maritimum* yielded four fractions with variable levels of activity the best was recorded in alcaloids fractions, it was 1.125 mg/mL against *P.aeruginosa* bacteria and 2mg/mL toward *E.faecalis* **(Table 5)**.

Table 5. Results of MIC and MBC of the alkaloid fraction (mg/mL).

Strains	Alkaloid fraction			Ciprofloxacin	
	MIC	MBC	MBC/MIC ratio	MIC	MBC
***Staphylococcus aureus* ATCC 6583**	2	4	2	0.031	0.125
***Pseudomonas aeruginosa* ATCC27583**	1.125	2.25	2	0.125	0.2
***Enterococcus faecalis* ATCC08152**	2	8	4	0.125	0.2
***Escherichia coli* ATCC 25922**	16	32	2	0.062	0.2

CIP: ciprofloxacin

Alcaloids extraction was established by many authors [16, 6, 37], bulbs and flowers of the amaryllidaceous plant *Pancratium maritimum* have been investigated [18] however alcaloids from the mixture of stems and leaves have not yet been studied.

A previous study [15] explored the antimicrobial activity of an alkaloid mixture from fruits and flowers that were inactive against *E.faecalis* bacteria. However, pure methanolic extract showed low antibacterial activity, which suggests that the alkaloid fraction effectively concentrates the active components responsible for antibacterial activity [38]. An alcaloid mixture of fruits and bulbs was tested for antimicrobial activity [39]. It was reported that Lycorine alkaloids showed antifungal activity against *C. albicans* [40]. However, higher MIC values were recorded in the alkaloid fraction (MIC=16 mg/mL against *E. coli* strain). Different pharmaceutical properties of alcaloids were identified from *P. maritimum* including anti-tumor, antiplasmodial, anti-inflammatory, antimicrobial, and antioxidant activities, which have been explored previously [41].

Previous studies demonstrated that alcaloids present in the bulbs of *P. maritimum* have therapeutic properties. As an example, for these molecules, Pancratistatin which was reported for its anticancer activity Recently alkaloid from *pancartium maritimum* has been explored for its antiviral activity against SARS COV2 virus [42].

Table 6. Results of MIC and MFC values of the alkaloid fraction (mg/mL).

Strains	Alkaloid fraction			Amphotericin B
	MIC	MFC	MFC/MIC Ratio	MIC
Candida tropicalis ATCC 3121	4	8	2	0.5
Candida albicans ATCC 942	4	8	2	0.5
Candida parapsilosis ATCC 924	0.125	0.25	2	0.5
Candida glabrata ATCC 913	0.031	0.0625	2	0.5

All the Alkaloid fractions were also evaluated for their anticandidal activities. Results are summarized in **Table 6**.

Our findings suggested fungicidal activities (MBC/MIC=2).

Alkaloid fractions demonstrated significant antibacterial and antifungal activity with MIC and MFC ranging from 0.031 to 4 mg/mL **(Table 6)**. Compared to fluconazole, the alkaloid extract was the most active (MIC=0.031 mg/mL against *C.glabrata* and MIC=0.125mg/mL against *C.parapsilosis*). A reduced MFC value was efficient in exhibiting *C.glabrata* yeast (MFC=0.0625 mg/mL).

Conclusion

According to our results, the stem and leaf methanolic extract of Tunisian *Pancratium maritimum* was rich in alcaloids notably lycorine, galanthamine, and hordenine which led us to investigate more alkaloid fraction, and showed excellent antibacterial and antifungal potentials of alkaloid fractions (MIC=1mg/mL against *P.aeruginosa*) and (MIC=0.125 and 0.031 mg/mL) against *C.parapsilosis* and *C.glabrata* respectively. Considering these findings, *P.maritimum* could be a potent anticandidal and antifungal agent.

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