



## Article

# Phytochemistry, Antioxidant Potential, and Antibacterial Activities of *Anacyclus pyrethrum*: Promising Bioactive Compounds

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**Abstract:** Secondary metabolites derived from plants have demonstrated significant biological activity and hold both edible and medicinal significance. These compounds play a crucial role in the development of pharmaceuticals, particularly in the context of inflammatory disorders and chronic diseases associated with oxidative stress. The objective of this study was to investigate the chemical characterization, antioxidant potential, and antibacterial properties of the aqueous extract of *Anacyclus pyrethrum* (AEAP). To achieve this, we employed various analytical techniques including HPLC–ESI–MS/MS, Fourier transform infrared, X-ray diffraction, scanning electron microscopy, and energy-dispersive X-ray spectroscopy. The antioxidant activity of the AEAP was assessed using DPPH (2,2-Diphenyl-1-picrylhydrazyl) and reducing power assays, while antibacterial activity was evaluated against both Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecium*) using the broth microdilution assay. Our findings demonstrated significant antioxidant activity of the AEAP, as well as broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria. HPLC analysis identified important bioactive compounds, notably pellitorine and 3,4-dihydroxybenzoic acid, known for their antioxidant and antibacterial properties. Overall, the AEAP demonstrated potent antioxidant and antibacterial activities, suggesting its potential as a valuable natural source of bioactive compounds with various therapeutic applications.

**Keywords:** antimicrobial phytochemicals; antioxidant activity; bioactive compounds; pellitorine; therapeutic applications



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## 1. Introduction

In recent years, there has been a growing interest in the use of herbal medicines for disease control due to their effectiveness, affordability, and limited side effects [1]. Natural products, particularly those derived from plants, have been a valuable source of raw materials for the discovery of bioactive compounds used in various industries, such as nutrition, cosmetics, and pharmaceuticals [2].

*Anacyclus pyrethrum* (L.) is one of wild species of flowering plant in the Asteraceae family and the *Anacyclus* genus, commonly known as pellitory, Aqar Qarha, Oud El Attas, and tigandizt. According to the Global Biodiversity Information Facility (GBIF) this plant is native to Morocco, Algeria, and Spain [3,4]. *A. pyrethrum* is a low-growing plant with finely divided, feathery leaves and produces daisy-like flowers with white petals and a yellow center. It is typically found in arid and rocky areas, particularly in mountainous regions, where it demonstrates remarkable adaptability to challenging and dry environments [5]. In Morocco, *A. pyrethrum* is commonly found in a variety of habitats, including clear-cut forests, scrublands, meadows, grasslands, lowlands, and mountainous regions, encompassing cold semi-arid, semi-humid, and humid climates [6]. This versatile species can be found at altitudes ranging from 1000 to 2500 m above sea level [6]. *A. pyrethrum* tends to flourish in well-drained soil and prefers open, unshaded areas with sparse tree and shrub cover within Mediterranean vegetation zones [6]. Reproduction in *A. pyrethrum* is primarily achieved through sexual means, mainly via seeds. However, asexual reproduction through plant parts or rhizomes is also feasible [3].

Extensive phytochemical screening of *A. pyrethrum* has unveiled a plethora of secondary metabolites, including alkaloids, tannins, coumarins, and flavonoids [7]. The root of *A. pyrethrum* is particularly rich in N-isobutyldienediylamide and polysaccharides [8]. Additionally, numerous experimental studies have demonstrated the wide range of biological effects associated with *A. pyrethrum*, including antioxidant and anti-inflammatory properties [9], antidepressant effects [10], and immunostimulatory capabilities [11]. Apart from its medicinal properties, *A. pyrethrum* has been traditionally used as a stimulant, cordial, and rubefacient [12]. It is recommended for gargling to alleviate symptoms of conditions such as rhinitis, neuralgia, rheumatic discomfort, and musculoskeletal pain. In Ayurvedic medicine, root of pellitory is combined with *Withania somnifera* and *Vitis vinifera* roots to treat epilepsy [13]. Furthermore, it has shown promising results in the remedy of conditions such as sciatica, paralysis, hemiplegia, and amenorrhea [14]. Anacycline, isobutylamide, inulin, and trace amounts of essential oil have been identified in the pellitory root [15]. Remarkably, there have been observations indicating that *A. pyrethrum* may lead to a reduction in insulin needs for those with insulin-dependent diabetes mellitus, along with the potential to decrease plasma glucose and serum cholesterol levels following oral administration for a duration of 3-6 weeks [16]. In Morocco, the roots of *A. pyrethrum* are known for their beneficial effects in traditional medicine, for various purposes, including the treatment of stomach diseases, stomatitis, cysts, articular rheumatism, and dental pain [17]. These attributes arise from a diverse array of phytochemical compounds, with over a hundred distinct compounds identified thus far. These compounds include phenolic compounds, flavonoids, and alkaloids [3,7,8].

Molecular studies on *A. pyrethrum* have provided valuable insights into its genetic makeup and the underlying mechanisms of its pharmacological properties. Manzanilla et al. (2022) showed the effectiveness of utilizing target capture genomic barcoding to identify and confirm the geographical source of specimens traded as *A. pyrethrum*, listed as a globally vulnerable medicinal plant in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species [18]. Usmani et al. [3], by using gas chromatography–mass spectrometry (GC–MS), identified various potential bioactive compounds in *A. pyrethrum*, including pellitorine, germacrene D, trans caryophyllene, and naphthalene isomers. An additional molecular investigation into the essential oil of *A. pyrethrum* revealed the presence of several compounds (ca. 32 compounds), constituting approximately 93% of the overall chemical makeup of the essential oil.

These findings offer valuable insights into the chemical composition of compounds within *A. pyrethrum*, shedding light on their potential as bioactive substances and their associated pharmacological activities. Pellitorine, isolated in 1895 by Dunstan and Garnett from the roots of *A. pyrethrum* [19], stands out as a key compound. It has been documented to exhibit a range of biological effects, including antimicrobial, antiviral, diuretic, antioxidant, and analgesic properties [5,20]. These studies have also revealed the biosynthetic

pathways and regulatory mechanisms that contribute to the plant medicinal properties [3]. Furthermore, molecular studies have also facilitated the identification and selection of high-yielding and disease-resistant cultivars of *A. pyrethrum* [21], thereby aiding in its cultivation and commercialization.

Expanding the therapeutic applications for *A. pyrethrum* could enhance treatment options, particularly in regions where traditional medicine plays a pivotal role in healthcare. In this context, this study aims to contribute to the valorization of *A. pyrethrum* from the Moroccan Middle Atlas. Our objective is to uncover novel therapeutic prospects while substantiating earlier research. We carried out a comprehensive investigation into the aqueous extract of *A. pyrethrum* (AEAP), delving into its phytochemical composition, antioxidant potential, antibacterial activities, and chemical profile.

## 2. Materials and Methods

### 2.1. Plant Samples and Extraction

#### 2.1.1. Plant Samples

The roots of *A. pyrethrum* were gathered from the wild in its natural habitat at Bin El Ouidan region, Morocco (32°7'48" latitude N/6°27'36" Longitude W). The plants were initially authenticated by botanist Professor A. Ouhammou and pharmacologist Professor A. Chait from the Faculty of Sciences at Semlalia, Cadi Ayyad University. The plants were then stored under voucher specimen MARK-1003 in the herbarium of the Department of Biology, Faculty of Sciences Semlalia, Cadi Ayyad University, Marrakech, Morocco.

#### 2.1.2. Plant Extraction

The powder was obtained by crushing the washed and dried roots, which were then extracted with distilled water (1 g/10 mL) under agitation for 12 h. The aqueous macerate was centrifuged (1200 rpm), filtered, and lyophilized using a Christ instrument. The extract underwent a microbial assessment, and no signs of microbial contamination (*Escherichia coli*, *Salmonella*, *Staphylococcus aureus* or *Pseudomonas aeruginosa*) were detected. The lyophilized dry powder was then aseptically stored in amber bottles at 4 °C until it was ready for utilization.

### 2.2. Phytochemical Study

A phytochemical analysis was conducted to assess the presence of secondary metabolites, including total phenols, flavonoids, and tannins in the extract. The results were reported as the average  $\pm$  standard deviation (SD) of three independent experiments.

#### 2.2.1. Determination of Total Phenolic Content (TPC)

The extract TPC was assessed using an adapted Folin-Ciocalteu method [22]. A 0.4 mL aliquot of the diluted extract was combined with 1.5 mL of the Folin-Ciocalteu reagent. Subsequently, 1.6 mL of a 7.5% sodium carbonate solution was introduced. The resultant mixture was then left to incubate in darkness at room temperature for 2 h. The absorbance was read at a wavelength of 765 nm. Gallic acid served as the reference standard, and the outcomes were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g DW).

#### 2.2.2. Determination of Total Flavonoid Content (TFC)

The extract TFC was assessed using a method described in a previous study [23]. A 200  $\mu$ L extract was diluted with 1 mL of distilled water. Subsequently 60  $\mu$ L of 5% NaNO<sub>2</sub> and 60  $\mu$ L of 10% AlCl<sub>3</sub> were introduced into the solution. After 5 min, 400  $\mu$ L of 1 M NaOH was added to the mixture. The absorbance was read at 510 nm, and the TFC was expressed as milligrams of catechin equivalent per gram of dry matter (mg CE/g DM).

### 2.2.3. Quantification of Condensed Tannins

The total condensed tannin content was assessed following a method previously outlined [24]. Briefly, diluted samples (400 µL) were blended with 3 mL of a 4% methanol vanillin solution and 1.5 mL of concentrated HCL. The mixture was allowed to incubate for 15 min, and the absorbance was gauged at 500 nm. The total condensed tannin content was quantified and expressed as milligrams of catechin equivalent per gram of dry matter (mg CE/g DM).

## 2.3. Antioxidant Activity

### 2.3.1. Determination of DPPH Radical Scavenging Activity

The antioxidant activity of the extract was determined using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay, as previously outlined [25] with certain modifications [26]. Specifically, 1.5 mL of a methanolic DPPH solution ( $6 \times 10^{-5}$  M) was mixed with 60 µL of the extract at varying concentrations (1, 2, 4, 6, and 8 mg/mL) of AEAP. The mixture was then shielded from light and allowed to stand at room temperature for 30 min. Following this incubation, the absorbance was recorded at 515 nm, using a negative control consisting of 1.5 mL of the DPPH solution and 60 µL of methanol. The study also included positive controls, such as butylated hydroxytoluene (BHT) and quercetin. The percentage of inhibition was calculated using the formula:

$$\%Inhibition(mg/mL) = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

where  $A_{control}$  is the absorbance of the control and  $A_{sample}$  is the absorbance of the test compound.

The sample concentration that induces 50% inhibition (IC<sub>50</sub>) was determined from a graph plotting the percentages of inhibition against the sample concentrations.

### 2.3.2. Determination of Ferric Reducing Ability Power (FRAP) Activity

The FRAP test was carried out in accordance with a previously established method [27]. The assay involves the prevention of the formation of Fe(II)–ferrazine complexes when samples interact with ferrous iron. In the procedure, a mixture of distilled water (1 mL), phosphate buffer (2.5 mL; 0.2 M; pH = 6.6), and potassium ferricyanide ( $K_3[Fe(CN)_6]$ ) (2.5 mL; 1%) was combined with 0.5 mL of solutions of varying concentrations. After incubating for 30 min, the mixture was supplemented with 2.5 mL of distilled water, 2.5 mL of 10% trichloroacetic acid, and 0.5 mL of  $FeCl_3$ . The absorbance was read at 700 nm. Quercetin and BHT were used as positive controls.

## 2.4. Antibacterial Activity

### 2.4.1. Microbial Strains and Culture Conditions

The antibacterial activity of AEAP was assessed against a panel of Gram-positive bacteria, including *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecium* (ATCC 4602), as well as Gram-negative bacteria, such as *Pseudomonas aeruginosa* (ATCC 27653) and *Escherichia coli* (ATCC 25922). The bacterial cultures were incubated in Mueller–Hinton broth (MHB) for 24 h, and the final inoculum sizes were standardized to  $1 \times 10^8$  CFU/mL (0.5 McFarland standard).

To assess the antibacterial activity, three essential in vitro assays were employed. Firstly, the agar well diffusion method was employed to measure the inhibition zones (ZOI) induced by the plant extract. Secondly, the microdilution assay was performed to determine the minimal inhibitory concentration (MIC), which represents the lowest concentration of the extract that inhibits visible bacterial growth. Finally, the minimum bactericidal concentration (MBC) was established, which represents the minimal concentration of the extract lethal to the bacteria. All tests were conducted in triplicate for each bacterial strain to ensure accuracy and reproducibility of the results.

#### 2.4.2. Agar Well Diffusion Inhibition Zones Test

For the antibacterial assessment, Petri dishes containing approximately 25 mL of Mueller–Hinton agar (MHA) were prepared and appropriately labeled. The bacterial inoculum was evenly spread across the agar surface using sterile cotton swabs, with the plate rotated 90° after each pass to ensure uniform distribution. Wells, each with a 4 mm diameter, were created in a sterile manner using a cork-borer. Subsequently, 100 µL of the plant extract, prepared at a concentration of 100 mg/mL, was dispensed into each well. As a positive control, gentamicin (10 µg/disc) was employed, while 100 µL of a 4% DMSO was used as a negative control to confirm that any observed antibacterial activity was not attributed to the solvent. The plates were left at room temperature for 30 min to allow for the proper diffusion of the plant extract. After incubation at 37 °C, the zones of bacterial inhibition surrounding each well were measured in mm, and the mean value was calculated to assess the extent of antibacterial activity [28].

#### 2.4.3. MIC and MBC/MFC Determination

The antibacterial activity of AEAP was assessed using a broth dilution micro-method carried out in 96-well microtiter plates. This followed the protocols established by the Clinical and Laboratory Standards Institute (CLSI), with few modifications [29]. The AEAP and their polyphenolic fractions were first dissolved in distilled water to create stock solutions at a concentration of 100 mg/mL. From these stock solutions, serial twofold dilutions were prepared in Mueller–Hinton broth (MHB), resulting in a range of final concentrations from 50 to 0.19 mg/mL. Microbial cultures were then inoculated into the wells to reach a final concentration of  $5 \times 10^5$  CFU. Each determination was carried out in duplicate, with control wells containing only MHB and MHB + distilled water. Gentamicin served as a positive reference standard drug. After incubation for 24 h, the MIC was established as the lowest concentration of the extract that entirely inhibited visible bacterial growth. To determine the MBC, 20 µL from all clear MIC wells were plated onto agar plates. The MBC was defined as the lowest concentration of the plant extracts that resulted in the death of 99.9% of the bacterial inoculum. Each determination was performed in three independent experiments, and the results were calculated based on the mode.

### 2.5. Chemical Characterization

#### 2.5.1. High-Performance Liquid Chromatography with Photodiode Array Detection-Tandem Mass Spectrometer (HPLC–PDA–MS/MS)

The LC system employed in this study was a Thermo Finnigan instrument (Thermo Electron Corporation, Waltham, MA, USA), featuring a reversed-phase column (Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 3.5 µm, Agilent, Santa Clara, CA, USA). The LC instrument was connected to a mass spectrometer (LCQ-Duo ion trap) equipped with an electrospray ionization source. The mobile phase consisted of water and acetonitrile (ACN), both infused with 0.1% formic acid. A gradient flow of the mobile phase was initiated, starting with 5% ACN and gradually increasing to 30% over a span of 60 min. In the next 30 min, the ACN concentration was elevated to 90%. The flow rate was set at 1 mL/min with a split ratio of 1:1. Employing the full scan acquisition method, spectra were scanned within the range of  $m/z$  50–2000 in the negative mode. Data evaluation was carried out using the X-calibur software (X-calibur™ 2.0.7, Thermo Fisher Scientific, Waltham, MA, USA).

#### 2.5.2. Fourier Transform Infrared (FTIR)

Finely ground potassium bromide particles were mixed with *A.pyrethrum* powder at a ratio of 1:100. The resulting mixture were analyzed using an infrared spectrometer to investigate molecular vibrations within the sample particles. Infrared spectra were collected in the range of 400–4000 cm<sup>-1</sup>, enabling the identification and characterization of chemical bonds and functional groups present in the silver nanoparticles.

### 2.5.3. X-ray Diffraction (XRD)

The XRD patterns was acquired using a PAN analytical X'pert PRO X-ray diffractometer from JEOL (Tokyo, Japan). Measurements were conducted within the  $2\theta$  range of  $20\text{--}80^\circ$ , employing a fixed current of 40 mA and a voltage of 45 kV. Analysis involved depositing a suspension of myogenic AgNPs into a glass substrate. XRD data enabled scrutiny of the crystal structure and phase composition of the silver nanoparticles. The average crystal size was determined using Scherrer's equation, which relates size (D) to wavelength ( $\lambda$ ), the full width at half maximum of the diffraction peak ( $\beta$ ), Scherrer's constant (K), and Bragg's angle ( $\theta$ ).

### 2.5.4. Scanning Electron Microscope (SEM)

Using a SEM, we conducted an analysis of the morphology, size, and shape of *A. pyrethrum* powder particles across magnification levels ranging from  $\times 1,000$  to  $\times 1,000,000$ , following the methodology previously described [30]. Particle size determination was accomplished by processing acquired SEM images using the Image J program 1.53a.

### 2.5.5. Energy-Dispersive X-ray Spectroscopy (EDX)

Elemental composition of samples was ascertained through EDX spectrometry. The EDX analysis provided information about element presence and distribution within the samples. A transmission electron microscope (TEM) of the JEM-2100 model (JEOL, Tokyo, Japan) was used to evaluate the particle structure and size distribution. TEM offers detailed insights into the particles morphology, structure, and size distribution.

## 2.6. Statistical Analyses

The data were presented in the form of mean values along with the standard error of the mean (SEM) by utilizing Graphpad Prism 09. A one-way analysis of variance (ANOVA) was conducted and post hoc Tukey's tests were performed to evaluate variations among the groups. Statistical significance was acknowledged at a  $p < 0.05$ .

## 3. Results

### 3.1. Phytochemical Screening

Our findings revealed the presence of total polyphenols ( $21.98 \pm 0.05$  mg GAE/g), flavonoid ( $8.1 \pm 0.16$  mg QE/g), and condensed tannins ( $5.49 \pm 0.08$  mg CE/g) within the AEAP (Table 1).

**Table 1.** Mean values of total polyphenol, flavonoid, and tannin contents in the AEAP.

	Total Polyphenol Content mg GAE/g DM	Flavonoids mg CE/gDM	Tanins mg CE/g DM
Crude extracts (mg/g)	$21.98 \pm 0.05$	$8.1 \pm 0.16$	$5.49 \pm 0.08$

GAE: gallic acid equivalent, CE: catechin equivalent, DM: dry matter.

### 3.2. Antioxidant Activity

AEAP exhibited antioxidant activity using the DPPH and reducing power methods. The IC<sub>50</sub> value was  $1.6 \pm 0.04$  mg/mL, indicating its capability to convert the stable purple-colored DPPH radical into the yellow-colored DPPH-H form (Table 2). Additionally, AEAP showed reducing power with an IC<sub>50</sub> value of  $1.4 \pm 0.04$  mg/mL.

**Table 2.** The antioxidant activity of the AEAP by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP).

Antioxidant Assay	Plant Extract (IC <sub>50</sub> = mg/mL)	Standard Antioxidant (IC <sub>50</sub> = mg/mL)	
		Quercetin	BHT
DPPH	1.6 ± 0.04	0.1 ± 0.0	0.2 ± 0.0
FRAP	1.4 ± 0.04	0.1 ± 0.0	0.1 ± 0.0

### 3.3. Antibacterial Activity

The AEAP displayed significant antibacterial activity as demonstrated by the inhibition zone diameter against the tested bacteria. When tested against *S. aureus* (ATCC 25923) at a concentration of 100 mg/mL, AEAP exhibited a mean inhibition zone of 17.65 ± 1.14 mm. Similarly, comparing AEAP to 10 µg/disc gentamicin against *E. faecium* (ATCC 4602), both agents showed comparable inhibition zones of 10.9 mm and 12.0 mm, respectively. Moreover, the antibacterial activity of AEAP was determined to be equally effective against Gram-negative as it was against Gram-positive bacteria. The inhibition zone diameter against *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27653) was measured at 17.7 mm and 15.5 mm, respectively. Comparatively, the positive control gentamicin exhibited inhibition zones of 23 mm and 22 mm, respectively (Table 3).

**Table 3.** Inhibition zones of the AEAP (100 mg/mL) against microbial strains.

Bacteria Group	Bacterial Strains	Reference	Diameter Inhibition Zone (mm)	
			AEAP	Gentamicin
Gram-positive bacteria	<i>Staphylococcus aureus</i>	ATCC 25923	17.65 ± 1.14	23
	<i>Enterococcus faecium</i>	ATCC 4602	10.87 ± 0.69	12
Gram-negative bacteria	<i>Escherichia coli</i>	ATCC25922	17.73 ± 0.61	23
	<i>Pseudomonas aeruginosa</i>	ATCC 27653	15.49 ± 2.01	22

In order to determine the effective concentration of AEAP, broth microdilution tests were conducted, along with determining MIC and MBC. As illustrated in Table 4, AEAP exhibited bactericidal effect against the tested bacteria, including *S. aureus* (1.95 mg/mL), *E. faecium* (3.91 mg/mL), *E. coli* (8.51 mg/mL), and *P. aeruginosa* (2.5 mg/mL). These MIC values denoted the lowest extract concentration at which no visible bacterial growth occurred, indicating thereby its inhibitory potential. However, the MBC was found to be greater than 50 mg/mL.

**Table 4.** Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the AEAP (50 mg/mL) against the microbial strains.

Bacteria Group	Bacterial Strains	Reference	MIC (mg/mL)	MBC (mg/mL)
Gram-positive bacteria	<i>Staphylococcus aureus</i>	ATCC 25923	1.95	>50
	<i>Enterococcus faecium</i>	ATCC 4602	3.91	>50
Gram-negative bacteria	<i>Escherichia coli</i>	ATCC25922	8.51	>50
	<i>Pseudomonas aeruginosa</i>	ATCC 27653	2.50	50

### 3.4. Characterization of the Extract

#### 3.4.1. HPLC Analysis

The AEAP underwent analysis using HPLC–PDA–MS/MS, where the phytoconstituents were identified based on their MS/MS fragments and retention times, revealing a total of 24 secondary metabolites encompassing various phenolic compounds and organic acids (Figures 1 and 2 and Table 5).

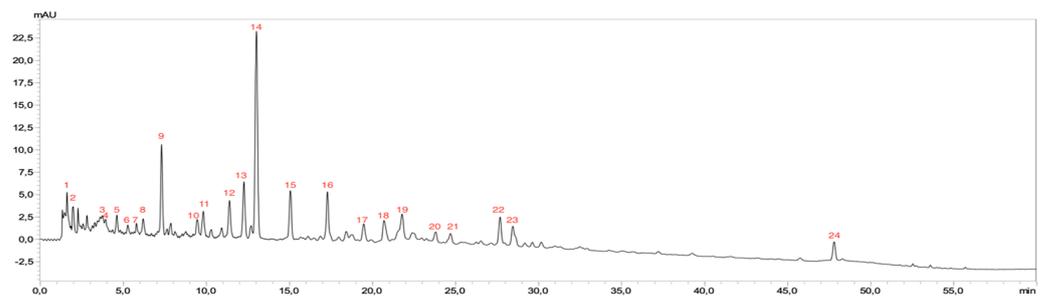


Figure 1. HPLC-MS/MS profile of AEAP.

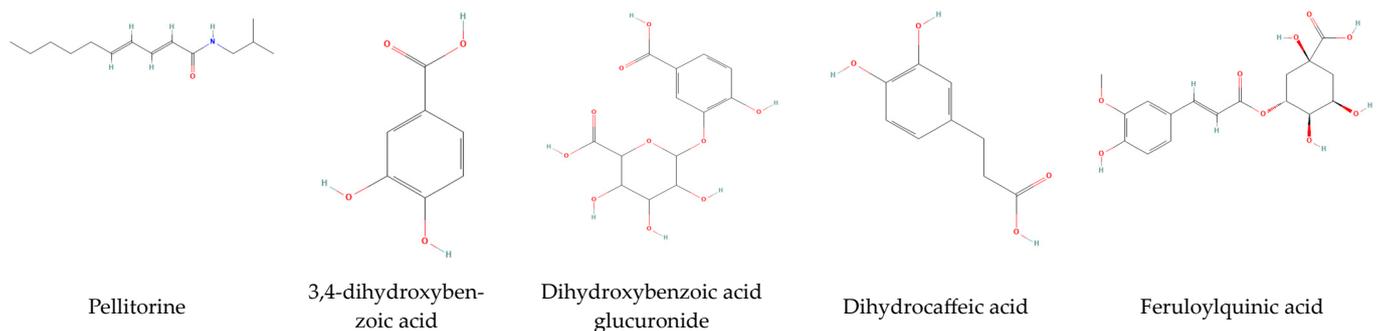


Figure 2. Chemical structure of the most frequent secondary metabolites in AEAP.

Table 5. Tentatively identified compounds in the AEAP using HPLC-ESI-MS/MS analysis.

	Rt (min)	MW	MS/MS	Compound Name
1	1.516	191	111	Citric acid
2	1.935	335	169	Gallic acid derivative
3	3.523	267	108, 153	Dihydroxybenzoic acid derivative
4	3.642	299	153	Dihydroxybenzoic acid rhamnoside
5	4.42	315	108, 153	Dihydroxybenzoic acid glucoside
6	5.243	371	135, 191	Caffeoylglucaric acid
7	5.862	169	125	Gallic acid
8	6.338	341	108, 167	Vanillyl quinic acid
9	7.124	153	108	3,4-dihydroxybenzoic acid
10	9.452	225	137	Hydroxybenzoic acid glycerol
11	10.921	353	135, 191	Chlorogenic acid
12	11.676	311	137	Hydroxybenzoic acid quinylyl ester
13	12.289	329	153	Dihydroxybenzoic acid glucuronide
14	13.102	223	133,81	Pellitorine
15	15.075	181	107, 135	Dihydrocaffeic acid
16	17.482	367	191	Feruloylquinic acid
17	19.437	353	179, 191	Cryptochlorogenic acid
18	20.607	179	135	Caffeic acid
19	21.88	163	119	<i>p</i> -Coumaric acid
20	23.919	337	191	<i>p</i> -Coumaroylquinic acid
22	24.853	161	133	Hydroxycoumarin
22	27.796	515	179, 191	Isochlorogenic acid b
23	28.521	447	301	Quercetin rhamnoside
24	48.907	515	179, 191	Isochlorogenic acid C

Rt: retention time; MW: molecular weight; MS/MS: Tandem Mass spectrometry.

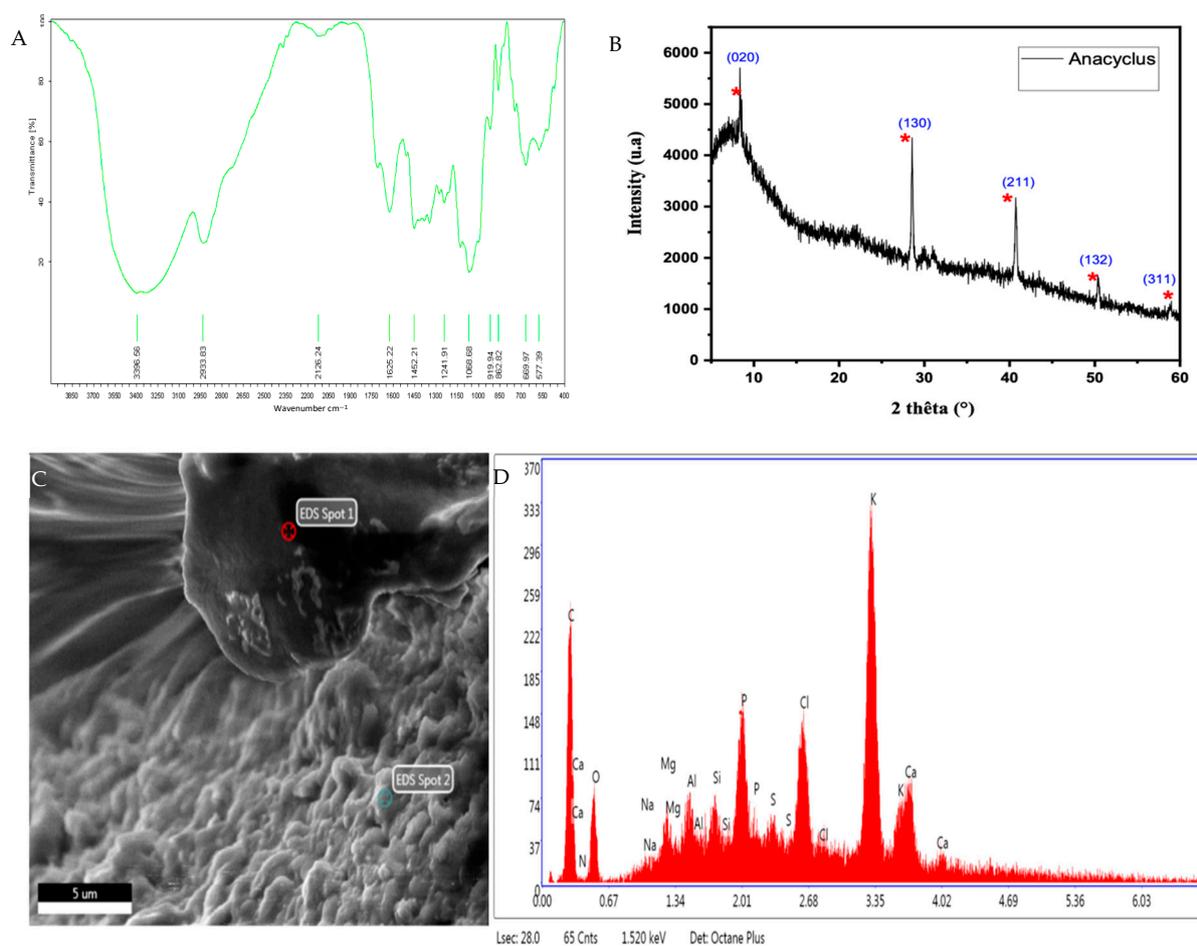
HPLC analysis of AEAP unveiled several compounds of interest. Predominantly, pellitorine emerged as the most abundant compound, featuring a retention time of 13.102 min and a molecular weight of 223. Pellitorine exhibited MS/MS fragmentation peaks at  $m/z$  133 and 81. Another substantial compound, 3,4-dihydroxybenzoic acid, eluted at 7.124 min with an MW = 153, and an MS/MS fragmentation peak at  $m/z$  108. Dihydroxybenzoic acid glucuronide, with a retention time of 12.289 min and an MW = 329, displayed an MS/MS fragmentation peak at  $m/z$  153. Similarly, dihydrocaffeic acid, detected at 15.075 min with an MW of 181, demonstrated MS/MS fragmentation peaks at  $m/z$  107 and 135. Feruloylquinic acid was observed at 17.482 min, possessing an MW of 367, and

exhibited an MS/MS fragmentation peak at  $m/z$  191. A recurrent appearance of dihydroxybenzoic acid glucuronide was noted at 12.289 min with an identical MW and MS/MS fragmentation pattern. Finally, hydroxybenzoic acid quiny ester was found at 11.676 min, with an MW of 311, alongside an MS/MS fragmentation peak at  $m/z$  137 (Table 5).

The HPLC–PDA–MS/MS analysis provided insights into the specific phytoconstituents present in AEAP. Notably, we identified 24 secondary metabolites, with pellitorine and 3,4-dihydroxybenzoic acid emerging as the most abundant components. Pellitorine, characterized by a molecular formula of  $C_{14}H_{25}NO$  and a molecular weight of 223.35 g/mol, and 3,4-dihydroxybenzoic acid, known as protocatechuic acid, with a molecular formula of  $C_7H_6O_4$  and a molecular weight of 154.12 g/mol, stood out as significant constituents within the extract (Figure 2).

### 3.4.2. EDX–SEM Analysis

The FTIR analysis of AEAP is depicted in Figure 3A, revealing a robust band within 3350 and 3460  $cm^{-1}$ , indicative of the stretching of the  $-NH_2$  and  $-OH$  group bonds. Notably, a band emerged at 2869–2938  $cm^{-1}$ , attributed to C-H vibration of  $-CH_3$ . Additionally, a peak at 1641.07  $cm^{-1}$  was credited to stretching and vibration of the carbonyl group,  $C=O$ , present in the extract. Furthermore, an absorption peak at ca. 1068.87  $cm^{-1}$  indicated the stretching vibration of the  $-C-O-C$  bridge of the ring, suggesting that presence of cyclic molecules in the extract. Distinct bands associated with the phosphate group were observed within the range of 862.66 and 919.70  $cm^{-1}$ , indicating the presence of minerals in the extract.



**Figure 3.** Physicochemical characterization of the AEAP: (A) Fourier Transform Infrared Spectroscopy, (B) X-ray Diffraction, (C) Scanning Electron Microscopy, and (D) Energy-Dispersive X-ray Spectroscopy.

Figure 3B showed the diffractogram of the AEAP, revealing a halo lies between 5° and 12° at 2θ, corresponding to a reflection plane of (020). Additional peaks were identified at 2θ around 28°; 41.5°; 50.9°, and 59.6°, aligning with (130), (211), (132) and (311) crystal reflection planes, respectively. These reflections could be attributed to a range of molecules present in the extract, including the specific active ingredient pellitorine, which is one of the most abundant molecules in the extract.

The coupling of EDX with SEM enabled microanalysis of AEAP, offering insight into its chemical composition in terms of atomic and weight percentages. The SEM image and EDX analysis (Figure 3C) illustrated the amorphous organization of molecules within the extract, visually represented by globular fibers stabilized by certain mineral elements as per EDX analysis. Moreover, elemental analysis results (Figure 3D) highlighted a substantial mineralogical composition.

Elements including K, Cl, P and Ca predominated in both weight and atomic percentages, as detailed in Table 6. Additionally, other elements were detected at lower levels, including S, Si, Al, Mg, Na and Fe.

**Table 6.** Elemental analysis of the AEAP.

Sample/Element		K	Ca	Cl	P	S	Si	Al	Mg	Na
AEAP	Weight %	33.62	12.42	10.60	8.02	4.00	3.26	2.86	1.86	0.52
	Atomic %	21.87	7.88	7.60	6.59	3.17	2.95	2.70	1.95	0.57

#### 4. Discussion

The purpose of this study was to present valuable insights into the phytochemical composition, antioxidant activity, and potential health benefits of the aqueous extract of *A. pyrethrum*. We aim to discuss the implications of our findings, their significance in the context of medicinal plants, and their potential applications in various fields. To provide insights into the chemical composition and mineralogical properties, the AEAP has been characterized using various techniques, including EDX–SEM, FTIR, diffractogram and HPLC analysis.

Polyphenols play a crucial role in shaping the biological characteristics of natural substances. In the current study, phytochemical screening revealed the presence of  $21.98 \pm 0.05$  mg GAE/g of total polyphenols. This finding aligns with a previous investigation utilizing a hydroethanolic extract of *A. pyrethrum*, which reported a similar content ( $25.96 \pm 1.93$  mg GAE/g) obtained from Timehdite region, Morocco [31]. A study conducted in India on the hydromethanolic extract of *A. pyrethrum* reported a notably higher total phenolic content ( $62.89 \pm 0.43$  mg GAE/g) [12]. The concentrations of these polyphenols are significantly influenced by various factors, including the growing region, harvest time, extraction solvent, and storage conditions [32]. *A. pyrethrum* is known for its rich content of flavonoids, which are well-regarded for their antioxidant properties. In our research, we determined that the roots contain  $8.1 \pm 0.16$  mg QE/g of flavonoids. In contrast, a previous study reported a lower level of flavonoids at  $0.88 \pm 0.02$  mg QE/g [31]. Strikingly, the leaf examination revealed a significantly higher flavonoid content of  $13.53 \pm 0.05$  mg QE/g [31]. Moreover, Kalim and colleagues found an even more substantial flavonoid content, measuring  $38.89 \pm 0.52$  mg QE/g [12]. It is worth noting that recent research has shed light on the variability in assessing antioxidant activity due to the distinct mechanisms of action exhibited by different antioxidants [33]. Herein, we demonstrated remarkable antioxidant effects in *A. pyrethrum* roots, with an  $IC_{50}$  of  $1.6 \pm 0.04$  mg/mL in the DPPH and  $1.4 \pm 0.04$  mg/mL in the FRAP test. Notably, a study by Manouze et al. (2017) reported varying values in the DPPH test:  $12.38 \pm 0.28$  µg/mL for the aqueous extract and  $13.41 \pm 0.67$  µg/mL for the methanolic extract of *A. pyrethrum* sourced Oukaimed region, Morocco [9]. The high antioxidant activity observed in *A. pyrethrum* can be attributed to its rich phytochemical composition, particularly the presence of phenolic compounds. These phenolic compounds are believed to directly contribute to the plant antioxidative properties by playing a crucial

role in mitigating the harmful effects of free radicals in the body, effectively safeguarding cells and tissues against oxidative damage [34]. Additionally, the ability of AEAP to effectively neutralize free radicals, as indicated by its low IC<sub>50</sub> values in the DPPH and reducing power assays, highlights its potential in preventing cellular damage and mitigating the risk of oxidative stress-related diseases [35]. These findings align with the growing interest in plant-based antioxidants as alternatives to synthetic antioxidants [36,37].

The presence of polyphenols in the extract, such as hydroxybenzoic acid quinyll ester, dihydrocaffeic acid, feruloylquinic acid, caffeic acid, p-coumaric acid, p-coumaroylquinic acid, hydroxycoumarin, along with the alkylamide pellitorine, is of significance. These compounds have been previously confirmed to possess a wide range of beneficial activities, including, immunomodulatory, antithrombotic, antibacterial, antiviral, antioxidant, anti-inflammatory, analgesic, anticancer, antidiabetic, and antiprotozoal [20,38,39]. Additionally, the compounds in the extract have been reported to exhibit various pharmacological activities, such as antioxidant, antibacterial, neuroprotective, and nematocidal properties [40–42]. The identification of these compounds may pave the way for further research into their bioactivity and potential medicinal applications [14,43].

The HPLC analysis has successfully identified the presence of various bioactive compounds in the extract. Phenolic compounds may directly contribute to the antioxidative effects [44,45]. Flavonoids have the capability to effectively scavenge various reactive oxygen species (ROS), including superoxide and nitric oxide radicals [46,47]. The existence of these compounds and organic acids also plays a role in the overall bioactivity of *A. pyrethrum*.

The EDX–SEM analysis provided further insight into the characterization of AEAP, shedding light on its mineral composition and amorphous structure. The analysis revealed the presence of various mineral elements in *A. pyrethrum* extract, including K, Cl, P, Ca, S, Si, Al, Mg, Na, and Fe. These mineral elements exhibited varying weight % and atomic %, with K, Cl, P, and Ca being the predominant ones. The SEM microscopic image vividly depicted the amorphous arrangement of all the molecules within the extract, stabilized by select mineral elements as per EDX analysis. The data confirmed the presence of these mineral elements, while FTIR analysis revealed distinct bands in the spectrum of the aqueous extract. Notably, the FTIR analysis showcased the existence of specific functional groups, including -NH<sub>2</sub>, -OH, -CH<sub>3</sub>, and C=O, characteristic of flavonoids, tannins, and other secondary metabolites. The diffractogram analysis further revealed the presence of various crystal reflection planes, likely attributed to a range of molecules within the extract, including the active ingredient. The high content of elements and the amorphous structure of AEAP suggest potential benefits for its solubility and bioavailability, positioning it as a promising candidate for a range of applications [48].

Our results demonstrated significant antibacterial activity of *A. pyrethrum* against both Gram-positive and Gram-negative bacteria. Notably, we observed a substantial inhibition zone measuring 17.65 mm ± 1.14 against *S. aureus*, notably surpassing a previous finding of a 17 mm inhibition zone. For *P. aeruginosa*, our study recorded an inhibition zone of 15.49 mm ± 2.01, closely aligning with the value of 15.65 mm observed in the same study [31]. This underscores the broad-spectrum antibacterial potential of *A. pyrethrum*. Furthermore, the observed bactericidal effect at relatively low concentrations underscores its efficacy in inhibiting bacterial growth. These findings align with previous studies [3,49,50] that have highlighted the antibacterial effects of *A. pyrethrum* against other bacterial strains (*Listeria monocytogenes* and *Candida albicans*), reinforcing its effectiveness in combating bacterial infections [51]. According to Elazzouzi et al. [7], *A. pyrethrum* aqueous extract exhibited relatively high antibacterial activity compared to ethanol and alkylamide extract. Jawhari et al. reported bactericidal effects across all components of *A. pyrethrum*, including the roots, capitula, seeds, and leaves, with variations noted across different bacterial strains, plant extracts, and plant parts employed in the study [31]. These findings attribute the observed antibacterial activity to the presence of secondary metabolites within the extract, particularly phenols and flavonoids, which contribute to the antibacterial effects [52].

Altogether, these findings suggest that *A. pyrethrum* holds promise for the development of natural antimicrobial agents, a crucial consideration in light of increasing antibiotic resistance. It is worth noting that, although this study did not include a toxicity assessment, prior research has consistently reaffirmed the non-toxic nature of *A. pyrethrum* extracts [14,53,54]. With an LD50 value exceeding 5000 mg/kg, indicating low acute toxicity and aligning with the Organization for Economic Co-operation and Development (OECD) guidelines for acute systemic toxicity classification, AEAP exhibits a robust safety profile [54].

## 5. Conclusions

This study highlights the remarkable potential of AEAP as a rich reservoir of bioactive compounds, boasting substantial antioxidant and antibacterial properties. AEAP exhibited robust antioxidant capabilities, effectively scavenging free radicals, and demonstrated a broad-spectrum antibacterial effectiveness against both Gram-positive and Gram-negative bacteria. The phytochemical profiling identified key bioactive compounds, with pellitorine and 3,4-dihydroxybenzoic acid standing out for their antioxidant and antibacterial effects. Furthermore, the comprehensive composition analysis of AEAP revealed its intricate composition, replete with diverse functional groups and molecules. These findings support further exploration of *A. pyrethrum* as a natural source of bioactive compounds with potential applications in various pharmacological interventions. Nevertheless, future studies should prioritize the isolation and characterization of individual bioactive compounds within AEAP and elucidate their mechanisms of action. Additionally, conducting clinical and preclinical trials to validate the health benefits of AEAP in humans is imperative. These investigations hold the promise of advancing our understanding of AEAP's therapeutic potential and its prospective contributions to healthcare.

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