Phytochemical Characterization of Natural Leaf Extracts from *Achillea ligustica :* HRMS Analysis, Antioxidant Activity, and Brine Shrimp Lethality Testing

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ABSTRACT

This research aims to enhance the composition of phenolic compounds, the antioxidant and cytotoxic activities of the methanol, ethyl acetate, and n-butanol extracts produced from the leaves of *Achillea ligustica*, collected in the northeast of Algeria. The measure of extracts' total phenolic and flavonoid contents was performed by the aluminum nitrate and Folin-Ciocalteu colorimetric techniques, respectively. The chemical composition was detected using the Liquid Chromatography–High Resolution Mass Spectrometry method. Moreover, the antioxidant activity was investigated by four methods (DPPH, ABTS, FRAP, and Phenanthroline method), while the cytotoxic effect was evaluated on the phenolic compounds extracted from *Achillea ligustica* using the brine shrimp lethality test. The obtained data showed that both the highest phenolic (334.29 ± 1.06 µg GAE/mg extract) and the highest flavonoid (92.85 ± 0.15 µg QE/mg extract) contents were identified in the methanolic extract. Also, large spectrum of phenolic acids, flavonoids, and anthocyanins was detected. Besides, in all realized tests highest antioxidant activity was detected in the ethyl acetate extract, while the lowest toxicological potential (LC_{50} = 502.73 µg/mL) was demonstrated in the n-butanol extract. Finally, the methanolic extract induced the highest mortality rate in *Artemia salina* larvae, reaching 65% at 400µg/mL. The findings of our research revealed that the leaves of *Achillea ligustica* represent a noteworthy medicinal plant species that merits additional investigation.

Keywords: *Achillea ligustica*, Antioxidant activity, LC-HRMS analyses, Brine shrimp, phenolic compounds.

1. Introduction

The therapeutic properties of botanical species have been recognized by humans since ancient times. These traditional knowledges were gradually abandoned due to the emergence of modern medicine and the perceptions of inefficacy. However, numerous studies demonstrating the efficacy and reliability of plant-based preparations have reversed this trend. Phenolic compounds, a large and diverse class of natural substances with over 50,000 identified representatives (Tacias-Pascacio *et al.* 2022), are particularly noteworthy. They are associated with preventing, treating, or alleviating the symptoms of diseases, offering a more accessible and cost-effective alternative to chemically synthesized products, which are often more expensive due to technological patents (Kumar and Goel. 2019). The toxicological and pharmacological properties of medicinal plants have garnered the attention of scientists seeking to verify their therapeutic potential, isolate activeconstituents, and investigate possible toxicities (Naeeri and Shirzad. 2013).

The *Achillea* genus consists of more than 150 species that are distributed throughout North Africa, Europe, Asia and North America (Orhan. 2019). In traditional medicine, many ailments, including as gastrointestinal disorders, stomach and duodenal ulcers, anemia, sensitive skin, and as an anthelmintia, have been treated using *Achillea* species (Cecchini *et al.* 2012; Conforti *et al.* 2005; Muselli *et al.* 2009). Moreover, several recent studies indicate that the *Achillea* genus has a great number of beneficial characteristics, such as hemostatic, hypoglycemic, wound-healing, anti-inflammatory, antioxidant, antispasmodic, anti-allergic, and bactericidal activities. These therapeutic applications are attributed to biologically active compounds isolated and identified by various researchers (Tuberoso *et al.* 2009; Saeidnia *et al.* 2011; Barda *et al.* 2021).

The current research aimed to investigated the chemical composition of polyphenols of the extracts of the Algerian medicinal plant *Achillea ligustica* (Asteraceae), traditionally known for its therapeutic properties using LC-HRMS analyses. Moreover, the proprieties of these extracts such as the antioxidant activity using DPPH, ABTS, phenanthroline, and FRAP (Ferric reducing

antioxidant power) assays and the cytotoxic activity, using the brine shrimp lethality bioassay, were performed.

2. Materials and methods

2.1.Plant material

Achillea ligustica (Asteraceae) were sampled from the Mila region, located in the northeast of Algeria (36°30′14.51″ N, 6°24′45.41″ E, at an altitude of 315 m). A representative plant sample was identified by Prof. Sakhraoui Nora (University of 20 August 1955-Skikda). The leaves of *Achillea ligustica* were air-dried at room temperature for one month under natural ventilation and then ground with an electric mill (Sayona SZJ-R14 (350W, 50g).

2.2. Preparation of extracts of different polarities

After drying and grinding the plant material, 100 g of the powder was subjected to exhaustive maceration with 1L of 70% methanol (MeOH/H2O: 70/30) at room temperature (25°C) under magnetic agitation for 24 hours, repeated for three consecutive days with daily solvent replacement. To remove the methanol, the extract was subjected to a filtration and concentration under reduced pressure at 40°C, then to a liquid-liquid extraction with a series of solvents of progressively increasing polarity. Finally, in order to remove the solvent, each fraction obtained was concentrated at 40°C and then conserved at 4°C.

2.3. Total phenolic (TPC) and total flavonoid (TFC) content

The TPC was realized using the protocol of Singleton et al. 1999 as Müller *et al.* (2010) describe. In a 96-well plate, the following mixture was prepared: 20 µL of extract (diluted to 1 mg/mL in methanol), 75 µL of 7.5% (w/v) Na_2CO_3 solution and 100 µL of 10% (v/v) Folin-Ciocalteu reagent. Methanol was utilized as the blank instead of the extract. After adding all reagents, an incubation of the plate was released for 2 hours in the dark. Total Phenolic Content was calculated and expressed as µg GAE/mg after measurement of absorbance using a 96-well microplate reader at 765 nm.

The determination of TFC was realized following the protocol developed by Topçu et al. (2007) and adapted for microplate analysis. For this 50 µL of sample, 130 µL of methanol, 10 µL of potassium

acetate and 10 µL of aluminum nitrate (10%) were mixed in a 96-well ELISA plate. For the blank, methanol (150 µL) was added to replace the extract. A calibration curve was created using different concentrations of quercetin (from 25 to 200µg/ml). Absorbance readings were taken at 415 nm in microplate reader (Perkin Elmer, Enspire) and TFC was calculated and expressed as µg QE/mg.

2.4. Phytochemicalidentification by HRMS analysis

Before the analysis, all extracts were subjected to filtration by using a 0.45 µm filter. An Exploris 120 mass spectrometer (Thermo Fisher Scientific, San José, CA, USA) with an electrospray source and an Orbitrap analyzer was used for molecular identification. A reversed-phase C18 column with a flow rate of 1 mL/min and a runtime of 28 minutes was used for chromatographic separation. The mobile phase consisted of 0.1% formic acid in water (solvent A) and methanol (solvent B).

The mass spectrometer was coupled with a Vanquish HPLC system and autosampler (Thermo Fisher Scientific, San José, CA, USA). The MS analyses were performed both in negative and positive ionization mode (3500 V and - 4000) V. The working condition were the following: spray voltage 3500 V (positive polarity) and 4000 V (negative polarity), sheath gas 20 a. u., aux gas 8 a. u., sweep gas 1 a. u., ion transfer tube temperature 300°C, and vaporizer temperature 290°C.Full scan data were acquired in the mass range 100 - 1000 m/z, the RF lens was set to 70% of the maximum value and the orbitrap resolution was set at 60000. The MS/MS experiments were carried out in untargeted mode, subjected to MS fragmentation the ions with an intensity threshold of 2.1e5.

2.5. In vitro antioxidant assays

Four methods (DPPH, ABTS, phenanthroline, and ferric ion reducing power (FRAP) assays) were employed to offer a comprehensive evaluation of the extracts' antioxidant activity.

2.5.1. DPPH Radical Scavenging Test

The free radical scavenging activity was determined according to Blois (1958). The reaction involves the reduction of the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) by antioxidants through proton donation, leading to a decrease in absorbance at 517 nm due to the decolorization and

disappearance of the violet color (Conforti *et al.* 2005). The methanolic solution of DPPH (0.15 mM) was prepared. For each test, 40 µL of the extracts (MeOH, EtOAcor n-BuOH) at different concentrations (0,0078-0,5 mg/mL) was mixed with 160 µL of the DPPH solution. In parallel, the negative control was prepared by replacing the sample with methanol. The plates were incubated for 30 min at room temperature in the dark to determine the antioxidant activity (AA%), using the following equation:

AA (%) = [(A-control – A-sample) / A-control] × 100

Where

A-control: the absorbance of negative control.

A-sample: the absorbance of tested sample.

The IC_{50} values, indicating the concentration needed to inhibit 50% of DPPH radicals, were determined from the linear regression of the DPPH inhibition percentage versus sample concentration. Moreover, to evaluate antioxidant effectiveness, the IC_{50} values of the plant extracts were compared with those of the synthetic standard butylated hydroxyanisole (BHA).

2.5.2. Antioxidant activity against the ABTS radical

The antioxidant potential against the ABTS radical was investigated following the protocol described by Re *et al.* (1999). The $ABTS^{\cdot+}$ solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate in water, followed by a reaction in the dark for 12 to 16 hours. The solution was then diluted with ethanol (EtOH) to achieve an absorbance of 0.700 at 734 nm. For the experiment,40 µLof sample prepared atdifferentconcentrations (0,0078-0,5 mg/mL) was added to 160 µL of the $ABTS^{\cdot+}$radical solution. A negative control was prepared using the same procedure, replacing the samples with methanol. After incubation at room temperature for 10 minutes, the absorbance was determined at 734 nm.The percentage of $ABTS^{\cdot+}$radical inhibition was determined as follow:

%Radical scavenging effect = [(A-control – A-sample) / A-control] × 100

Where:

A-control: the absorbance of negative control.

A-sample: the absorbance of tested sample

Synthetic standards, such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene), were used to compare the inhibitory percentages and the concentration required to inhibit 50% of the $ABTS^{\cdot+}$ radical (IC_{50} values) was measured from the graph of ABTS inhibition versus sample concentration.

2.5.3. Phenanthroline activity

The phenanthroline activity was measured in the present study using the Szydlowska-Czerniak *et al.* (2008) methodology. In a 96-well microplate, the extract (10 µL) at different concentrations (0.0625–4mg/mL) was blended with $FeCl_3$ (50 µL, 0.2%), phenanthroline (30 µL, 0.5%) and MeOH (110 µL). The absorbance was taken at 510 nm after conservation of plate in the dark for 20 minutes at 30°C to enable the reaction to finish. The data were expressed as$A_{0.5}$ µg/mL.

2.5.4. Ferric reducing antioxidant power (FRAP)

The FRAP test, realized to evaluate the extracts' reducing power', was adapted from the protocol of Oyaizu. (1986). A mixture of 10 µL aliquot of standard or each sample at different concentrations, 50 µL of potassium ferricyanide (1%) and 40 µL of phosphate buffer (pH 6.6) was stored at 50°C for 20 minutes. Following the incubation, 10 µL of ferric chloride (0.1%) and 40 µL of distilled water were blended to the wells. The measurement of absorbance was taken at 700 nm and the data were expressed as $A_{0.5}$ µg/mL and compared to the antioxidant standards α-tocopherol and ascorbic acid.

2.6. Brine shrimp lethality test

The lethality test using brine shrimp was realized following the methodology of Meyer *et al.* (1982). *Artemia salina* cysts (10 mg) were hatched in saline solution (3.8%) at 25–28°C under fluorescent lighting for 48 hours. After hatching, 100 µL of a solution containing 10 A. salina larvae, 80 µL of seawater, and 20 µL of each extract or positive control (potassium dichromate,

0.1%) were added in triplicate to the wells of a 96-well plate. Extracts were tested at various concentrations prepared from a stock solution (4 mg/mL). Plates were incubated for 24 hours under fluorescent lighting. After incubation, the percentage mortality at each dose was calculated after counting the number of surviving larvae and the mortality was expressed as lethal concentration (LC_{50}) (Concentration required to kill 50% of larvae) using Probit analysis in IBM SPSS Statistics version 25. Substances with LC_{50} values below 1000 µg/mL were considered toxic.

2.7. Statistical analysis

Results of this research were treated using the version 25 of IBM SPSS Statistics. All results were shown as the mean ± SD and at $p < 0.05$ the differences were considered statistically significant Many tests were used such as the Kolmogorov-Smirnov test to check the normality of data and Levene's to analyze the homogeneity of variances. Also, the ANOVA and Tukey's test were used to compare the experimental groups, and the Probit analysis to calculate the lethal concentrations.

3. Results and Discussion

3.1. Total Phenolic and Flavonoid Content

Based on the absorbance results (Table 1) obtained using the Folin-Ciocalteu reagent, the phenolic content of methanolic extract exhibited a higher concentration (334.29 ± 1.06 µg GAE/mg Ext) compared to the ethyl acetate (127.03 ± 2.71 µg GAE/mg Ext) and n-butanol (151.25 ± 2.39 µg GAE/mg Ext) extracts. Similarly, the flavonoid concentrations in the crude extracts were also determined, as shown in Table 01, indicating highest flavonoid content (92.85 ± 0.15 µg QE/mg Ext) in methanolic extract, followed by the ethyl acetate (54.38 ± 0.29 µg QE/mg Ext) and the n-butanol extracts (27.85 ± 0.29 µg QE/mg Ext).

Table 1. Total phenolic and flavonoid contents in different extracts of *Achillea ligustica* (m ± SD; n=3). Values followed by different superscripts in the same column show a significant difference (Tukey's test, $p \leq 0.05$).

Extract	Total phenolic compounds content (µg GAE/mgExt)	Flavonoids content (µg QE/mg Ext)

MeOH extract	334.29 ± 1.06[c]	92.85 ± 0.15[c]
EtOAc extract	127.03 ±2.71[a]	54.38 ± 0.29[b]
n-BuOH extract	151.25 ± 2.39[b]	27.85 ± 0.29[a]

190

191 *3.2. Phytochemical Identification*

192 The qualitative phytochemical analysis of *Achillea ligustica* using High-Resolution Mass
193 Spectrometry (HRMS) confirmed the existence of several bioactive compounds, confirmed by their
194 characteristic masses and ionization patterns. Thirty-one (31) polyphenolic compounds were found
195 in the different extracts (methanol, ethyl acetate, and n-butanol). The resulting compounds owned to
196 three groups: phenolic acids, flavonoids, and anthocyanins. Table 2 provides a detailed list of the
197 identified compounds.

198

199 **Table 2.** Polyphenols detected and identified in different extracts of *Achillea ligustica*.
200

Compounds	**Molecular Formula**	**Mass [M- H]$^-$**	**Mode of Ionization**	**MeOH extract**	**EtOAc extrac**	**n-BuOH extract**
1/ Phenolic acids						
Chlorogenic acid	C_{16} $H_{18}O^-_9$	353.0878	[M-H]$^-$	d	d	d
Benzoic acid	C_7 H_5 O_3^-	137.0244	[M-H]$^-$	d	d	d
Caffeic acid	C_9 H_7 O_4^-	179.0346	[M-H]$^-$	d	d	d
Cinnamic acid	C_9 H_8 O_2^-	147.0451	[M-H]$^-$	d	nd	nd
P coumaric acid	C $_9H_7$ O_3^-	163.0404	[M-H]$^-$	d	d	d
Ferulic acid	C_{10} H_9 O_4^-	193.0508	[M-H]$^-$	d	d	d
Rosmarinic acid	C $_{18}$H $_{15}O_8^-$	359.0772	[M-H]$^-$	d	nd	nd
5-*p*- coumaroyl-hexoside	C $_{15}H_{18}$ O_8^-	325.0924	[M-H]$^-$	d	d	d
3-caffeoloquinic acid	C_{16} H_{18} O_9^-	353.0846	[M-H]$^-$	nd	d	d
5-*p*-coumaroylquinic acid	C_{16} H_{18} O_8^-	337.0942	[M-H]$^-$	d	d	d
2/ Anthocyanins						
Cyanidin-3-O-galactoside	C_{21} H_{21} O_{11}^-	483.8268	[M-H]$^-$	d	d	nd
3/ Flavonoïds						
Flavonols						
Quercetin 3-O-pentosyl hexoside	C_{26} H_{28} O_{16}^-	595.1308	[M-H]$^-$	d	d	d
Quercetin 3-O-(6’’-Malonyl-hexoside	C_{24} H_{22} O_{15}^-	549.0884	[M-H]$^-$	d	nd	nd
Isorhamnetin-3-O glucoside	C_{22} H_{22} O_{12}^-	477.1047	[M-H]$^-$	d	d	d
Flavan3-ols						

Catechin 3-O-hexoside	$C_{21}H_{24}O_{11}^-$	451.1256	$[M-H]^-$	d	d	nd
Catechin	$C_{15}H_{14}O_6^-$	289.0702	$[M-H]^-$	d	d	d
Flavonoids aglycone						
Quercetin aglycone	$C_{15}H_9O_7^-$	301.0356	$[M-H]^-$	d	d	nd
Luteolin aglycone	$C_{15}H_9O_6^-$	285.2289	$[M-H]^-$	d	nd	nd
Apigenin	$C_{15}H_9O_5^-$	269.2295	$[M-H]^-$	d	d	d
kaempferol	$C_{15}H_9O_6^-$	285.0405	$[M-H]^-$	d	nd	d
Flavonoids glycone						
Isoquercetine glucose	$C_{21}H_{19}O_{12}^-$	463.0882	$[M-H]^-$	nd	d	d
Rutine glucoside	$C_{27}H_{29}O_{16}^-$	609.1461	$[M-H]^-$	d	d	d
Rutindiglucose	$C_{27}H_{29}O_{16}^-$	609.1461	$[M-H]^-$	d	d	d
Apigetrin	$C_{21}H_{19}O_{10}^-$	431.0984	$[M-H]^-$	d	d	d
Puerarin	$C_{21}H_{19}O_9^-$	415.1035	$[M-H]^-$	d	nd	nd
Apigenin 6,8 diglucoside	$C_{27}H_{29}O_{15}^-$	593.1512	$[M-H]^-$	d	nd	nd
Kaempferol 3,7,4 triglucoside	$C_{33}H_{39}O_{21}^-$	771.1978	$[M-H]^-$	d	nd	nd
Luteolin-7-O glucoside	$C_{21}H_{19}O_{11}^-$	447.0933	$[M-H]^-$	d	nd	d
Luteolin-7- Rutinoside	$C_{27}H_{29}O_{15}^-$	593.1501	$[M-H]^-$	d	d	d
Apigenin -7-Oglucoside	$C_{21}H_{19}O_{10}^-$	431.0984	$[M-H]^-$	d	d	d
Phloridzin	$C_{21}H_{24}O_{10}^-$	435.1297	$[M-H]^-$	nd	nd	d

d= detected, nd= not detected

3.2.1. Phenolic Acids

The pharmacological effects of phenolics acids including antioxidant, antimicrobial and anti-inflammatory properties are well documented (Khatri *et al.* 2019), making plants a valuable resource for medicinal applications. In current study, ten (10) phenolic acids; Chlorogenic acid, *p*-coumaric acid, caffeic acid, 5-*p*-coumaroyl hexoside, ferulic acid and 5-*p*-coumaroylquinic acid were detected in our extracts. These compounds enhance the plant's antioxidant capacity, which could help protect the body from oxidative stress and chronic diseases. Among them, chlorogenic acid and caffeic acid are particularly well-known for their antimicrobial (Espíndola *et al.* 2019), antioxidant and anti-inflammatory properties (Gu *et al.* 2023). Indeed, Rosmarinic acid and cinnamic acid, identified in the methanolic extract, exhibit antibacterial and antioxidant characteristics (Kernou *et al.* 2023) and contribute to the prevention of oncological diseases (Ruwizhi and Aderibigbe. 2020). Besides, 3-caffeoylquinic acid, present in both extracts (ethyl acetate and n-butanol extracts) and benzoic acid, found in the methanolic and ethyl acetate extracts are recognized for their antioxidant (Makori *et al.* 2021) and antifungal activities (Berne *et al.* 2015).

3.2.2. Flavonoids

Flavonoids and their glycosides are commonly associated with the antioxidant, antidiabetic, anticancer, and anti-inflammatory effects of medicinal plants (Panche *et al.* 2016). The studied medicinal plant exhibits a significant diversity of flavonoids, with twenty (20) compounds identified, including derivatives of flavonols such as isorhamnetin-3-*O*-glucoside, widely distributed in all extracts, which serves as an anti-inflammatory and antimicrobial agent (Gong *et al.* 2020). Quercetin-3-*O*-(6’’-malonyl-hexoside) and quercetin-3-*O*-pentosyl hexoside, highly biologically active compounds (BAC), are identified in the methanolic extract and knowing for their essential roles in antioxidant and anti-inflammatory activities (Lesjak *et al.* 2018). Additionally, catechin and catechin-3-*O*-hexoside, belonging to flavan-3-ol derivatives, possess significant antioxidant activity, which can be useful in protecting the body against oxidative stress and chronic diseases (Coșarcă *et al.* 2019).

In terms of composition, methanol and ethyl acetate extracts from *Achillea ligustica* contain flavonoid aglycones such as quercetin, luteolin, apigenin, and kaempferol, which are also detected in the n-butanol extract, along with their glycosides. These compounds are known for their significant implications in inflammation, heart disease, and cancer (Middleton *et al.* 2000). This group includes notable glycosides such as rutin derivatives (rutin glucoside and rutin diglucose), identified in all extracts and isoquercetin glucose, a strong antioxidant detected in ethyl acetate and n-butanol extracts. The cited compounds contribute to the plant's antidiabetic, anti-Alzheimer, and antimicrobial potential (Ganeshpurkar and Saluja. 2017).

The Apigenin derivatives (apigetrin, apigenin-7-O-glucoside, found in all extracts, and apigenin-6,8-diglucoside, identified in the methanol extract) are biologically active substances that showed antioxidant, anti-tumor, anti-inflammatory, cardioprotective, neuroprotective properties (Li *et al.* 2023).Kaempferol derivatives (kaempferol 3,7,4-triglucoside, found in the methanol extract) have gained recognition for their properties as an antioxidant, anti-inflammatory, bacteriostatic and neuroprotective (Parveen *et al.* 2023). Additionally, luteolin derivatives (luteolin-7-O-glucoside,

detected in methanol and n-butanol extracts, and luteolin-7-rutinoside, found in all extracts) display numerous pharmacological properties, including antioxidant and antimicrobial activities (Lopez-Lazaro. 2009).Puerarin, detected in the methanol extract, is a powerful antioxidant capable of effectively removing toxins from the body, with notable antimicrobial and neuroprotective properties (Liu *et al.* 2023). Phloridzin, observed in the n-butanol extract, contributes significantly to the plant's high antioxidant potential. By reducing inflammation and oxidative damage, phloridzin plays a crucial role in the therapy of many illnesses, including cardiovascular and cancer disease (Khanam *et al.* 2022).Only one anthocyanin derivative (cyanidin-3-O-galactoside) was identified, exclusively in methanol and ethyl acetate extracts. This critical bioactive component is well-recognized for its antioxidant, cardioprotective (Kong *et al.* 2003), anti-inflammatory (Cui *et al.* 2021) and anti-thrombotic properties (Rechner and Kroner. 2005).

This study confirms that the bioactive compounds composition is induced by the extraction solvents. Methanol appears to be the most effective solvent, detecting 28 out of 31 compounds, suggesting its higher extraction potential. However, ethyl acetate and n-butanol extracts, both detecting 11 compounds, exhibit lower extraction efficiency, likely caused by variations in solvent polarity and the solubility of specific polyphenols in each solvent.

The obtained results are consistent with the literature, where several studies have employed modern chromatographic and spectroscopic techniques, to identify phenolic compounds in different *Achillea* species extracts. Agar *et al.* (2015) discovered many phenolic components in the methanol extracts of *Achillea coarctata*, *Achillea kotschyi*, and *Achillea lycaonica*, highlighting the phenolic richness of *Achillea spinulifolia* and *Achillea goniocephala* and identifying compounds including quinic acid, chlorogenic acid, rosmarinic acid, rutin, quercetin, luteolin, and apigenin. On the other hand, Dias *et al.* (2013) studied the methanolic extract of *Achillea millefolium* and found components belonging to two classes of natural compounds: phenolic acids (including derivatives of caffeoylquinic acid and dicaffeoylquinic acid) and flavonoids (including derivatives of apigenin, quercetin, luteolin, kaempferol, and isorhamnetin). Regarding previous studies on *Achillea*

ligustica, Boubertakh *et al.* (2024) identified many substances including chlorogenic acid, luteolin, quercetin, apigenin, and rosmarinic acid in the hydroethanol extract. Additionally, Tzakou *et al.* (1995) identified apigenin, luteolin, and santin in methanolic and aqueous extracts. These outcomes are consistent with those of Tuberoso *et al.* (2009), who discovered many flavonoids, including quercetin, luteolin, and 6-hydroxykaempferol, in ethanolic extracts. These findings were further supported by the research of Venditti *et al.* (2015) on methanolic extracts, identifying various compounds, including luteolin, 7-O-methyl apigenin, caffeic acid, chlorogenic acid, apigenin-7-O-glucuronide, and quercetin-3-O-glucuronide. These results highlight the potential of *Achillea* species, including *Achillea ligustica*, as a source of various bioactive substances with significant therapeutic characteristics.

3.3. Antioxidant Activity

The table 3 illustrates the findings of antioxidant activity against of the standards and the three extracts *Achillea ligustica*.

3.3.1. DPPH Assay

From the data reported in this work, increased antioxidant activity is indicated by a lower IC_{50}. Consequently, the ethyl acetate extract's antioxidant potential (IC_{50} = 4.18 ± 0.06 µg/mL), obtained by the DPPH radical method, is higher than that of the n-butanol extract (IC_{50} = 9.14 ± 0.11 µg/mL) and the commercial antioxidant BHA (IC_{50} = 5.73 ± 0.41 µg/mL). These expected data can be explained by the higher content of flavonoids in the ethyl acetate extract (54.38 ± 0.29 µg QE/mg Ext), which is significantly lower in the n-butanol extract (27.85 ± 0.29 µg QE/mg Ext). The methanolic extract showed lower antioxidant activity (IC_{50} = 21.14 ± 0.93 µg/mL) while presenting good flavonoid content (92.85 ± 0.15 µg QE/mg Ext).

3.3.2. ABTS Assay

In relation to the ABTS radical, the both extracts ethyl acetate and n-butanol demonstrated the highest antioxidant activity (IC_{50} = 4.06 ± 0.04 µg/mL and IC_{50} = 6.96 ± 0.28 µg/mL, respectively), which is close to the antioxidant activity of the standards BHA (IC_{50} = 1.03 ± 0.00 µg/mL) and BHT

296 (IC_{50} = 1.59 ± 0.03 µg/mL). This is possibly due to the capacity of these extracts to stabilize
297 themselves by donating electrons or hydrogen to the ABTS radical. The methanolic extract showed
298 lower antioxidant activity (IC_{50} = 30.21 ± 2.70 µg/mL).

299 *3.3.3. Phenanthroline Assay*

300 The results indicated significant antioxidant activity of ethyl acetate extract and the methanolic
301 extract with $A_{0.5}$ = 3.93 ± 0.78 µg/mL and $A_{0.5}$ = 4.60 ± 0.53 µg/mL, respectively, close to the
302 antioxidant activity of BHT ($A_{0.5}$ = 2.24 ± 0.17 µg/mL) and BHA ($A_{0.5}$ = 0.93 ± 0.07 µg/mL).
303 While, the n-butanol extract showed lower antioxidant activity ($A_{0.5}$ = 11.04 ± 0.20 µg/mL).

304 *3.3.4. Reducing Power Assay*

305 Using the reducing power assay method, the extracts' antioxidant capacity was statistically
306 comparable to that of α-tocopherol and ascorbic acid. However, the reducing capacity of all extracts
307 was significantly different. In the FRAP assay, the ethyl acetate extract yielded the best values. The
308 data indicated that the antioxidant potential of all extracts was found to be significantly higher than
309 that of the reference α-tocopherol ($A_{0.5}$ = 34.93 ± 2.38 µg/mL), with the values; methanol ($A_{0.5}$ =
310 29.53±0.13 µg/mL), ethyl acetate ($A_{0.5}$ = 7.96 ± 0.92 µg/mL), and n-butanol ($A_{0.5}$ = 10.20±0.43
311 µg/mL). Moreover, the antioxidant activity ($A_{0.5}$ = 7.96 ± 0.92 µg/mL) of the ethyl acetate extract
312 being close to the inhibition capacity of the standard ascorbic acid ($A_{0.5}$ = 6.77 ± 1.15 µg/mL).

313

314 **Table 3.** Antioxidant activity of various extracts of *Achillea ligustica* (m ± SD; n = 3). Means
315 followed by the same letter in the same column do not differ statistically by Tukey's test.

Extracts and standards	**DPPH IC_{50} (µg/mL)**	**ABTS IC_{50} (µg/mL)**	**Phenanthroline $A_{0.5}$ (µg/mL)**	**Reducing power $A_{0.5}$ (µg/mL)**
MeOH extract	21.14 ±0.93^{d}	30.21 ± 2.70^{c}	4.60±0.53^{c}	29.53±0.13^{c}
EtOAc extract	4.18±0.06^{a}	4.06±0.04^{a,b}	3.93±0.78^{c}	7.96±0.92^{a}
n-BuOH extract	9.14±0.11^{c}	6.96±0.28^{b}	11.04±0.20^{d}	10.20±0.43^{b}
BHA	5.73±0.41^{b}	1.03±0.00^{a}	0.93±0,07^{a}	NT
BHT	NT	1.59±0.03^{a}	2.24±0,17^{b}	NT
α-Tocopherol	NT	NT	NT	34.93±2.38^{d}

Ascorbic acid	NT	NT	NT	6.77±1.15[a]

NT: not tested, BHA: Butylatedhydroxyanisole, BHT: Butylatedhydroxyltoluene

The wide variety of phenolic acids (such as caffeic acid, ferulic acid and chlorogenic acid) and flavonoids (including quercetin derivatives and rutin glycosides) identified in *Achillea ligustica* confirms it's potential as a therapeutic agents and a promising source of antioxidants. Indeed, the same phenolic compounds can exhibit different antioxidant effects depending on their concentration in the extracts studied (Shi *et al.* 2022), this can explain the differences in antioxidant activity in the different assays and extracts. Additionally, phenolic compounds display varying degrees of antioxidant activity depending on the assay used (e.g., DPPH, ABTS, FRAP, phenanthroline, and others) (Tabart *et al.* 2009). Numerous in vitro studies demonstrate that phenolic compounds obtained from *Achillea* species act as non-enzymatic antioxidants, serving as excellent free radical scavengers. Conforti *et al.* (2005) observed that methanolic extracts obtained from *Achillea ligustica* using the DPPH assay had strong antioxidant activity (IC_{50} of 50 µg/mL), highlighting the plant's significant antioxidant properties. Similarly, several studies have used the DPPH reducing assay to investigate the antioxidant activity of other *Achillea* species. For instance, Varasteh-Kojourian *et al.* (2017) found that *Achillea biebersteinii* exhibited stronger antioxidant activity (IC_{50} = 0.276 ± 0.003 mg/mL) compared to *Achillea eriophora* (IC_{50} = 0.703 ± 0.023 mg/mL), though both were less effective than ascorbic acid.

To evaluate the capacity of *Achillea* species to scavenge ABTS radicals, Gevrenova *et al.* (2021) studied *Achillea aleppica* and *Achillea santolinoides* and found that methanolic extracts exhibited significantly stronger ABTS radical scavenging activity compared to ethyl acetate extracts. Specifically, the methanol extracts of *A. aleppica* and *A. santolinoides* showed values of 88.93 ± 0.79 mg TE/g and 42.06 ± 0.40 mg TE/g respectively, demonstrating methanol's superior ability to extract antioxidant compounds. In contrast, Ertaş *et al.* (2014) reported that compared to α-tocopherol, the standard antioxidant, which showed almost 90% inhibition, the moderate ABTS

radical scavenging activity of *Achillea cappadocica* metanolic extract reaching 70% inhibition at 100 µg/mL.

Several studies have assessed the reducing power of *Achillea* species using the FRAP assay. Özgen *et al.* (2004) found that Achillea wilhelmsii's methanolic extract showed a concentration-dependent increase in reducing power, with values ranging from 2.1 ± 0.3µg/mL at 50 µg/mL to 13.1 ± 1.2 µg/mL at 500 µg/mL. However, the reducing power was relatively moderate compared to strong standard antioxidants. Georgieva *et al.* (2015) investigated *Achillea millefolium* and found that the decoction method provided the highest FRAP value (132.71 ± 1.29 µM TE/g dw), outperforming microwave-assisted extraction (76.41 ± 0.53 µM TE/g dw), highlighting the influence of extraction methods on the plant's antioxidant capacity.

In another study using the phenanthroline assay, Boubertakh *et al.* (2024) examined the hydro-ethanolic leaf extract (HEAL) of *Achillea ligustica*, which demonstrated moderate antioxidant activity ($A_{0.5}$ = 5.25 ± 0.65 µg/mL). However, HEAL was less effective compared to the standard antioxidant BHT. Furthermore, Amira *et al.* (2023) studied the antioxidant potential of the hydro-methanolic extract of *Achillea odorata* and found it exhibited strong antioxidant properties, with a phenanthroline $A_{0.5}$ value of 6.22 ± 0.04µg/mL comparable to BHA value. These studies confirm that *Achillea* species, particularly *Achillea ligustica*, are promising sources of natural antioxidants with potential medicinal applications for combating oxidative stress.

3.4. Brine Shrimp Lethality Test

Due to its ease of application, affordability, and reliability, the brine shrimp Artemia salina is widely used as test organisms in short-term toxicity investigations. This test provides preliminary information about the biological activities (cytotoxic, antitumor, and pesticidal properties) of natural products, guiding researchers toward more in-depth studies (Sleet and Brendel. 1983). The use of microplate technology allows for efficient testing of multiple samples and their respective dilutions (Solis *et al.* 1992). Meyer *et al.* (1982) established a relationship between toxicity and the average lethal dose (LC_{50}) of plant extracts on *A. salina* larvae. Values above 1000 µg/mL are

considered non-toxic. This study marks the first time extracts from *Achillea ligustica* were subjected to acute toxicity testing using *A. salina* larvae, along with determining the LC_{50} of each. At 400 µg/mL, the methanol extract demonstrated the highest larval mortality in *A. salina*, reaching 75%, while the ethyl acetate extract resulted in a 45% mortality rate. However, the n-butanol extract exhibited the lowest percentage of mortality, recorded at 35% at the same concentration (**Figure 1**). In the lethal concentration bioassay, the number of dead larvae was proportional to the increase in concentration.

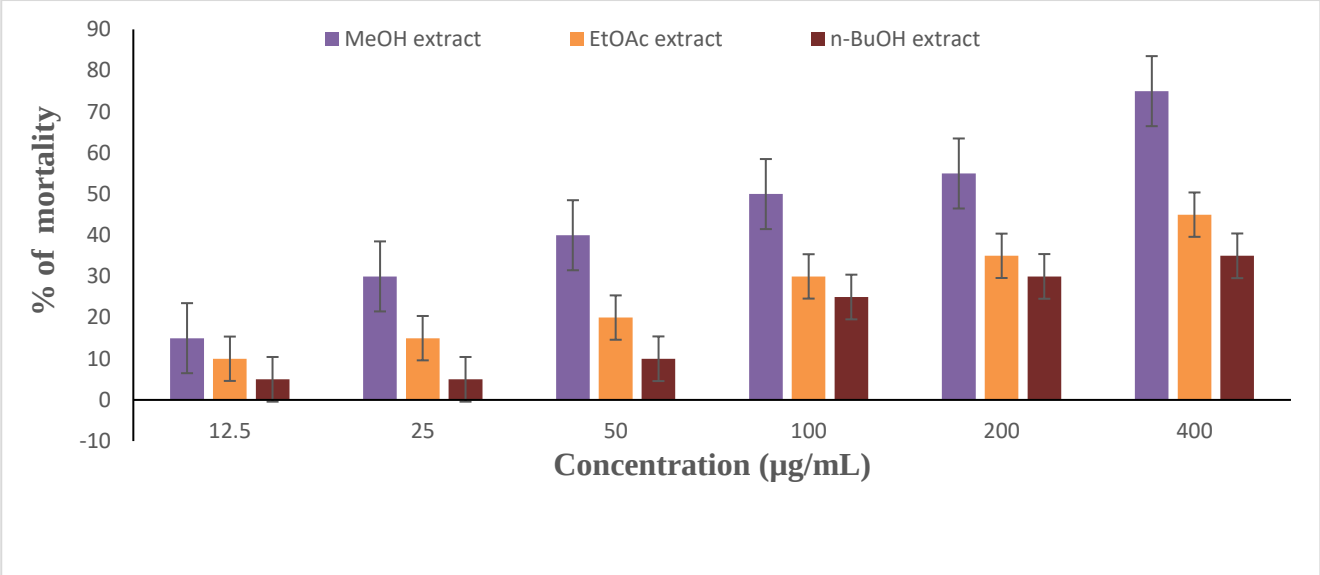

Figure 1. Percentage of mortality at different concentrations of the crude extracts of *Achillea ligustica* in Brine shrimp lethality assay.

The results of the crude extract and commercial cytotoxic drug potassium dichromate ($K_2Cr_2O_7$) toxicity test for brine shrimp are shown in **Table 4**. As signaled by Clarkson *et al.* (2004) and Meyer *et al.* (1982), and by comparing the results with the highly cytotoxic positive control potassium dichromate (LC_{50}= 26.49 ± 1.34 µg/mL), the methanolic extract showed high toxicity against *A. salina* with a value of LC_{50} = 105.03 ± 3.30 µg/mL. However, the LC_{50} values of both extracts ethyl acetate (582.78 ± 0.67 µg/mL) and the n-butanol (664.25 ± 3.54 µg/mL) showed low

toxicity (1000 µg/mL >LC_{50} ≥ 500 µg/mL). In this context, compounds in *Achillea* species, including *A. ligustica*, responsible for toxicity are possibly alkaloids (Raeisi *et al.* 2024) and saponins (Barda *et al.* 2021). These compounds are well-known for their toxic effects (Masi *et al.* 2022; Zong *et al.* 2015). Further studies on this species are needed to identify the compounds responsible for its potential toxicity and to validate its traditional use, ensuring public safety. It is also worth noting that many studies have shown extracts with cytotoxic effects to exhibit diverse biological activities, including antifungal (Niño *et al.* 2006), antibacterial (Brasileiro *et al.* 2006) and anti-cancer characteristics (Meyer *et al.* 1982).

Table 4. Effect of methanol, ethyl acetate and n-butanol extracts of *Achillea ligustica*and the positive control bichromate de potassium ($K_2Cr_2O_7$), on Brine shrimp.

Extract	**Concentration (µg /mL)**						
	12.5	**25**	**50**	**100**	**200**	**400**	LC_{50} **µg/mL**
	% Mortality						
MeOH extract	15 ± 0.71	30 ± 1.41	40 ± 0.00	50 ± 0.00	55 ± 0.71	75 ± 0.71	105.03 ±3.30[b]
EtOAc extract	10 ± 0.00	15 ± 0.71	20 ± 0.00	30 ± 0.00	35 ± 0.71	45 ± 0.71	582.78 ± 0.67[c]
n-BuOH extract	5 ± 0.71	5 ± 0.71	10 ± 0.00	25 ± 0.71	30 ± 0.00	35 ± 0.71	664.25 ±3.54[d]
$K_2Cr_2O_7$	**Concentration (µg /mL)**						
	10		**20**	**40**	**80**		LC_{50} **µg/mL**
% Mortality	0.00 ± 0.00		50 ± 10.00	80 ± 0.00	100 ± 0.00		26.490 ±1.34[a]

4. Conclusion

The current research examined the chemical composition, the antioxidant and cytotoxic activities of *Achillea ligustica* sampled from Mila region (Algeria). The HRMS analysis revealed the richness of this plant on bioactive substances, including phenolic acids, flavonoids, and anthocyanins.

Depending on the extraction solvent, the extracts' levels of antioxidant activity varied, in certain assays, such as the reducing power assay, the highest antioxidant activity was demonstrated in ethyl acetate extract and often it outperforming commercial antioxidants. This demonstrates *A. ligustica's* potential as a beneficial natural antioxidant source with potential uses in both industry and health. In terms of cytotoxicity, the brine shrimp lethality tests revealed that the n-butanol extract exhibited the lowest toxicological potential, followed by the ethyl acetate extract with moderate toxicity, and the methanolic extract, which showed the highest toxicity. The cytotoxic activity observed can be attributable to specific bioactive substances such as alkaloids and saponins, which warrant further investigation. These findings suggest that *Achillea ligustica* has a great potential as a source of bioactive compounds with potential medical applications. However, to gain more knowledge of the mechanisms of action, safety, and possible applications of specific compounds, future research should concentrate on their isolation and structural clarification. For this herb to be used safely in both traditional and modern medicine, more toxicological analyses are also necessary.

Acknowledgements

This study was supported by the Algerian Fund for Scientific Research (Laboratory for the Optimization of Agricultural Production in Subhumid Areas) and the Ministry of Higher Education and Scientific Research of Algeria (PRFU Projet to Pr. N. ZAIDI). The authors thank the Prof. SAKHRAOUI N. (University of Skikda) for the identification of the botanical species.

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