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Acinos alpinus and *Ziziphora hispanica*: Phenolic Profile, Antioxidant and Antibacterial Properties of Hydromethanolic Extracts from Aerial Parts

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Abstract: *Acinos alpinus* and *Ziziphora hispanica* belonging to the Lamiaceae family are well-known for their biological properties. The phenolic composition was characterized using HPLC coupled to photodiode array and electrospray ionization mass spectrometry. The antioxidant capacity was assessed by 2,2′-diphenyl-1-picrylhydrazyl radical and ferric reducing antioxidant power assays. The antibacterial activity was evaluated by disc-diffusion and broth micro-dilution methods. A total of 53 phenolic compounds were identified in both extracts, with the predominance of caffeoylquinic acid isomers with a content of 2.09 mg/g in *A. alpinus* extract, while coumarylquinic acid isomers with a content of 17.33 mg/g was determined in *Z. hispanica* extract. The *A. alpinus* extract displayed the highest radical scavenging activity (IC_{50} DPPH = 3.226±0.035 mg/mL) and reducing power (EC_{50} FRAP = 3.792±0.001 mg/mL), followed by *Z. hispanica* extract with IC_{50} DPPH value of 7.265±0.209 mg/mL, and EC_{50} FRAP value of 24.689± 0.106 mg/mL. Furthermore, both *A. alpinus* and Z. *hispanica* extracts exhibited a bactericidal effect against six pathogenic bacteria with a MIC value from 4.16 to 33.33 µg/mL. On the basis of the results achieved, the aerial parts of Moroccan *Acinos alpinus* and *Ziziphora hispanica* might be used as natural agents in the food and pharmaceutical field.

Keywords: *Acinos alpinus; Ziziphora hispanica;* phenolic compounds; HPLC-PDA-ESI/MS; biological activity.

1. Introduction

For centuries, indigenous plants have been used in herbal medicine to treat various diseases (Cowan, 1999). Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to available antibiotics have led scientists to study the antimicrobial activity of medicinal plants (Srinivasan *et al*., 2001). In Morocco, phytotherapy is one of the most important components of traditional medicine, particularly in rural areas, for the treatment of a variety of physical, physiological, and mental illnesses. It is vital to explore the phenolic composition of medicinal plants as well as their antioxidant and antibacterial capabilities in order to promote proper use and establish their potential as new sources of medicines. On the one hand, oxidative stress is responsible for many diseases, due to oxidation products recognized as reactive oxygen species (ROS). These latters cause damage to cellular structures, nucleic acids, lipids and proteins, leading to chronic diseases (Atta-ur-Rahman, 2021). Several mechanisms are involved in the removal of ROS, including enzymatic (catalase, peroxidases, superoxide dismutase, glutathione reductase, and minerals, which act as enzymatic cofactors, such as copper, iron, and zinc) and non-enzymatic (vitamins B, C, and E, phenolic compounds, flavonoids, carotenoids, and α tocopherols) (Atta-ur-Rahman, 2021). Therefore, there is a growing demand for the use of plant-based antioxidants due to their safer nature, to replace synthetic antioxidants with potential side effects on human health such as: butylated hydroxytoluene (BHT), butylated hydroxy anisole (BHA), propyl gallate (PG), ethylenediaminetetraacetic acid (EDTA) and nordihydroguaiaretic acid (NDGA) (Haddou *et al.*, 2023; Atta-ur-Rahman, 2021; El-Ouariachi *et al.*, 2014; El-Ouariachi *et al.*, 2011;). On the other hand, the emergence of multidrug-resistant bacteria poses a global health problem due to the ineffectiveness of some antibiotics (Bhattacharjee, 2019). In the United States, more than 2.8 million people have been infected with antimicrobial-resistant bacterial and fungal infections, with more than 35,000 deaths per year (GLASS, 2021). There are many natural plant products that have emerged as potential potent natural antimicrobials (Katanić *et al*., 2019; Valle *et al.*, 2015; Valle *et al*., 2015) and thus they can be considered as additional treatments with herbal antibiotics. In this regard, phenols from many medicinal and food herbs have been studied as a promising source of effective antioxidant and antimicrobial agents. A growing scientific and consumer interest in phenols is the recognition of their high abundance in our diet, their antioxidant properties and their important role in the prevention of various diseases associated with oxidative stress, such as cancer, cardiovascular and neurodegenerative diseases (Teanpaisan *et al*., 2016; Barbouchi *et al.*, 2019; Elmsellem *et al.*, 2019; Mohammed *et al*., 2024; Zejli *et al.*, 2024).

The Lamiaceae family is found in a variety of traditional remedies, cosmetics, and meals (Atta-ur-Rahman, 2021). The species included in the genera *Ziziphora* and *Satureja* (*Acinos* = *Clinopodium*) are mostly used in the world, especially in Morocco, for their potential health benefits. Regarding the genus *Ziziphora*, about 17 species are distributed in open and often xeric habitats of southern and eastern Europe, northwestern Africa and Asia to the Himalayas and Altai Mountains (First, 2017). Species of this genus have been used in the pharmaceutical, cosmetic and food industries. Indeed, many studies have shown various activities for this genus as antioxidant (Meral et al., 2002), antibacterial (Ozturk *et al.*, 2007), antifungal (Tabatabaiee, 2006), insecticidal and ovicidal (Lolestani & Shayesteh, 2009) and vasodilator activities (Senejoux *et al.*, 2012). *Ziziphora* also shows inhibitory effect on gastric acid production under basal and vagal stimulation conditions (Niazmand *et al.*, 2010), on performance, blood biochemical and immune parameters of laying hens (Oroji *et al.*, 2021; Nobakht *et al.*, 2012; Shahbazi *et al.*, 2016;), and it acts as a yogurt starter (Carlsen *et al.*,

2010). In Morocco, this genus is represented by a single species: *Z. hispanica* (Fennane, 2018) and in common people it is called "Tifliout tbourayt", and it has been employed in traditional medicine for various purposes such as stomach and intestinal ailments, heart disorders, migraine, cough, jaundice as reported by Meratate *et al.* (2015).

As far as the genus *Acinos*, belonging to the family Lamiaceae (Labiatae) subfamily Neptoidae and tribe Menthae, contains about 200 species of aromatic herbs and shrubs that are cultivated in the Middle East, the Mediterranean region of Europe, West Asia, North Africa, the Canary Islands and South America (Jennan *et al.*, 2018). More than 30 species of this genus are distributed in the eastern parts of the Mediterranean area (Momtaz & Abollahi, 2010) and about 20 species and subspecies mainly present in Morocco (Fennane, 2018). Many species of this genus are well known for their aromatic and medicinal characters. The aerial parts of these species have particular tastes and can be added to stuffing (Jennan *et al.*, 2018; Golubović, 2020). The goal of this study was to investigate the phenolic profile, antioxidant, and antibacterial activities of hydromethanolic extract from aerial parts of *A. alpinus* and *Z. hispanica* gathered in the Middle Atlas of Morocco (Ifrane and Boulemane respectively). Whereas the phenolic content has been investigated in a few studies (Meratate *et al.* 2015; Jennan *et al.*, 2018; Golubović *et al.*, 2014), in Morocco, no data are available for the characterization of phenolic compounds from *A. alpinus* and *Z. hispanica* extracts.

2. Methodology

2.1 Plant material

During June 2020, the aerial parts of *A. alpinus* and *Z. hispanica* were harvested from Ifrane and Boulmane area of the Middle Atals of Morocco, respectively. The plants were identified by the Scientific Institute of Rabat (Morocco). The voucher specimens are deposited at the Herbarium of the Department of Botany at the Scientific Institute. The plants were protected from light and dried at room temperature for 15 days. Then the plants were crushed and stored at +4°C until analysis.

2.2 Sample preparation

1 g of powder was dissolved in 14 mL of MeOH/water (80/20 *v/v*). The mixture was afterwards shaken in an ultrasonic bath at room temperature for 3 min, centrifuged at 3000 RCF for 20 min, and the recovered pellet was evaporated using EZ-2. Afterwards, a volume of MeOH/ water (LC/MS) was added in the dried extract and filtered through a 0.2 µm Acrodisc nylon membrane (Merck Life Science, Merck KGaA, Darmstadt, Germany) prior to HPLC-PDA-ESI/MS (Bouymajane *et al.*, 2022).

2.3 HPLC-PDA/ESI-MS analyses

Analysis of phenolic compounds was performed using a high-performance liquid chromatography coupled with photodiode array detector and electrospray ionization-mass spectrometry (HPLC-PDA/ESI-MS) (Shimadzu, Kyoto, Japan). Chromatographic separation was carried out on an Ascentis Express C18 column (150×4.6 mm, 2.7 µm; Merck Life Science, Merck KGaA, Darmstadt, Germany) using as mobile phase 0.1% (v/v) acid formic in water (mobile phase A) and 0.1% (v/v) acid formic in acetonitrile (mobile phase B) (Lechhab *et al.*, 2021; Arena *et al.*, 2022). PDA range: from 190 to 400 nm. The gradient elution applied was: 0 min – 0% B, 15 min – 15% B, 30 min – 20% B, 60 min – 50% B, 70 min – 100% B at a flow rate of 0.8 mL/min.

2.4 Antioxidant activity

Two assays based on multiple processes, namely 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP), were used to assess the *in vitro* antioxidant activity of the hydromethanolic extracts from aerial parts of *A. alpinus* and *Z. hispanica*. DPPH test is commonly used to assess natural antioxidants' ability to scavenge free radicals, it is a persistent radical with a rich purple color and a prominent absorption band in the 515–520 nm region. The DPPH radical can receive either a hydrogen atom or an electron from the antioxidant scavenger molecule to be transformed to a more stable reduced form, which is yellow (hydrogen atom transfer HAT mechanism).

The reducing power assay assesses antioxidant substances' ability to donate an electron to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions (single-electron transfer SET based method); this reduction can be evaluated spectrophotometrically at 700 nm.

2.4.1 DPPH assay

Using the DPPH radical, the free radical scavenging activity of different concentrations of extracts (0.0625–2 mg/mL) was evaluated. Briefly, 0.5 mL of each sample was combined with 3 mL of methanol DPPH solution (0.1 mM) and stored at room temperature for 20 minutes in the dark. The acid ascorbic was used as a control. The absorbance was then measured at 517 nm using UV spectrophotometer (Perkin Elmer).

2.4.2 FRAP assay

The ferric reducing power of extracts was assessed according to a previously described (Bouymajane *et al.*, 2022; González-Palma *et al.*, 2016). Briefly, 2,5 mL of tampon phosphate (0,2 M, pH 6,6), 2,5 mL of potassium ferricyanure (1%) [$K_3Fe(CN)_6$], and 1 mL of each sample (0.0625–2 mg/mL) were mixed. Then, 2.5 mL of 10% trichloroacetic acid was added after the mixture had been incubated at 50°C for 20 minutes, and the mixture was then centrifuged at 3000 rpm for 10 minutes. Then 2.5 mL of supernatant was blended with 2.5 mL of distillation water and 0.5 mL of ferric chloride at a 0.1% concentration ($FeCl_3$). The samples were incubated at room temperature for 10 min at the dark. The absorbance was spectrophotometry measured at 700 nm. The ascorbic acid was served as a control. The results were expressed as the mean absorbance values with standard deviation. The EC_{50} was also calculated.

2.5 Antibacterial activity

This activity was carried out based on a hydromethanolic extract (MeOH/water 80/20, *v/v*), subsequently evaporated by rotavapor to eliminate any trace of methanol that could influence the results.

2.5.1 Pathogenic bacterial strains

Antimicrobial activity was assessed against Gram-negative bacteria: *Escherichia coli*, *Pseudomonas aeroginosa* and *Salmonella typhimurium*, and Gram-positive bacteria: *Staphylococcus aureus*, *Enterococcus faecalis* and *Listeria monocytogenes* (Diass *et al.* 2023). The antimicrobial assays were carried out by the disc-diffusion and microdilution method in order to determine the antibacterial activity of extracts against the pathogenic bacteria following the method described by Bouymajane *et al.* (2022).

2.5.2 Disc-diffusion method (Aromatogram method)

The disc diffusion method was done as a preliminary screening test to detect for the presence of antimicrobial activity with the extracts using sterile filter disc (6 mm in diameter). Bacteria were cultured overnight at 37 ºC in Muller Hinton agar and then adjusted with sterile water to a concentration of 1×10^{8} cfu.mL^{-1} using 0.5 McFarland as reference. 1 mL of bacterial suspension was seeded on Petri dishes containing Muller Hinton agar. After, the impregnated sterile filter disc with 10 µL of extracts of *A. alpinus* and *Z. hispanica* was placed on Petri dishes. The antibiotics used as a reference were chosen according to their uses among the most used to eradicate the bacterium, according to the European committee on antibiograms (EUCAST). After incubation of Petri dishes at 37 ºC for 24 h, the diameter of the inhibition zone was measured in millimeters and expressed as means ± (SD).

2.5.3 Microdilution method

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using flat-bottom 96-well microplates. Briefly, 50 µL of sterile distillated water sterile were added to all well microplate. Then, 50 µL of aqueous extracts (100 µg/mL) were added to first well plate and mixed, in order to determine a serial dilution. Furthermore, 50 µL of Muller Hinton broth and 50 µL of bacterium suspensions were added to each well. The well containing bacterium suspension with Mueller Hinton broth and the well containing sterile distilled water and aqueous extract were served as a control and a blank respectively. After microplate incubation at 37° C for 24 h, 40 µL of TTC (2, 3, 5-triphenyl tetrazolium chloride) were added to each well and reinsulated at 37°C for 30 min.

MIC was determined at the lowest concentration of aqueous extracts at which the bacterial growth was not observed. Whereas MBC was determined at the lowest concentration of aqueous extract that did not produce any bacterial colony, by plating 2 µL of samples from the wells in which no growth was observed on the Muller Hinton agar medium and incubated at 37° C for 24 h. The ratio MBC/MIC is used to determine the bacteriostatic and bactericidal effect of aqueous extracts. If the MBC/MIC ratio is below 4, the effect is bactericidal, and if MBC/MIC is greater than 4, the effect is bacteriostatic.

2.6 Statistical analysis

The main effects of temperature, salt stress and pH on germination rates were assessed through a one-way ANOVA. The comparisons of means were performed at a 5 % level of significance using Duncan's multiple-range mean separation test. All experimental data were analyzed using SPSS statistical software (Version 21, IBM SPSS Statistics for Windows).

3. Results and Discussion

3.1 Characterization of phenolic compounds in the Acinos alpinus and Ziziphora hispanica extracts using HPLC-PDA/ESI-MS

Using HPLC-PDA/ESI-MS, the phenolic profiles of extracts derived from aerial parts of *A. alpinus* and *Z. hispanica* were analyzed (**Figure 1**). Compounds detected are listed in **Table 1**, along with retention times, maximum, mass spectrometry, and literature data. A total of 53 phenolic compounds were detected in the examined extracts; in particular, the *A. alpinus* extract has been shown to be qualitatively richer than the *Z. hispanica* one, while the latter was quantitatively richer. Among the

identified molecules, the predominance of hydroxycinnamic acid derivatives in both species (**Figure 2**), followed by flavonoids, can be appreciated. The flavonoids (apigenin and luteolin derivatives, quercetin derivatives, chrysoeriol, eriodictyol, naringenin derivatives, and dihydroxy-tetramethoxyflavone) and hydroxycinnamic acids (caffeic acid derivative, salvianolic acid isomers and rosmarinic acid) were attributed to the *A. alpinus* extract. In particular, a predominance of caffeoylquinic acid isomers among the hydroxycinnamic acids was noted, while naringenin and luteolin derivatives were the most representatives among flavonoids. With regards to *Z. hispanica* extract, the identified phenolic compounds belong to flavonoids (apigenin and luteolin derivatives, quercetin derivatives, chrysoeriol, and jaceosidin) and hydroxycinnamic acids (coumaric and caffeic acid derivative and rosmarinic acid). Among these, the coumaroylquinic acid isomers were the most abundant, while its flavonoidic content was quantitatively poorer in regard to the *A. alpinus* one. It is worth noting that the salvianolic acid, apigenin, and luteolin derivatives have been reported as widespread compounds widely found in the Lamiaceae family.

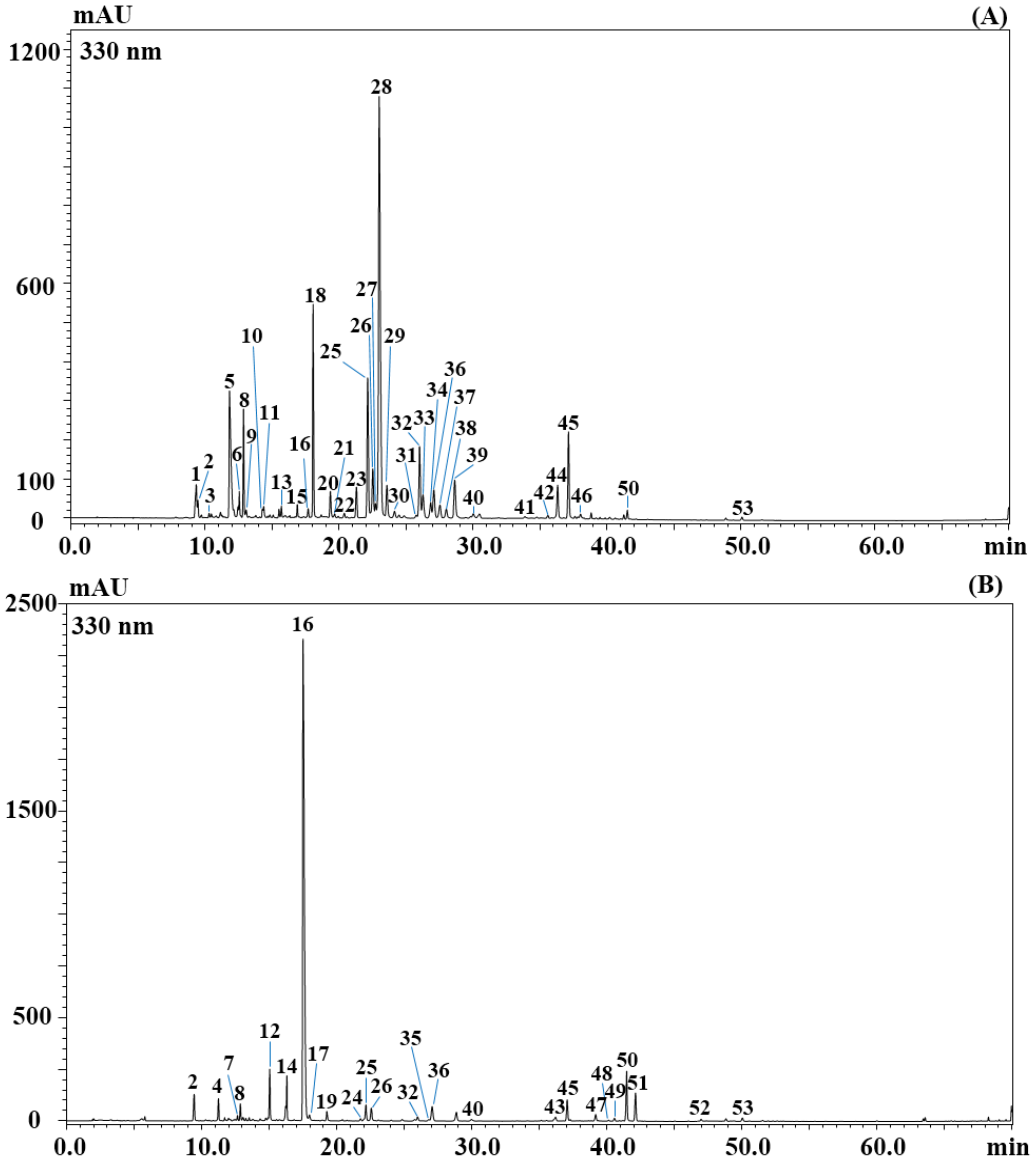

Figure 1. Phenolic profile in the hydromethanolic extracts obtained from the aerial parts of A) *A. alpinus*, B) and *Z. hispanica* (330 nm) using HPLC-PDA/ESI-MS.

Table 1. Characterization of phenols in the hydromethanolic extracts obtained from the aerial parts of *A. alpinus* and *Z. hispanica* L. quantification of phenolic compounds was reported in mg/g of dried extract ± SD (n = 3).

N°	Compounds	t_R (min)	UV max (nm)	[M-H]$^-$	*A. alpinus* (mg/g)	*Z. hispanica* (mg/g)	Ref.
1	Caftaric acid	9.35	327	311, 179	0.48 ± 0.004		(Cirlini et al., 2016)
2	1-*O*-Caffeoylquinic acid	9.47	324	353, 179, 161	0.55 ± 0.009	0.53 ± 0.006	Abu-Reidah et al., 2019)
3	Caffeoyl hexoside	10.3	327	341	0.05 ± 0.001		Abu-Reidah et al., 2019)
4	1-*O*-Coumarylquinic acid	11.24	310	337, 163		0.53 ± 0.003	Abu-Reidah et al., 2019; Marzouk et al., 2018)
5	Unknown	11.83	328		x		-
6	3-*O*-Caffeoylquinic Acid	12.56	325	353, 191	0.39 ± 0.006		Abu-Reidah et al., 2019)
7	Feruloylquinic acid	12.66	324	367		0.41 ± 0.007	(Petreska et al., 2011)
8	4-*O*-Caffeoylquinic Acid	12.87	326	353, 179, 161	1.06 ± 0.016	0.46 ± 0.005	Abu-Reidah et al., 2019)
9	5-*O*-Caffeoylquinic Acid	13.07	325	353, 179, 191	0.09 ± 0.002		Abu-Reidah et al., 2019)
10	Tuberonic acid hexoside	14.29	313	387, 225, 207	x		Abu-Reidah et al., 2019)
11	Quercetin 3-glucuronide-7-glucoside isomer	14.38	256, 327	639, 477, 301	x		Database
12	Unknown	15.04	328			x	-
13	Quercetin 3-glucuronide-7-glucoside isomer	15.69	260, 356	639, 477, 301	x		Database
14	Vicenin (Apigenin 6,8-di-C-glucoside)	16.31	270, 334	593		0.17 ± 0.002	(Marzouk et al., 2018; Jovanović et al., 2017; Martins-Gomes et al., 2018)
15	Unknown	16.88	324		x		-
16	4-*O*-Coumarylquinic acid	17.69	314	337, 173, 163	0.10 ± 0.002	16.67 ± 0.145	Abu-Reidah et al., 2019)
17	5-*O*-Coumarylquinic acid	17.99	311	337, 173		0.13 ± 0.001	Abu-Reidah et al., 2019)

18	Caffeic acid derivative	18.06	326	449, 179, 161	1.85 ± 0.046		Database
19	Feruloylquinic acid	19.29	327	367		x	(Petreska et al., 2011)
20	Eriocitrin (Eriodictyol-7-O-rutinoside)	19.35	283, 327sh	595, 287	x		(Marzouk et al., 2018)
21	Eriodictyol-O-hexoside	19.66	286, 310sh	449, 287	x		(Martins-Gomes et al., 2018)
22	Luteolin dihexoside	20.38	264sh, 344	609, 285	0.02 ± 0.001		(Marzouk et al., 2018)
23	Unknown	21.28	312		x		-
24	Rutin (Quercetin 3-O-rutinoside)	21.78	255, 344	609, 301		0.42 ± 0.001	(Hossain et al., 2010)
25	Luteolin-rutinoside	22.12	255, 348	593, 447, 285	0.63 ± 0.001	0.17 ± 0.001	Abu-Reidah et al., 2019; Yahia et al., 2019)
26	Luteolin-hexoside	22.50	255, 348	447, 285	0.21 ± 0.007	0.15 ± 0.010	Abu-Reidah et al., 2019)
27	Luteolin-glucuronide	22.71	252, 344	461, 285	0.13 ± 0.010		(Afonso et al., 2018)
28	Narirutin (Naringenin-7-O-rutinoside)	22.98	288, 329sh	579, 417, 271	x		(Yahia et al., 2019; Taamalli et al., 2015)
29	Kaempferol-rutinoside	23.56	265, 347	593, 447, 285	0.39 ± 0.002		Abu-Reidah et al., 2019)
30	Naringenin-O-glucoside	24.13	283, 327sh	433, 271	x		(Afonso et al., 2016)
31	Lithospermic acid	25.75	282, 335	537, 493, 359	x		(Marzouk et al., 2018)
32	Apigenin-rutinoside	26.01	266, 337	577, 431, 269	0.05 ± 0.002	x	Abu-Reidah et al., 2019; Marzouk et al., 2018)
33	Hesperidin	26.24	283, 327sh	609, 447, 301	x		(Rita et al., 2016)
34	Apigenin 6-C-glucosyl-8-C-arabinoside	26.82	266, 336	563, 431, 269	x		(El-Ansar et al., 2019)
35	Apigenin O-hexoside	26.83	266, 332	431		0.03 ± 0.001	Abu-Reidah et al., 2019)
36	Rosmarinic acid	27.06	328	359, 179, 161	0.23 ± 0.003	x	(Ziani et al., 2019)
37	Apigenin-rutinoside + apigenin-glucuronide	27.51	266, 336	577, 445, 269	x		Abu-Reidah et al., 2019; Marzouk et al., 2018; Ziani et al., 2019)
38	Dicaffeoylquinic acid	27.98	327	515, 353, 191, 179	0.16 ± 0.001		Abu-Reidah et al., 2019)

39	Salvianolic acid A isomer	28.62	253, 290, 309	493, 295	x		(Cirlini et al., 2016; Martins-Gomes et al., 2018; Rita et al., 2016)
40	Diosmin/Chrysoeriol-7-O-rutinoside	30.46	266, 344	607, 299	x	0.31 ± 0.001	(Yahia et al., 2019; (Rita et al., 2016)
41	Salvianolic acid B	33.85	285, 308sh	717, 519	x		(Rita et al., 2016)
42	Unknown	35.56	285, 333		x		-
43	Luteolin	36.22	263, 344	285		0.07 ± 0.001	(Marzouk et al., 2018)
44	Isosakuranetin-O-rutinoside/Isosakuranetin-O-neohesperidoside	36.29	283, 329sh	593, 285	x		(Taamalli et al., 2015)
45	Acacetin−7-O-rutinoside	37.10	267, 333	591, 283	0.19 ± 0.003	0.24 ± 0.001	(Abu-Reidah et al., 2019; Taamalli et al, 2015)
46	Naringenin	38.00	286, 327sh	271	x		Abu-Reidah et al., 2019)
47	Jaceosidin isomer	39.21	283, 344	329		x	Marzouk et al., 2018)
48	Jaceosidin isomer	40.15	292, 331	329		x	Marzouk et al., 2018)
49	Apigenin	40.58	266, 334	269		0.01 ± 0.001	Marzouk et al., 2018; Taamalli et al., 2015)
50	Jaceidin isomer	41.50	288, 344	359	x	x	(Taamalli et al., 2015)
51	Chrysoeriol	42.15	250, 266sh, 291sh, 344	299		x	(Tadić et al., 2015)
52	Dihydroxy-tetramethoxyflavone isomer	47.02	289, 344	373		x	Marzouk et al., 2018)
53	Acacetin	50.07	267, 340	283	x	x	(Cirlini et al., 2016; Hossain et al., 2010; Pacifico et al, 2015)

x: Not quantified; sh: wavelength shoulder.

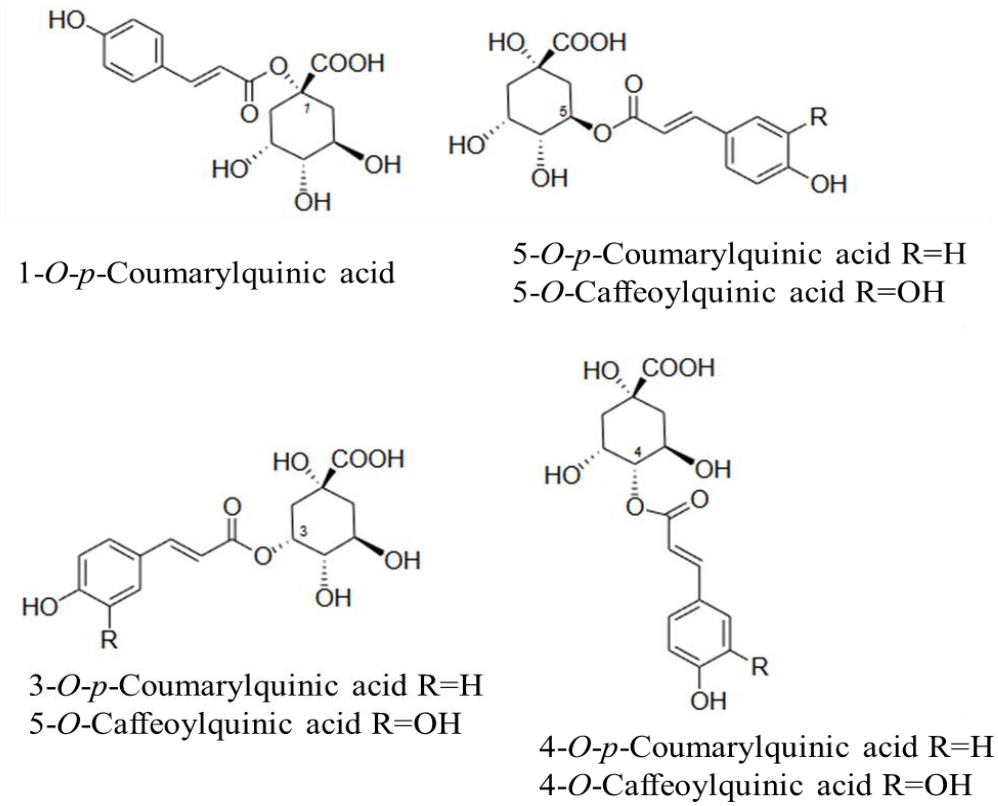

Figure 2. Structures of the most abundant hydroxycinnamic acids detected in the hydromethanolic extracts obtained from the aerial parts of A) *A. alpinus*, B) and *Z. hispanica* (330 nm) using HPLC-PDA/ESI-MS.

3.2 Antioxidant activity

The antioxidant activity of hydromethanolic extracts of *A. alpinus* and *Z. hispanica* was evaluated using DPPH and FRAP (**Table 2** and **Figure 3**). Regarding the DPPH assay, the results showed that both extracts exhibited good radical scavenging activity, which was quite comparable to that of ascorbic acid standard over the entire concentration range. Indeed, *A. alpinus* exhibited the best activity, which was found to be still similar to that of ascorbic acid at lower and high concentrations (0.0625, 0.125 and 2 mg/mL). The calculated IC_{50} values confirmed this trend; such, the IC_{50} of *A. alpinus* (3.23 mg/mL) was close to that of ascorbic acid (2.56 mg/mL).

For reducing power test (FRAP), the best performance came from a *A. alpinus* extract (EC_{50} = 3.79±0.100); however, it was less effective than the ascorbic acid used as a reference. According to the absorbance and the effective concentration values, the activity of the extracts decreases in the following order: Asc.ac. > *A. alpinus* > *Z. hispanica*. According to same findings, for the two-crude methanolic extracts of *A. alpinus* and *Z. hispanica*, the percentage of free radical inhibition increases with increasing concentration.

3.3 Antibacterial activity

The antibacterial activity of *A. alpinus* and *Z. hispanica* extracts was assessed (**Tables 3, 4** and **Figure 4**). The broth micro-dilution method classified both *A. alpinus* and *Z. hispanica* extracts with bactericidal effect against *Escherichia coli, Salmonella typhimurium, Pseudomonas aeroginosa, Staphylococcus aureus, Enterococcus faecalis,* and *Listeria monocytogenes*. The obtained results from disc diffusion assay showed that the diameter of the inhibition zone varied from 12.1 to 26.1

mm for *A. alpinus* extract and from 14.07 to 25.53 mm for *Z. hispanica* extract. *S. aureus* shows a very noticeable sensitivity to extracts of *A. alpinus* with diameters of 26.1±0.1 mm. However, *L. monocytogenes* is the least sensitive to the same extract with 12.1±0.46 mm. For *Z. hispanica* extract, *S. aureus* and *E. coli* represent the most resistant strains with 14.07 and 15.1 mm respectively.

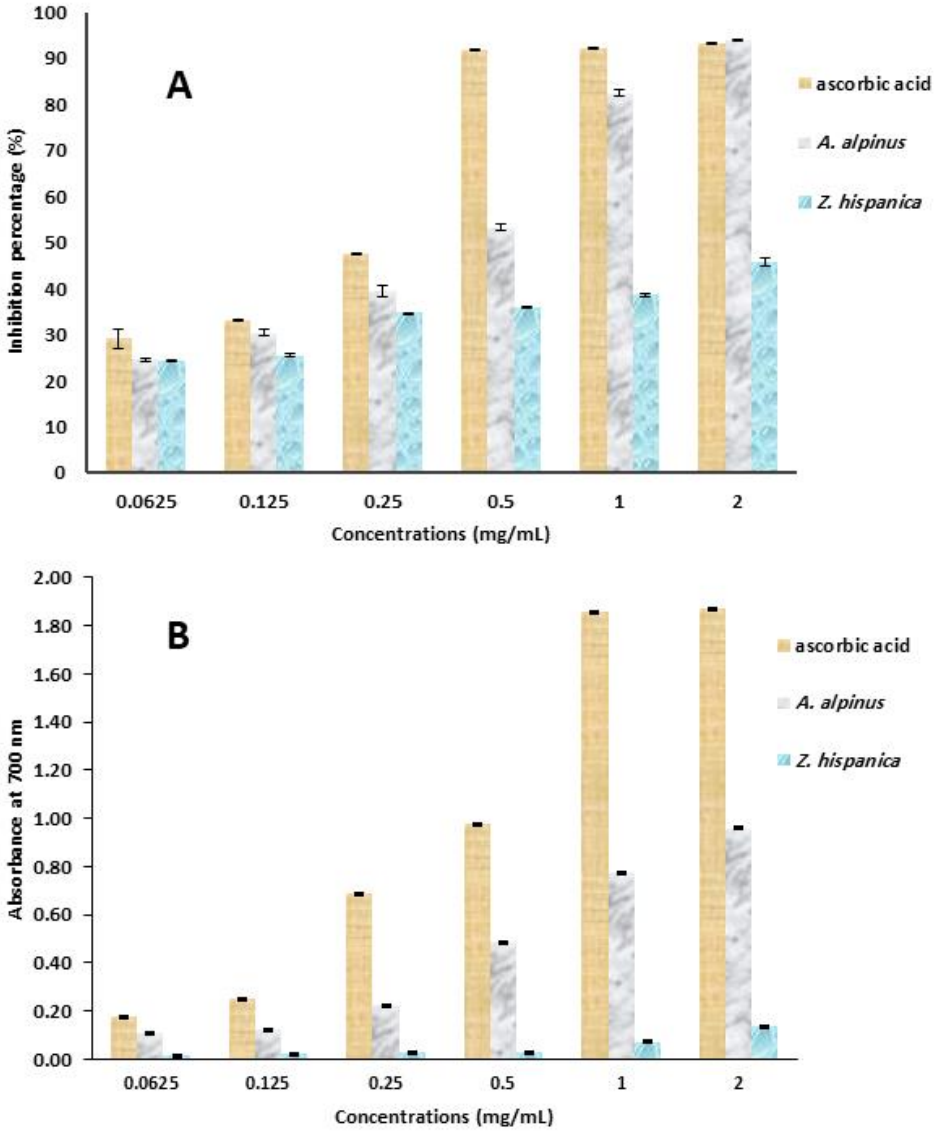

Figure 3. Free radical scavenging activity (A) and reducing power (B) of the hydromethanolic extracts obtained from the aerial parts of *A. alpinus* and *Z. hispanica*. Values are expressed as the mean ±SD (n=3) ($p < 0.05$).

Table 2. Antioxidant activity of hydromethanolic extracts obtained from the aerial parts of *A. alpinus* and *Z. hispanica* determined using DPPH and FRAP

Concentration (mg/mL)	Inhibition rate DPPH (%) (mg/mL)	IC_{50} DPPH (mg/mL)	EC_{50} FRAP (mg/mL)
Ac. Asc. (reference)	93.24±0.01^{b}	2.56±0.036^{c}	2.29±0.003^{c}
A. alpinus	93.92±0.04^{b}	3.23±0.035^{b}	3.79±0.001^{b}
Z. hispanica	45.78±0.81^{a}	7.26±0.209^{a}	24.69±0.106^{a}

$^{a-c}$ Values are mean (n=3) ± standard deviations. Values followed by the same letter in each column are not significantly different in the analysis of variance and Ducan's multiple range test ($p < 0.05$).

Our findings also reveal that *A. alpinus* extract has a much stronger effect on both *S. aureus* and *E. coli* than *Z. hispanica* extract. As compared to *A. alpinus* extract, the *Z. hispanica* extract displayed remarkable antibacterial properties against *S. typhimurium* and *L. monocytogenes*. In addition, we observed that the two plants had the same statistical effect on *P. aeruginosa* and *E. faecalis*.

The results of the tests comparing the inhibition dimensions of the hydromethanolic extract with those of different antibiotic discs tested show that the extract of *A. alpinus* causes significantly greater inhibition on five strains (*E. coli*, *P. aeruginosa*, *S. aureus*, *S. typhimurium* and *E. faecalis*) than Ampicillin, greater than Erythromycin on *E. faecalis* and significantly greater inhibition than Erythromycin and Imipenem on *S. aureus*. For *Z. hispanica*, its extract inhibits four strains (*P. aeruginosa*, *S. aureus*, *S. typhimurium*, *L. monocytogenes* and *E. faecalis*) significantly more than Ampicillin, we also noticed a stronger effect than Erythromycin on *E. faecalis*, even stronger than Tetracycline on *L. monocytogenes*.

For the extracts tested, it appears that all the bacterial strains show sensitivity but with different degrees, which is reflected in the difference in the MICs. The *S. aureus* strain is the most sensitive to the methanol extracts of *A. alpinus*, with a MIC of 4.16 µg/mL. The highest MIC for the *Z. hispanica* methanolic extract was 33.33 µg/mL, which was achieved with regard to the *E. coli* and *P. aeroginosa* strains. Except for this last case, all the extracts show a very significant inhibitory effect with a concentration equal to or less than 16.66 µg/mL. As the content of the plant extract grew in the experimental tubes, the intensity of the cloudiness caused by bacteria growth decreased gradually.

For all pathogenic bacteria and for both extracts, it was found that the MBC varies between 16.66 µg/mL and 33.33 µg/mL. S. aureus is more sensitive to both extracts with MBC of 16.66 µg/mL. Only two strains (*S. aureus* and *L. monocytogenes*) showed a bacteriostatic effect with a MBC/MIC value equal to 4 for the *A. alpinus* hydromethanolic extract. For the *Z. hispanica* extract, all MBC/MIC ratios are lower than 4, where the effect is bactericidal. The achieved findings suggest that *A. alpinus* extract include antioxidants that are efficient against DPPH free radicals compared to *Z. hispanica*. Pîrvu *et al.* (2019) found that hydroethanolic extracts of Romanian *A. alpinus* aerial parts have a potent anti-radical action with an IC_{50} of 2.36 µg/mL, when compared to gallic acid. Golubović *et al.* (2014; 2020) found that the methanolic and ethanolic extracts of the aerial parts of the same species from Serbia showed a remarkable efficiency to scavenging free radicals, with IC_{50} values of roughly 24.1 µg /mL and 37.51 µg /mL, respectively. The greatest antioxidant activity was reported on six species of the same genus (*Satureja*) by the same authors and in the same study (*A. alpinus* with a value IC_{50} of 24.1 µg/mL). A correlation between the antioxidant power and phenolic component concentration has been further reported (Ruberto & Baratta, 2000; Gómez-Maldonado *et al.*, 2020; Tohidi *et al.*, 2017). Analyses on the antioxidant effect of various organic extracts of the species *Acinos* have been conducted e.g. Stojanović *et al.* (2009) reported that *Satureja* species are a promising source of natural antioxidants due to the high content of flavonoids and Linoleic acid in their extracts. Concerning the antioxidant activity of *Z. hispanica*, this is quite rare; a work done by Meratate *et al.* (2015) found that IC_{50} varies depending on the solvents used from 0.33 mg/mL for the dichloromethane extract to 1.06 mg/mL for the *n*-butanol extract. Other studies showed a very powerful antioxidant activity by other species belonging to the same genus such as 30.7 µg/mL recorded by *Z. cliopodioide* (Vijayakumar *et al.*, 2020), 43.17 µg/mL by *Z. tenuior* (Gholivand *et al.*,

2014) and 206.6 µg/mL by *Z. capitata* (Mohammadhosseini *et al.*, 2016). According to Heim, (2002), this strong antioxidant power is probably due to the flavonoid compounds, which are known as antioxidant substances having the ability to trap radical elements and reactive forms of oxygen. In the FRAP assay and on six *Acinos* species, Golubović *et al.*, (2014), found that the highest antioxidant activity was recorded by the extract of *A. alpinus* (7.0 mmol Fe^{2+}/g), which contained the highest amount of total polyphenols and flavonoids (**Table 1**). For reducing power test, the best performance came from a *A. alpinus* extract (3.79±0.100); however, it was less effective than the ascorbic acid used as a reference. According to the absorbance and the effective concentration values, the activity of the extracts decreases in the following order:

Asc.ac. > *A. alpinus* > *Z. hispanica.*

Researchers have shown a strong correlation between plant extracts' antioxidative properties and their phenolic components, which have the potential to interact with free radicals by donating hydrogen or electrons (Ložiene *et al.*, 2007; Menković *et al.*, 2013). It also is well established that antioxidant activity is positively correlated with the structure of polyphenols. Generally, polyphenols (especially flavonoids) with a high number of hydroxyl groups exhibit the highest antioxidant activity (Heim *et al.*, 2002) due to their ability to donate more atoms to stabilize free radicals (Torres de Pinedo *et al.*, 2007), which may partly explain the high antioxidant activity of *A. alpinus* with a large amount of flavonoids (481.15 mg/kg) compared to *Z. hispanica* which has few elements with a hydroxyl group (-OH) in its structure. The body of research indicates that Caffeoylquinic Acids (the main component of *A. alpinus)* have a variety of biological actions, including anti-inflammatory, antioxidant, anti-bacterial, anti-parasitic, neuroprotective, anti-cancer, antiviral, and antidiabetic properties (Liu *et al.*, 2020). Many works have shown that caffeoylquinic acid is one of the main chlorogenic acids of great biological importance due to its antioxidant, antimicrobial, neuroprotective, anticancer, anti-inflammatory and other properties (Kalinowska *et al.*, 2018). On the other hand, the main components of *Z. hispanica* contributing to the antioxidant impact of the plant would be coumaroylquinic acid derivatives, which have strong antioxidant activity (Hammoda *et al.*, 2013).

In terms of antimicrobial activity, the inhibitory diameters varied from bacterium to bacterium, extract to extract. These differences could be attributed to differences in the nature and/or concentration of chemical inhibitors in various plant species, as well as their respective solubilities in extraction solvents. Water and methanol can extract pollutant compounds that may be responsible for this activity (Talibi *et al.*, 2015). This suggests that the active components of the extracts may be among the polar compounds as they are found in aqueous and methanolic extracts. These findings are consistent with those of Golubović et al. (2016), who discovered that the ethanolic extract of *A. alpinus* from Serbia and Montenegro had strong antibacterial activity with the following inhibition diameters: 23, 17, 23, 26 and 24 mm respectively for *E. coli, S. enteritidis, S. aureus, P. aeruginosa, and Enterococcus sp.*. Similarly, the same countries' methanolic extract of *A. alpinus* is effective against the same microbial pathogens with a diameter of 23, 23, 23, 26 and 25 mm (Golubović *et al.*, 2014) which confirms our results. Studies have suggested that polyphenols and flavonoids are characterized by antimicrobial properties (Mulinacci *et al.*, 2001). Several works have highlighted the high sensitivity of Gram (+) bacteria compared to Gram (-) (Burt, 2004), and this can be attributed to the difference in the outer layers of Gram (-) and Gram (+) bacteria. The resistance of Gram (-) is attributed to their hydrophilic outer membrane which can block the penetration of hydrophobic compounds (Kalemba & Kunicka, 2003).

Table 3. Diameter of the zone of inhibition (mm) of *A. alpinus* and *Z. hispanica* extracts

	E. coli	*P. aeroginosa*	*S. typhimurium*	*S. aureus*	*E. faecalis*	*L. monocytogenes*
AA-M	23.03±0.4^{c}	23.03±0.35^{b}	23.03±0.45^{b}	26.1±0.1^{d}	25.10±0.17^{c}	12.1±0.46^{a}
ZH-M	15.1±0.1^{a}	23.1±0.46^{b}	25.03±0.25^{c}	14.07±0.6^{a}	25.5±0.44^{c}	25.53±0.35^{e}
Ampicillin	18.97±0.45^{b}	16.97±0.55^{a}	18.1±0.17^{a}	19.03±0.25^{b}	17.97±0.45^{b}	14.03±0.55^{b}
Erythromycin	29.93±0.4^{e}	27.9±0.56^{d}	28.97±0.65^{e}	25.03±0.06^{c}	13.07±0.12^{a}	24.07±0.06^{d}
Tetracycline	29.97±0.15^{e}	26.97±0.75^{c}	27.97±0.55^{d}	28.98±0.53^{e}	28.03±0.55^{d}	21.13±0.12^{c}
Imipenem	27.93±0.5^{d}	41.83±0.29^{e}	29.97±0.15^{f}	25.07±0.12^{c}	36.93±0.21^{e}	27.97±0.15^{f}

$^{a-g}$ Values are mean (n = 3) ± standard deviations. Values followed by the same letter in each column are not significantly different in the analysis of variance and Ducan's multiple range test ($p < 0.05$).

Table 4. Minimum inhibitory and minimum bactericidal concentrations (MIC, MBC) of *A. alpinus* and *Z. hispanica* extracts (µg/mL). Values are mean (n = 3) ± standard deviations in the analysis of variance and Ducan's multiple range test ($p < 0.05$).

		E. coli	*P. aeroginosa*	*S. typhimurium*	*S. aureus*	*E. faecalis*	*L. monocytogenes*
AA-M	**MIC**	16.66±0.02	16.66±0.06	16.66±0.05	4.16±0.04	16.66±0.04	8.33±0.07
	MBC	33.33±0.03	16.66±0.05	16.66±0.01	16.66±0.08	33.33±0.02	33.33±0.03
	MBC/MIC	2	1	1	4	2	4
ZH-M	**MIC**	33.33±0.03	33.33±0.02	16.66±0.03	16.66±0.05	16.66±0.02	16.66±0.06
	MBC	33.33±0.07	33.33±0.08	33.33±0.02	16.66 ± 0.01	33.33±0.01	16.66±0.04
	MBC/MIC	1	1	2	1	2	1

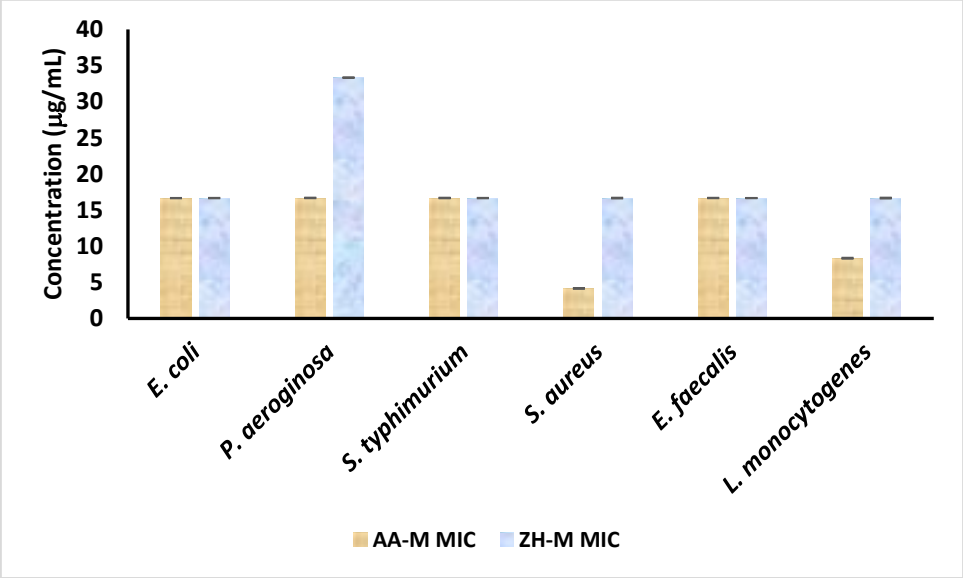

Figure 4. Minimum inhibitory concentration of *A. alpinus* and *Z. hispanica* extracts (µg/mL). Values are mean (n = 3) ± standard deviations in the analysis of variance and Ducan's multiple range test ($p < 0.05$).

On the other hand, the results obtained from the disk diffusion method performed by Meratate *et al.* (2015) by *Z. hispanica* on the same strains indicated that the dichloromethane extract showed moderate antimicrobial activity against all microorganisms tested. (Inhibition zone between 8 and 12 mm for four strains tested) which may be due to the extraction solvent with a low yield. The *Acinos*

extracts investigated in this study revealed higher antibacterial activity in general, which can be linked to its crucial constituent. For all plant species examined, ethanol and methanol extracts are considered not selective (about equally active against Gram-negative and Gram-positive bacteria). The research results of such extracts coincide mainly with those for methanol (Golubović *et al.*, 2014) and ethanol (Golubović *et al.*, 2020) on the same selected plant species.

Selected studies have reported the identification and characterization of caffeoylquinic acids as pump inhibitors with potential to target efflux systems in a wide panel of Gram-positive human pathogenic bacteria (Fiamegos *et al.*, 2011). Caffeoylquinic acid was tested for its minimum inhibitory concentration (MIC) against a panel of bacteria and fungi. The MIC values obtained were between 5 and 10 µg/mL (Bajko *et al.*, 2016). On the other hand, a work demonstrated that coumaroylquinic acid derivatives exhibited antibacterial activity in vitro against five bacterial strains tested with MICs equal to or less than 177.6 µM (Zhang *et al.*, 2013).

Conclusion

In this study, the phenolic composition, the antioxidant and the antimicrobial activities of hydromethanolic extracts, obtained from the aerial part of Moroccan *A. alpinus* and *Z. hispanica*, were studied for the first time. A total of 39 and 25 phenolic compounds were detected, respectively. *A. alpinus* was found to be a more effective antioxidant in both in vitro assays (DPPH and FRAP) due to the flavonoid diversity. In terms of antimicrobial activity, *A. alpinus* extract showed a much stronger effect on both *S. aureus* and *E. coli* than *Z. hispanica* extract. As compared to *A. alpinus* extract, the *Z. hispanica* extract displayed a remarkable antibacterial property against *S. typhimurium* and *L. monocytogenes*. On the basis of the achieved results, both plants and especially *A. alpinus* might have an interesting prospect for a potential application in medical practice.

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Compliance with Ethical Standards: Not applicable.

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