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Phytochemical screening and evaluation of the antioxidant and anti-bacterial activity of Woundwort (*Anthyllis vulneraria* L.)

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Abstract

For millennia, medicinal plants have been used to prevent and cure diseases. Up to now there is a growing interest in their use in several areas as conducted for *Anthyllis* in our investigation. Actually the present research aims to investigate the biochemical characterisation of a medicinal plant collected from Tunisia named Woundwort [*Anthyllis vulneraria* L. (Jones and Turrill 1933)] by determining the mineral content, phenolic compound contents and biological activities of its leaf and flower extracts. On the one hand, the results obtained showed that *A. vulneraria* accumulated minerals at different amounts with significant differences between leaves and flowers extracts. On the other hand, the data revealed that the hydroethanolic flower extract contained the highest content of total polyphenols, flavonoids and condensed tannin, as it exhibited the strongest antioxidant activity. The flower extract also showed better antibacterial effect than leaf extract. These results support the exploitation of active compounds extracted from the leaves and especially the flowers of *A. vulneraria,* which can provide new alternatives to the use of certain drugs, additives, among others, as they can be used as structure–activity models for the development of new products.

Keywords Biological activity · Medicinal plant · Minerals · Phenolic compounds

Abbreviations

$AlCl_3$	Aluminium chloride
ATCC	American Type Culture Collection
EtOH	Ethanol
$FeCl_2$	Iron(II) chloride
H_2O_2	Hydrogen peroxide
MeOH	Methanol
UV-B	Ultraviolet (280–315 nm)

1 Introduction

Anthyllis vulneraria [*A. vulneraria* (Jones and Turrill 1933)], commonly named "Woundwort" is a mediterranean medicinal plant that belongs to the Fabaceae family (Nartowska et al. 2001). The term "*Anthyllis*" comes from the Latin words "*Anthos*" and "*ioulos*", which mean "flower" and "downy", respectively (as are the undersides of leaf), while "*vulneraria"* in Latin is "*vulnus*", which means "injury" referring generally to wounds healing (Halabalaki et al. 2011). As its name suggests, *A. vulneraria* is a popular remedy for burns and skin rashes. In the traditional medicine, *A. vulneraria* flowers were used to heal wounds, low the high blood pressure, treat inflammation, vomiting, acne and purify the body by promoting the elimination of toxins (Nartowska et al. 2001). They were also used to heal mouth and throat pain, to limit hair loss and promote hair growth (Menković et al. 2011). In recent years, *A. vulneraria* has attracted the attention of researchers to quantify its phenolic compounds and prove its potential as a source of bioactive molecules with effective biological properties. Different environmental factors, including biotic (microbial invasion, insect pests and herbivores) and abiotic (cold and drought) factors stimulate the production of a wide variety

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of bioactive substances in plants to protect themselves (Yang et al. 2018) and which also play a crucial role in preventing and curing human diseases (Sales de Oliveira et al. 2020).

In response to these external changes, plants vary their chemical composition, which explains the qualitative and quantitative differences in their metabolites during a given season and, consequently, the variation in their pharmacological property (Yang et al. 2018). Antioxidant components, such as phenolic compounds are generally received by the human organism as food, drugs and supplements (Lushchak 2014). The antioxidant activity is one of the most important biological properties that characterises bioactive substances produced by plants (Stagos 2020). Plants rich in phenolic compounds have an important pharmacological potential because of their antioxidant activity that allows them to adsorb, neutralise and eliminate free radicals (Stagos 2020) and thus, to defend against some cancer, infections, inflammation, microorganisms and cardiovascular disorders (Pandey and Rizvi 2009; Ncube et al. 2012). Plants also have long been known by their antimicrobial property. For this aim, many scientists looking for new antimicrobial agents are paying particular attention to the plant kingdom (Gorlenko et al. 2020).

The main target of the present study was to quantify mineral content in powdered *A. vulneraria* leaves and flowers and to determine the phenolic compound contents, antioxidant activity and antibacterial property of the leaf and flower ethanol extracts. Hence, in addition to previous findings, this investigation gives further information about the nutritive value of *A. vulneraria* as dietary supplements rich in natural antioxidants and could be also a supportive data for using the specie as a new industrial crop in cosmetic, pharmaceutical and food industries.

2 Material and methods

Chemicals and reagents – Catechin, EtOH, $FeCl_2$, ferrozine, gallic acid, vanillin, ascorbic acid and quercetin were purchased from Sigma-Aldrich Chimie (*S.Q.F*, *France*). $AlCl_3$, H_2O_2, phosphate buffer, nitric acid and perchloric acid were acquired from Loba Chemie Pvt. Ltd (*Mumbai*, *India*). Mueller Hinton agar and Penicillin-Streptomycin (Pen-Strep) were bought from Thermo-Fisher Scientific (*Barcelona*, *Spain*).

Plant sampling – *Anthyllis vulneraria* was collected at the flowering stage (beginning of April) from Zaghouan located in the North of the Tunisian ridge (latitude 36°, 24 min, 10 s North; longitude 10°, 08 min, 34 s East). Once brought back to the laboratory, *A. vulneraria* leaves and flowers were allowed to dry in the shade at room temperature. The weight was measured every 2 days until a constant weight (dry weight) was obtained. Then, dry samples were ground into a fine powder and stored in amber glass bottles for further analysis.

Determination of mineral content – Mineral content was determined in powdered *A. vulneraria* leaves and flowers by hot mineralisation with nitric and perchloric acids (Larsson et al. 1998; Rezgui et al. 2017). After the complete evaporation of the mixture and obtaining a white haze dry residue, 20 mL of nitric acid (N7) were added. The determination of iron (Fe), copper (Cu) and zinc (Zn) contents was carried out using an atomic absorption spectrophotometer (*Varian SpectrAA 220FS, Canada*), whereas potassium (K) and calcium (Ca) contents were determined using a photoelectric flame photometer (*Model 410 Flame Photometer Range, Cambridge, United Kingdom*). The results obtained are expressed as microgram per gram of Dry Weight (μg g^{-1} DW).

Phenolic compound contents in *A. vulneraria* leaf and flower extracts –

Extracts preparation. The preparation of *A. vulneraria* leaf and flower extracts was assessed using two different methods, including cold maceration extraction and ultrasound assisted extraction.

To perform the cold maceration extraction, 1 g of each dry sample was mixed with 10 mL of different concentrations of EtOH (absolute, 75% and 50%) and extracted during 24 h at 4 °C under stirring using a multi-position magnetic stirrer (*Ovan, MM90E, Barcelona, Spain*). Then, the extracts were centrifuged (*Orto Alresa Mod. Consul, Ajlvir, Madrid, Spain*) at 1500×*g* for 10 min. The different supernatants were concentrated using a sample concentrator (*Techne FSC496D sample concentrator, Madrid, Spain*) under a jet of moderate nitrogen gas, then lyophilised using a freeze dryer (*Unicryo MC2L, UniEquip Laborgerätebau & Vertr. GmbH, Munich, Germany*) for 2 days.

The ultrasound assisted extraction method also was carried out using different concentrations of EtOH. Shortly, 1 g from each powdered leaf and flower was mixed with 10 mL of EtOH at different concentrations (absolute, 75% and 50%). The different mixtures were stirred using a vortex (*FALC Instruments, A121498, Italy*) for 1 min and extracted in an ultrasonic bath (*COXO Medical Instrument CO., LTD, DB4820, Medical World Company*) for 15 min at 25 °C at a frequency of 40 kHz and a power of 100 W. Then, the extracts were filtered with filter paper (Wattman no. 4) and the supernatants obtained were concentrated then lyophilised for 2 days.

Anthyllis vulneraria leaf and flower freeze-dried extracts were dissolved in absolute EtOH, 75%-aqueous EtOH and 50%-aqueous EtOH and stored in darkness at 4 °C until their use.

Extraction yield. The final dry weight of each lyophilised extract was used to calculate the extraction yield according to the following formula:

$$\text{EY}\,(\%) = \frac{W_1}{W_2} \times 100$$

where W_1 represents the weight of the dry extract after the lyophilisation and W_2 represents the weight of the dry ground plant material.

Total polyphenol content. Total polyphenol content (TPC) in the leaf and flower extracts of *A. vulneraria* was determined following the method described by Singleton et al. (1965) and reported by Segovia Gómez and Almajano Pablos (2016). The absorbance reading was recorded at 765 nm using a spectrophotometer (*MAPADA spectrophotometer, UV-1600. Shanghai Mapada Instruments co., Ltd*). The calibration curve was prepared with gallic acid at different concentrations ranging from 100 to 1700 µM ($R^2 = 0.992$). The results of TPC are expressed as milligram of Gallic Acid Equivalent per gram of Dry Weight (mg GAE g^{-1} DW).

Total flavonoid content. Total flavonoid content (TFC) was determined using the $AlCl_3$ colorimetric method as described by Skowyra et al. (2014). The absorbance was measured at 405 nm, and the calibration curve was prepared with quercetin at increasing concentrations from 50 to 500 µM, ($R^2 = 0.998$). Results are expressed as milligram of Quercetin Equivalent per gram of Dry Weight (mg QE g^{-1} DW).

Condensed tannin content. Condensed tannin content (CTC) was determined by the vanillin method described by Julkunen-Tiitto (1985). The absorbance was measured at 550 nm, and the calibration curve was prepared with catechin at different concentrations ranging from zero to 1000 µg mL^{-1}, ($R^2 = 0.997$). Results are expressed as milligram of Catechin Equivalent per gram of Dry Weight (mg CE g^{-1} DW).

Antioxidant activity of *A. vulneraria* leaf and flower extracts –

Total antioxidant capacity by phosphor-molybdenum method. Total antioxidant capacity (TAC) of *A. vulneraria* leaf and flower extracts was determined as reported by Zengin et al. (2015). The absorbance of the mixtures obtained was measured at 695 nm. The calibration curve was prepared with ascorbic acid at different concentrations ranging from zero to 100 µg mL^{-1}, ($R^2 = 0.998$) and the TAC results are expressed as milligram of Ascorbic Acid Equivalent per gram of Dry Weight (mg AAE g^{-1} DW).

Ferrous ion chelating assay. Ferrous ion chelating (FIC) assay was assessed following the method of Dinis et al. (1994). Shortly, 100 µL of each diluted extract were mixed with 0.2 mL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 0.4 mL of 5 mM ferrozine and the mixtures were adjusted to 4 mL with EtOH. After shaking, the mixtures were incubated in the dark at room temperature for 10 min. The absorbance of the extracts was measured at 562 nm and the percentage of Fe^{2+} chelating effect (FIC effect (%)) was calculated as follows:

$$\text{FIC effect}\,(\%) = \left(1 - \frac{\text{As}}{\text{Ac}}\right) \times 100$$

where Ac is the absorbance of the blank (containing $FeCl_2$—ferrozine complex) and As is the absorbance of the extract.

Hydrogen peroxide scavenging assay. Hydrogen peroxide scavenging (HPS) assay was determined as described by Ruch et al. (1989). A solution of 43 mM H_2O_2 was prepared with phosphate buffer (0.1 M, pH = 7.4), then 100 µL from each extract were mixed with 0.6 mL of H_2O_2 solution. The percentage of H_2O_2 scavenging effect (HPS effect (%)) was calculated using the following formula:

$$\text{HPS effect}\,(\%) = \left(1 - \frac{\text{As}}{\text{Ac}}\right) \times 100$$

where Ac is the absorbance of the blank (containing sodium phosphate buffer without H_2O_2) and As is the absorbance of the extract.

Determination of antibacterial activity of *A. vulneraria* leaf and flower extracts –

Bacterial strains tested. Six different microbial strains, causing infective and toxic food poisoning, were provided by the "Departament de Biologia, Sanitat i Medi Ambient" of the Universitat de Barcelona to be tested; including *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 15,313), *Micrococcus luteus* (ATCC 4698), *Escherichia coli* (ATCC 25922) and *Salmonella paratyphi* (ATCC 9150).

Inhibitory zone assay (disc diffusion method). To perform the disc diffusion method, 15 mL of Mueller Hinton agar was inoculated with 200 µL of bacterial suspension (0.4×10^4 CFU mL^{-1}) then kept in culture dishes until solidification. Oxford discs were placed in the inoculated plates and impregnated with each extract (100 µg mL^{-1}), and then the plates were incubated at 30 °C. The penicillin (100 µg mL^{-1}) was used as a positive control, while the sterile EtOH 50% was used as a negative control (Fan et al. 2019). The plates were read from the back against a dark

background. The diameter of the zone inhibition measured in millimetre (mm) correlates to the sensitivity of the strain to the extract.

Minimum inhibitory zones (MIC). The minimum inhibitory concentration (MIC) of the extracts (50%-aqueous EtOH) was determined using the broth dilution method reported by Manandhar et al. (2019) with some modifications. Two-fold serial dilutions of the antibiotic (penicillin) and the different extracts were prepared (0.062, 0.125, 0.25, 0.5 and 1 mg mL^{-1}), then 0.2 mL of the different bacterial suspensions were added to each test tube except the negative control (CTR-) and tubes were incubated for 24 h at 37 °C. The MIC was calculated following the formula described below:

$$\mathrm{MIC}\left(\mathrm{mg\,mL^{-1}}\right) = \frac{\mathrm{Lc} + \mathrm{Hc}}{2}$$

where Lc represents the extract's lowest concentration inhibiting the growth of microbial strains and Hc represents the extract's highest concentration allowing the growth of microbial strains.

Statistical analysis – For all the parameters studied below, extracts were analysed in triplicate ($n = 3$). Statistical analysis was performed using the one-way analysis of variance (ANOVA) in Minitab software (*Version 18, München, Germany*), where Tukey test was used at a significance level of $p < 0.05$.

3 Results

Mineral content – Minerals content was determined in powdered *A. vulneraria* leaves and flowers and the results obtained are presented in Table 1.

The results obtained showed that powdered *A. vulneraria* leaves and flowers accumulated important minerals contents with significant differences among extracts ($p < 0.05$). Flowers contained higher contents of Ca, Cu and Zn than leaves extract with significant differences estimated at 17.5, 37.13 and 21.79%, respectively. Additionally, Fe content in flowers was two folds higher than Fe content determined in leaves extract. Contrariwise, *A. vulneraria* leaves contained the highest K content estimated at 12,320 µg mg^{-1} DW, while flowers contained only 9462 µg mg^{-1} DW.

There are various reports in literature about minerals amounts analyses in different species, meanwhile there is no data concerning the mineral composition of Tunisian *A. vulneraria*, which prompt us to conduct these analyses. For instance, Butkut et al. (2018) determined the mineral composition of two *Astragalus* species from the Fabaceae family, including *Astragalus glycyphyllos* and *Astragalus cicer*, and reported that K, Ca, Zn and Fe contents in the leaves of *Astragalus glycyphyllos* were lower than contents in leaves of *A. vulneraria* with values estimated at 89.7, 22.2, 27 and 226.6 µg mg^{-1} DW, respectively, and were lower in flowers with contents equal to 78.8, 29.4, 47.6 and 141 µg mg^{-1} DW, respectively. Mineral contents were also lower in *Astragalus cicer* leaves and flowers compared with mineral contents in *A. vulneraria* leaves and flowers.

Phenolic compound content in *A. vulneraria* leaf and flower extracts –

Extraction yield of extracts. The extraction solvent and method efficiency to extract phenolic compounds from *A. vulneraria* leaf and flower extracts was determined and the results are represented in Table 2.

Extraction yield values were significantly different ($p < 0.05$) depending on the extraction method and the solvent concentration used for the extraction. From the perspective of the extraction method, the highest extraction yields were found in the leaf and flower extracts extracted using ultrasound assisted extraction method, which were 1.5-, 2.4- and 2.5-folds higher in leaf extract and 1.5, 1.8- and 1.4- in flower extract compared with leaf and flower extracts extracted using cold maceration method when extracted with absolute EtOH, 75% and 50%-aqueous EtOH, respectively.

The results depicted in Table 2 also revealed a significant influence of the extraction power of the solvent on the yield. Hydroethanolic solvent was the most suitable for better extraction of phytochemical components and flower extract presented the highest extraction yield. The leaves and flowers extracted with 50%-aqueous EtOH showed better extraction yield estimated at 58.15% and 66.19% when extracted by ultrasound assisted extraction method and 22.72% and 46.14% when extracted by cold maceration

Table 1 Minerals content in powdered *A. vulneraria* leaves and flowers

	Minerals content (µg mg^{-1} DW)				
	K	Ca	Cu	Fe	Zn
Leaves	$12{,}320 \pm 35.2^{b}$	$16{,}320 \pm 36.9^{a}$	51.82 ± 1^{a}	792.27 ± 3.1^{a}	155.81 ± 1.3^{a}
Flowers	9462 ± 23.2^{a}	$19{,}190 \pm 11.3^{b}$	71.06 ± 0.6^{b}	1546.66 ± 26.1^{b}	199.24 ± 3^{b}

Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD. Different letters in each column indicate significant differences between extracts at $p < 0.05$

Table 2 Extraction yield of *A. vulneraria* leaf and flower extracts

Extract	Extraction yield (%)					
	Maceration extraction			Ultrasound assisted extraction		
	EtOH	75% EtOH	50% EtOH	EtOH	75% EtOH	50% EtOH
Leaf	8.61[b]	14.21[b]	22.72[b]	12.72[b]	34.41[b]	58.15[b]
Flower	16.48[a]	27.12[a]	46.14[a]	24.98[a]	49.26[a]	66.19[a]

Results represent the mean of three replicates ($n=3$) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between extracts at $p<0.05$

Table 3 Phenolic compounds contents of *A. vulneraria* leaf and flower extracts

50%-EtOH extract	TPC (mg GAE g^{-1} DW)	TFC (mg QE g^{-1} DW)	TCTC (mg CE g^{-1} DW)
Leaf	93.27 ± 0.21[b]	37.88 ± 0.18[b]	22.72 ± 0.11[b]
Flower	147.77 ± 0.11[a]	48.83 ± 0.37[a]	24.24 ± 0.14[a]

Results represent the mean of three replicates ($n=3$) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between extracts at $p<0.05$

method, respectively, followed by the leaves and flowers extracted with 75%-aqueous EtOH. Extracts prepared with absolute EtOH presented the lowest extraction yield values.

Since it showed the best extraction yield, EtOH 50%-aqueous extracts obtained by ultrasound assisted extraction were used to determine the phenolic compound contents, antioxidant activity and antibacterial properties of *A. vulneraria* leaves and flowers.

Spectrophotometric determination of phenolic compounds. The different phenolic compounds contents determined spectrophotometrically are represented in Table 3.

The phenolic compound contents found in *A. vulneraria* leaf and flower extracts varied among extracts with significant differences at $p<0.05$. The highest phenolic compound contents were found in the flower extract and were estimated at 147.77 mg GAE g^{-1} DW for TPC, 48.83 mg QE g^{-1} DW for TFC and 24.24 mg CE g^{-1} DW for TCTC, while the leaf extract contained TPC and TFC 1.6- and 1.3-folds, respectively, lower and a TCTC broadly similar to that determined in the flower extract.

Quantitative and qualitative variation of phenolic compound contents in *A. vulneraria* extracts was observed in several recent studies. For example, Moradi et al. (2018) determined TPC and TFC in *A. vulneraria* leaves collected from the south west city of Iran and extracted in EtOH. The results obtained were two folds higher than contents found in the present study. Csepregi et al. (2016) measured also TPC, TFC and CTC in *A. vulneraria* extract and found contents very lower than contents found in our study estimated at 6.8, 1.7 and 4.5 g GAE g^{-1} DW, respectively. Such trend has been previously studied on Fabaceae family plants. For instance, Al-Dabbagh et al. (2018) determined TPC and TFC in the leaves extract of *Trigonella foenum-graecum* and *Cassia acutifolia* extracted with 70% EtOH and found TPC lower than *A. vulneraria* estimated at 9.7 and 10.5 mg GAE g^{-1} DW, respectively, and TFC almost similar to TFC found in our study estimated at 14.6 and 20.8 mg QE g^{-1}, respectively. Chen et al. (2018) quantified also TPC and TFC in the flowers of *Pueraria lobate* and *Sophora japonica* and reported TPC of 23.99 and 81.17 mg GAE g^{-1} DW, respectively, and TFC estimated at 14.59 and 42.88 mg RE g^{-1} DW, respectively.

Table 4 Antioxidant activity of *A. vulneraria* leaf and flower extracts

	Leaf	Flower
TAC (mg AAE g^{-1} DW)	219.7 ± 0.02[b]	293.3 ± 0.01[a]
FIC (%)	48 ± 0.03[b]	65 ± 0.06[a]
HPS (%)	27 ± 0.02[b]	38 ± 0.08[a]

Results represent the mean of three replicates ($n=3$) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between extracts at $p<0.05$

Antioxidant activity – The evaluation of the antioxidant activity of *A. vulneraria* leaf and flower extracts was carried out in vitro by different analytical methods and the results are in Table 4.

Radical scavenging activity was significantly higher in *A. vulneraria* flower extract than in leaf extract ($p<0.05$). On the one hand, the flower extract showed a significantly higher TAC ($p<0.05$) compared to the leaf extract estimated at 293.3 mg AAE g^{-1} DW. Furthermore, the flower extract of *A. vulneraria* exhibited as well FIC and HPS activities 1.3- and 1.4-folds higher than the leaf extract, respectively.

Antioxidant activity of medicinal plants belonging to different families was determined in previous studies. For example, antioxidant activity of medicinal plants belonging to different families was determined in previous studies. For

example, Sharma and Vig (2014) found better TAC values in *Parkinsonia aculeate* extract than TAC obtained in the present study estimated at 360 mg g^{-1} of extract. Osman et al. (2018) also determined TAC in *Dialium indum* extract and found values ranging between 104.52 and 1515.79 µmol TE/g Dry Extract.

Based on Tables 3 and 4 results, it can be suggested that antioxidant activities of extracts are linked to their polyphenol contents, which can support the hypothesis of a positive correlation between the effectiveness of extracts antioxidant capacities and their phenolic amounts as submitted by supportive studies of Pisoschi and Pop (2015) and Gabriela et al. (2016).

Antibacterial activity screening –

Inhibitory zone. The sensitivity of bacterial strains against *A. vulneraria* leaf and flower extracts was determined after 48 h of incubation at 37 °C. The area developed around the discs treated with extracts was measured and the results obtained are represented in Table 5.

According to the results obtained, the leaf and flower extracts of *A. vulneraria* (100 µg mL^{-1}) showed a significant inhibitory effect against the bacteria growth ($p < 0.05$). The flower extract showed better antibacterial activity than the leaf extract. The best antibacterial activity of the flower extract was against *S. aureus* strain with an inhibition zone estimated at 18 mm, while the best antibacterial activity of the leaf extract was observed against *M. luteus* strain with an inhibitory zone of 12 mm. However, the leaf and flower extracts did not show any antimicrobial activity against *S. paratyphi* and *E. coli* strains, respectively, as compared with the penicillin (100 µg mL^{-1}), which showed inhibition zones of 30 and 27 mm, respectively.

Table 5 Diameters of the inhibitory zones (mm) developed around discs treated with *A. vulneraria* leaf and flower extracts (100 µg mL^{-1})

	Inhibitory zone (mm)				
	Microorganism	50% EtOH	Penicillin	Leaf	Flower
Gram +	*S. aureus*	NS	24 ± 1.8^{c}	10 ± 0.6^{a}	18 ± 1.4^{b}
	M. luteus	NS	19 ± 1.2^{c}	12 ± 0.2^{a}	15 ± 0.5^{b}
	L. monocytogenes	NS	15 ± 0.9^{b}	10 ± 0.3^{a}	11 ± 0.6^{a}
	B. cereus	NS	10 ± 0.3^{a}	9 ± 0.2^{a}	14 ± 0.8^{b}
Gram –	*S. paratyphi*	NS	30 ± 1.6^{b}	NS	4 ± 1.5^{a}
	E. coli	NS	27 ± 1.8^{b}	5 ± 1^{a}	NS

Results are means of three different experiments ($n = 3$). Means in the same row with different letters are significantly different ($p < 0.05$). Penicillin (100 µg mL^{-1}) was used as a positive control. Sterile 50%-aqueous EtOH was used as negative control. No inhibition zone is indicated by NS (Not Sensitive)

Minimum inhibitory concentration. The minimum inhibitory concentration (MIC) assay was assessed for only the bacterial strains that showed a sensitivity to *A. vulneraria* leaf and flower extracts (100 µg mL^{-1}) in the disc diffusion method previously performed and the results obtained are in Table 6.

Table 6 shows the turbidity of the penicillin, *A. vulneraria* leaf and flower inoculations, and positive (CTR^{+}) and negative (CTR^{-}) controls after 24 h of incubation. The CTR^{+} containing the broth nutrient, bacterial culture and antibiotic or *A. vulneraria* extracts showed turbidity (bacterial growth) after the 24 h of incubation and was used to test the growing ability of the medium, while the CTR^{-} containing only the broth nutrient and the antibiotic or *A. vulneraria* extract did not show turbidity (no bacterial growth) after the 24 h of incubation and was used to test the sterility of the medium and equipment.

Bacterial growth (indicated by the presence of turbidity in the inoculum) was observed for all the bacterial strains tested at the concentrations of penicillin 0.062 and 0.125 mg mL^{-1} and only for *L. monocytogenes* and *E. coli* at the penicillin concentration 0.25 mg mL^{-1}. In one hand, the penicillin at 0.5 and 1 mg mL^{-1} inhibited totally the growth of all the bacterial strains. Likewise, *A. vulneraria* leaf extract at 0.062, 0.125 and 0.25 mg mL^{-1} was not enough to inhibit

Table 6 Turbidity of the Penicillin, *A. vulneraria* leaf and flower inoculations after 24 h of incubation at 37 °C

Cc (mg mL^{-1})	Penicillin					*A. vulneraria* leaf extract					*A. vulneraria* flower extract					CTR +	CTR –
	0.062	0.125	0.25	0.5	1	0.062	0.125	0.25	0.5	1	0.062	0.125	0.25	0.5	1		
S. aureus	+	+	–	–	–	+	+	+	–	–	+	+	–	–	–	+	–
M. luteus	+	+	–	–	–	+	+	–	–	–	+	+	–	–	–	+	–
L. monocytogenes	+	+	+	–	–	+	+	+	–	–	+	+	–	–	–	+	–
B. cereus	+	+	–	–	–	+	+	+	–	–	+	+	–	–	–	+	–
S. paratyphi	+	+	–	–	–	NT		+	+	–	–	+	–				
E. coli	+	+	+	–	–	+	+	+	+	+	NT	+	–				

(+) Turbidity indicating bacterial growth, (–) no turbidity indicating no bacterial growth and NT indicates microbial strain not tested

the growth of all the bacterial strains, except for *M. luteus*, which showed resistant at 0.25 mg mL^{-1}. No bacterial growth was observed at 0.5 and 1 mg mL^{-1} of *A. vulneraria* leaf extract, except for *E. coli*. In the other hand, *A. vulneraria* flower extract showed better antimicrobial activity than the penicillin and *A. vulneraria* leaves extract by inhibiting the growth of all the bacterial strains without exception at 0.25 mg mL^{-1}.

Based on results of Table 6, MIC of the different extracts tested against the different bacterial strains were calculated and the results obtained are represented in Table 7.

Anthyllis vulneraria flower extract was considered to have better antibacterial activity than penicillin and leaf extract. The flower extract of *A. vulneraria* had the lowest MIC values equal to 0.625 mg mL^{-1} to inhibit the growth of all the bacterial strains tested compared with the penicillin that showed similar results except for *L. monocytogenes* and *E. coli*, which showed sensitivity at a MIC value of 0.75 mg mL^{-1}. The leaves of *A. vulneraria* presented the lowest antimicrobial effect with MIC values higher than those recorded in the penicillin and *A. vulneraria* flowers inoculums estimated at 0.75 mg mL^{-1} against *S. aureus, L. monocytogenes* and *B. cereus* strains, except for *M. luteus* strain (0.625 mg mL^{-1}) and *E. coli* where MIC value was higher than the highest concentration of the penicillin used ($\geq$ 1 mg mL^{-1}).

4 Discussion

Plants frequently accumulate metabolites, mainly phenolic compounds, which represent an important source of molecules that can be used by humans for different purposes. These phenolic compounds, such as phenolic acids, flavonoids, stilbenes and lignans, etc., correspond to a very wide range of chemical structures and are characterised by an unequal qualitative and quantitative distribution. After several years of research, different studies have shown the biological virtues of these compounds in different fields (Lin et al. 2016; Cosme et al. 2020). The main property of these compounds is their antioxidant activity, which consists in neutralising free radicals that are harmful for living organisms. Beyond the antioxidant property, phenolic compounds are characterised by their powerful antimicrobial property as well as many other biological activities (Wang et al. 2009). In the present work, *A. vulneraria* was studied since it was widely used in folk medicine for centuries owing to its varied chemical composition and active ingredients. Several previous investigations have focused on the biological properties of *A. vulneraria* have demonstrated its efficiency to prevent and treat different diseases. For instance, the consumption of *A. vulneraria* aerial part as an infusion has been shown to treat vomiting, diabetes, and stomach disorder (Csepregi et al. 2020). The aerial part also have been used to treat wounds and swelling by direct application (Godevac et al. 2008). Moreover, *A. vulneraria* leaves have been proven to be effective in inhibiting human herpes-virus and poliovirus (Suganda et al. 1983). The flowers as well have a potent therapeutic efficacy against wounds, high blood pressure, heart failure, portal hypertension, vomiting, inflammation, acne and throat pain (Nartowska et al. 2001). The objectives of the present research work were to quantify the mineral content in *A. vulneraria* leaves and flowers, determine their phenolic compound contents and evaluate their antioxidant and antibacterial properties.

Essential minerals, like K and Ca, and trace elements, like Fe, Zn, and Cu, are important for plants metabolic processes, such as chlorophyll synthesis, respiration, as well as protein structure and function (Majdoub et al. 2017) and the consumption of plants rich in minerals is associated with numerous health benefits (Ryan-harshman and Aldoori 2005). Plants absorb minerals from the soil with their roots in varying amounts. This quantitative and qualitative absorption of minerals is influenced by environmental factors, such as the nature of the soil, aeration and temperature (Pallardy 2007). Each mineral nutrient plays a specific role in the development of the plant, for this reason some plants adopt different strategies to avoid the consequences related to the deficiency or excess of minerals and to cope with toxic heavy metals leading to serious physiological disorders (Rouached and Tran 2015). Several researchers showed that some Fabaceae species can tolerate high concentrations of heavy metals in the soil, such as Cu and Zn (Sujkowska-Rybkowska et al. 2020). In addition, owing to their ability to establish symbiotic association with nitrogen-fixing bacteria (nodulated rhizobia), Fabaceae species are capable to grow in metal contaminated sites (Ma et al. 2011; Karthik et al. 2017). Furthermore, Neubauer et al. (2000) reported that the root

Table 7 MIC values of the penicillin and *A. vulneraria* extracts against the different bacterial strains

	MIC (mg mL^{-1})		
	Penicillin	Leaf	Flower
S. aureus	0.625	0.75	0.625
M. luteus	0.625	0.625	0.625
L. mmonocytogenes	0.75	0.75	0.625
B. cereus	0.625	0.75	0.625
S. paratyphi	0.625	NT	0.625
E. coli	0.75	*	NT

*MIC is higher than the highest concentration of the antibiotic/extract in the first tube, NT indicates microbial strain not tested

exudates resulting from the rhizobium-legume symbiosis in Fabaceae plants can also immobilise metal ions and reduce their harmful effects on plants when they are present in very high contents. Seeing their high adaptability to sites contaminated with heavy metals, Fabaceae species like *A. vulneraria* are applied in re-vegetation and phyto-stabilisation of mine tailings in Europe and northern Africa (Mohamad et al. 2017; Fagorzi et al. 2018; Sujkowska-Rybkowska and Ważny 2018), which can explain the high levels of Cu and Zn found in the leaves and flowers of *A. vulneraria*. The richness of medicinal and edible plants in minerals confers them interesting biological properties provided when they are consumed as food or administered as drugs (Karppanen 1991). For instance, trace elements, such as Cu, Manganese (Mn), Selenium (Se), Fe and Zn, are indispensable co-factors for metabolic reactions of antioxidant enzymes like SOD, CAT and GPx to protect the human body from radicals (Leung 1998). Moreover, several researches demonstrated the anti-inflammatory property of the Zn and its ability to decrease oxidative stress biomarkers (Prasad 2014). An investigation carried out by Roughead et al. (1999) confirmed as well the antioxidant activity of Fe and its capacity to decrease risk of heart disease and cancer.

The phenolic content of the leaf and flower extracts of *A. vulneraria* was determined as well. EtOH solvent was chosen to extract phenolic compounds since it is considered one of the most powerful pure natural solvents that does not present a danger for human consumption. In addition, EtOH is labelled by the *Food and Drug Administration* (FDA) as *Generally Recognised As Safe* (GRAS) food substance products (Alzeer and Abou Hadeed 2016), hence the possibility of its safe use in pharmaceutical, food and cosmetic products without fear of intoxication. The results obtained showed that, for *A. vulneraria*, the best extraction yield value and the highest phenolics contents were observed in the flowers extracted with 50%-aqueous EtOH by ultrasound assisted extraction method. Being such an efficient and widely used extraction method on the industrial scale, the ultrasound assisted extraction method saves time as the chemical compounds, such as carotenoids and polyphenols, etc., diffuse more rapidly into the extraction medium, while producing high quality extracts that can be used for foods, supplements and pharmaceutical products (Safdar et al. 2017; Deng et al. 2017; Osorio-Tobón 2020). Moreover, Do et al. (2014) showed that increasing the water concentration in EtOH solvent improves the extraction efficiency and facilitates the extraction of chemical compounds that are soluble in both inorganic and organic solvents (Khaw et al. 2017) and thus, the extraction of the maximum contents of phenolic compounds. The results obtained also showed a significant difference ($p < 0.05$) in phenolics contents among the different extracts. The variability in phenolic compound contents observed in the different plant's parts may be due to biological (vegetative stage), environmental (climate) and technical (methods and extraction solvents polarity) factors (Ksouri et al. 2008; Sampaio et al. 2011; Liu et al. 2016). In our investigation, the estimated phenolic compounds varied significantly with respect to the used plant part and the extraction method and solvent, which is in line with the findings of Zengin et al. (2015) and Villasante et al. (2019). Researches about phenolic compounds, in particular flavonoids, seem to be very advanced by reason of their various physiological properties, such as anti-allergic, anti-inflammatory, antimicrobial, antiviral, antibacterial, anti-carcinogenic, antithrombotic, cardio-protective and vasodilator activities (Generali et al. 2019). The beneficial effects of polyphenols are of a particular interest in pharmaceutical, cosmetic and food industry. According to several researches that studied the positive impact of polyphenol consumption on health and prevention of diseases, manufacturers are now marketing polyphenol-enriched foods and dietary supplements (Martin and Apple 2009). In addition, their antioxidant activity ensures better preservation of cosmetic and food products by preventing lipid peroxidation (Chang and Kim 2018). In cosmetic industry, phenolic compounds are added in cosmetic products owing to their well-recognised properties, such as antioxidant, anti-inflammatory, antimicrobial, antimutagens and anti-aging activities, as well as their emollients, humectant, wound healing, protective agents against UV-B damage, and reducing skin discoloration effects (Halla et al. 2018).

Over the few last decades, several different analytical methods have been developed and improved in order to measure the antioxidant activity of plant's extracts (Cornelli 2009). In the present work, the antioxidant activity of *A. vulneraria* extracts was assessed by different analytical methods, including TAC, FIC and HPS assays and the results obtained showed as well a significant difference ($p < 0.05$) in antiradical assays among the different extracts. The results followed the same trend as the phenolics contents and showed that, for *A. vulneraria*, the strongest radical scavenging activities were exhibited by the flowers extracted with 50%-aqueous EtOH by ultrasound assisted extraction method. The significant difference observed among the values obtained by the different antiradical methods can be explained by the type of the methods and their chemical backgrounds (Lahue 1981; Apak et al. 2016).

In addition to their antioxidant activity, *A. vulneraria* leaf and flower extracts showed potent antibacterial activity. Foodborne infections caused by bacteria or their toxins, viruses or parasites or unconventional agents are considered one of the serious problems that threaten food industries and consumer's health. A strong correlation between phenolic compounds and antibacterial activity has been found to be

significant in several recent studies (Maddox et al. 2010). In this research work, the antimicrobial analysis revealed that *A. vulneraria* leaf extract inhibited the growth of *Staphylococcus aureus*, *Micrococcus luteus, Listeria monocytogenes*, *Bacillus cereus* and *Escherichia coli*, but it did not show any antimicrobial activity against *Salmonella paratyphi* strain, while flower extract had an antimicrobial activity against all the bacterial strains tested except *Escherichia coli* strain. Previous studies showed that the antibacterial activity of plant's extracts depended mainly on their richness on polyphenol contents (Maddox et al. 2010). Polyphenols are endowed with significant antimicrobial activity. Their activity is probably due to their ability to complex with extracellular proteins and makes complexes with the bacterial cell membrane. One of the most important functions of phenolic compounds, mainly flavonoids, is their role in protective effect against microbial invasion. This involves their accumulation as phytoalexins in response to microbial attack. Due to their ability to inhibit photogenic spore germination in plants, they have also been proposed for use against fungal pathogens in humans (Cho and Lee 2015). Several studies reported the regular presence of antimicrobial activity in flavonoids. The majority of flavonoids, recognised as antifungal constituents, are isoflavonoids, flavones and flavanones (Qiu et al. 2014). Additionally, the absence of antimicrobial activity in leaves extract against *S. paratyphi* strain and in flowers extract against *E. coli* strain could be explained by the fact that those strains developed resistance mechanisms or the concentration of *A. vulneraria* extracts are not high enough to inhibit bacterial growth.

To summarise, the results obtained in the present study suggest that the richness of *A. vulneraria* in minerals and phenolic compounds and their unequal distribution in its leaf and flower extracts confer it antioxidant and antibacterial activity and supported its involvement in pharmaceutical, cosmetic and food products to enhance their quality while insuring consumer health.

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Author's contributions MO contributed to the study conception, design, conceptualisation, methodology, software, investigation and data curation. The first draft of the manuscript was written by MO. Material preparation, data collection and analysis were performed by MO, NM, JA and MPA. LBK and MPA supervised and validated the work. All authors commented on previous versions of the manuscript, and read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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