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Analysis of polyphenolic compounds and biological activities evaluation of extracts from *Centranthus ruber*

Abderrahmane Mezrag[1.2], Aissaoui Meriem[1.2], Labib Noman[3*], Ahmed Boukeloua[4.5], Bougdia Abderrahmane[2], Ait-Messaoud Abdelhamid[2]

[1]Research Unit Development of Natural Resources, Bioactive Molecules and Physiochemical and Biological Analysis, Department of Chemistry, Constantine 1 University, Constantine 25000, Algeria
[2]chemistry department, Faculty of Science. Saad Dahlab Blida 1 University, Blida 09000, Algeria
[3]Department of Pharmacognosy & Medicinal Chemistry, Faculty of Clinical Pharmacy, 21 September University of Medical and Applied Sciences, Sana'a, Yemen
[4]Process Engineering Laboratory for Sustainable Development and Health Products, University Constantine 3 Salah Boubnider, Constantine 25000, Algeria
[5]Institute of Applied Sciences and Techniques, Faculty of Sciences, University of Oum EL Bouaghi, Oum EL Bouaghi 04000, Algeria

***Corresponding author**: Labib Noman Tel.: +96 774013060 E-mail ID: labibnomanali@gmail.com

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Abstract

This study aimed to evaluate the phytochemical components and biological properties of two *Centranthus ruber* extracts. The total polyphenols and flavonoids content were evaluated, and the highest total phenolic content was determined in ethyl acetate extract (337.7±7.8 µg EAG/mg). Significantly highest total flavonoid content value for the n-butanol extract at 63.7±3. 8µg EQ/mg. The n-butanol extract was analyzed by HPLC, and sixteen compounds were determined including flavonoids and phenolic acids. The results also show that n-butanol extract contains Chlorogenic acid (646.73 mg/kg) as a major concentration, followed by Hesperidin (341,09 mg/kg) and *P*-Coumaric acid (101.45 mg/kg). Using different in vitro assays including DPPH, FRAP methods, and β-carotene bleaching assay, the extracts exhibited significant antioxidant activity. The n-butanol extract showed higher activity of antioxidant assays than the ethyl acetate extract. The disk diffusion method was used to evaluate the antibacterial activity of the extracts against three microbial strains. The n-butanol extract exhibited higher zone inhibition 13±0.1, 17.1±0.05 and 15±0.13, respectively against the tested bacterial strains *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus*.

Keywords: *Centranthus ruber*, polyphenols and flavonoids content, HPLC analysis, antioxidant, antibacterial

Introduction

Caprifoliaceae is a family belonging to the order Dipsacales, it includes about 960 species in 41 genera. The family has an almost worldwide distribution, with centers of diversity in eastern North America and eastern Asia, but is absent from tropical and southern Africa (Wang et al., 2020). It is represented in Algeria with certain genera including; Lonicera (Meddour et al., 2021), Scabiosa (Barone et al., 2020), Lomelosia (Habib et al., 2020) and Centranthus (Richardson, 1975).

Centranthus ruber known as red valerian, is a flowering plant native to the Mediterranean and Balkan countries. as well as to southern France and Sardinia. It is also found in other parts of the world such as the USA, Australia, and New Zealand (Holmes et al., 2018).

Centranthus ruber exhibited inhibitory properties on protein denaturation and pancreatic lipase enzyme. Along with, antiarthritic and anti-obesity (Musolino et al., 2022). In addition, it is commonly used in traditional Italian cuisine (Musolino et al., 2022). Several compound types were detected in *Centranthus ruber* such as terpenoids, fatty acids, phytosterols, and iridoids (Musolino et al., 2017; Chami et al., 2018; Doyle et al., 2004).

Plants contain a diversity of biologically active compounds, and numerous studies have demonstrated that these phytochemicals exhibit various biological properties (Hirasa and Takemasa, 1998).

Many of these compounds extracted from plants have been identified as polyphenols and flavonoids that can contribute to antioxidant and antimicrobial activity (Ding et al., 2008).

The objective of this study is to evaluate the phytochemical contents and biological activity of the Algerian species *Centranthus ruber* extracts.

To our knowledge, no previous studies on both phytochemical contents and biological activity have been reported for the *Centranthus ruber* from Algeria.

Materials and Methods

Plant material

The ariel part of *Centranthus ruber* was collected during its maturation period in March, from the region of "Chiffa" to the west of Blida city.

Preparation of plant extracts

500 g of the dried plant was subjected to hydro distillation using a Clevenger-type apparatus. The essential oil was collected and stored in the bottle at 40°C. The aqueous phase of the extracted plant was subjected to a separating funnel for (liquid-liquid) extraction with Ethyl Acetate and Butanol solvents, respectively, and concentrated by rotavapor under reduced pressure.

Assay of total polyphenols

The content of total polyphenols in plant extracts is estimated by the Folin-Ciocalteu method described by Talbi et al. (2015). A volume of 200 µl of solutions of different extracts diluted in ethanol (1 mg/ml chloro-ethanolic, ethanolic, 0.125 mg/ml n-butanol and ethyl acetate) is mixed with 1 ml of Folin-Ciocalteu's reagent diluted in distilled water (1:10). After 4 min, 800 µl of an aqueous solution of sodium carbonate (75 mg/ml) are added. The tubes are kept in the dark at room temperature for 2 hours and the absorbance is measured at 765 nm against a blank which contains ethanol instead of sample. Gallic acid (0 - 75 µg/ml) is used as a standard, from which the concentration of total polyphenols in the different extracts is calculated by the following equation (Nickavar and Esbati, 2012):

Polyphenol content = C×V/M

Where: C: concentration of gallic acid (µg/ml); V: volume of extract solution (ml); M: mass of extract (mg)

Assay of total flavonoids

The determination of total flavonoids is carried out by the aluminum trichloride method ($AlCl_3$) described by Lin et al. (2007) with some modifications. 50 µl of a hydro-

ethanolic solution of $AlCl_3$ (10%) is added to 1 ml of the various extracts diluted in ethanol (0.5 mg/ml of n-butanol and ethyl acetate). Then, 50 µl of an ethanolic solution of sodium acetate (1 M) and 1.4 ml of distilled water are added. After 1 h of incubation at ambient temperature, the absorbances are measured at 420 nm against a blank that contains ethanol instead of the sample. Quercetin (0 - 30 µg/ml) was used as a standard, and the concentration of total flavonoids in the different extracts is calculated by the following equation (Nickavar and Esbati, 2012):

flavonoids content = C×V/M

Where: C: concentration of quercetin (µg/ml); V: volume of extract solution (ml); M: mass of extract (mg)

Analyses of Phenolic Compounds (HPLC)

The phenolic composition analyses of different extracts were made according to the Caponio, Alloggio13 approach, yet with slight modifications. The performance was assessed using an HP-Agilent 1290 Infinity HPLC equipped with a C18 column and diode array detector DAD. As a mobile phase, 3% acetic acid in (A) water and methanol (B) were used. Injection volumes were 1 µl, and extract concentrations were 20 mg/ml. The eluates were detected at 330 nm. The samples were prepared in methanol. Finally, injection volumes were 20 µl. The elution gradient applied at a flow rate of 0.8 ml/ min was: 93% A-7% B (0.1 min), 72% A-28% B (20 min), 75% A-25% B (8 min), 70% A-30% B (7min) and the same gradient for 15 min was 67% A-33% B (10 min), 58% A-42% B (2 min), 50% A-50% B (8 min), 30% A-70% B (3 min), 20% A-80% B (2 min) and 100% B in 5 min until the end of the experimental cycle. Gallic acid, catechin, chlorogenic acid, caffeic acid, hydroxybenzoic acid, Epicatechin, syringic acid, *P*-coumaric acid, trans-ferulic, sinapic acid, benzoic acid, acid hesperidin, rosmarinic acid, cinnamic acid, and quercetin were used as standards. Identification and quantitative analysis were made by comparison with standards. The amount of each phenolic compound was expressed as mg per gram of extract using external calibration curves obtained for each phenolic standard (Abdulqadir et al., 2018).

Antioxidant activity

DPPH free radical scavenging activity

The experimental protocol was followed to study the DPPH free radical scavenging activity according to Yen and Duh. (1994) with some modification. 400 µl of each extract (n-butanol, and ethyl acetate) at different dilutions in ethanol were added to 1600 µl of a freshly prepared ethanolic solution of DPPH (0.03 mg/ml). After incubating the mixture in the dark and at room temperature for 30 minutes, the absorbance was measured at 517 nm against the ethanol. The negative control contains all the reagents except the extracts which have been replaced by an equal volume of ethanol. The solution at different concentrations (0 – 0.2 mg/ml) of the reference antioxidant (ascorbic acid) represents the positive control which undergoes the same experimental condition. Each crude extract was analyzed in triplicate. The percentage of DPPH radical scavenging activity of each extract was calculated as follows (Kroyer and Hegedus, 2001):

$$\text{Inhibition\%} = Abs_{control} - Abs_{sample} / Abs_{control} \times 100$$

Where: $Abs_{control}$ is the absorbance of the negative control; Abs_{sample} is the absorbance of the positive control or sample

Ferric reducing antioxidant power (FRAP)

The test was carried out according to the method of Yıldırım et al. (2001) with some modifications. A volume of 200 µl of the extracts at different concentrations in ethanol was mixed with 500 µl of a 0.2 M phosphate buffer solution (pH=6.6) and 500 µl of an aqueous solution of $K3Fe(CN)_6$ at 1%. The tubes were incubated at 50°C in the dark for 20 minutes and then cooled to room temperature. Then, 500 µl of an aqueous solution of trichloroacetic acid 10% (TCA) was added to stop the reaction, and the tubes were centrifuged at 3000 rpm for 10 minutes. 1.5 ml of the supernatant is taken and added to 300 µl of distilled water, and at the end 60 µl of a freshly prepared aqueous solution of $FeCl_3$ is added. The reading of the absorbances is made at 700 nm against a blank that contains ethanol instead of a sample. BHT (Butylated hydroxytoluene) of a concentration range (0 – 0.125 mg/ml) is used as a positive control in this experiment, also subjected to the same experimental conditions.

β-carotene bleaching test

The β-carotene bleaching test is performed according to the method described by Lelono et al. (2009) with some modification. 2 mg of β-carotene is dissolved in 10 ml of chloroform, and this solution is added to 20 mg of linoleic acid and 200 mg of Tween 40 to form an emulsion. After evaporation of the chloroform under vacuum, 100 ml of distilled water was added with stirring. 4.8 ml of the emulsion obtained is poured into tubes containing 0.2 ml of different extracts diluted in ethanol (1 mg/ml of n-butanol and ethyl acetate). After shaking, the tubes are placed in a water bath at 50° C. for 2 hours and the kinetics of the discoloration of the emulsion is monitored by measuring the absorbance at 470 nm for intervals time: 30 min, 1 h, 1:30 h, and 2 h. The same steps are carried out for the positive control which is BHA (0.1 mg/ml) (butylated hydroxyanisole) and for the blank (negative control) which contains ethanol instead of sample.

The percentage of antioxidant activity is calculated using the following equation (Ismail and Hong 2002):

$$AA = [1– (A_0 – A_t) / (A^o_0 – A^o_t)] \times 100$$

Where: where A_0 and A^o_0 are the absorbance values measured at the initial time of the incubation for samples and control respectively, while A_t and A^o_t are the absorbance values measured in the samples or standards and control at t = 120 min.

Antibacterial activity

Antibacterial activity was tested in vitro by the disc diffusion method in which two extracts (n-butanol and ethyl acetate) were impregnated into sterile blank 6 mm discs and allowed to diffuse into inoculated agar medium (Ruangpan and Tendencia, 2004).

The experiments were performed using four reference bacterial strains (American Type Culture Collection): *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 10876, and *Staphylococcus aureus* ATCC 6538. All the bacterial strains were chosen by their high contamination frequencies and their dominant pathogenicity.

Results and Discussion

Phytochemical analysis

Total polyphenols and flavonoids content

According to the results obtained, we can conclude that the extracts contain amounts of polyphenols and flavonoids, and the extracts are particularly rich in these compounds, which will be further proven by their activity.

This variation in the polyphenol and flavonoid contents in the different extracts could be explained by the solvents chosen for the extraction, but also by the experimental conditions of the assays, where the Folin reagent for the polyphenols which can react with proteins, sugars and other sulfur compounds which may give inaccurate results (Singleton and Rossi, 1965; Musci and Yao, 2017).

The total polyphenols content of n-butanol and ethyl acetate extracts of *Centranthus ruber* were determined by the Folin-Ciocalteu method. The results are expressed in Table 1. in µg gallic acid equivalent per mg of extract (µg EAG/mg). According to the results, the extracts are rich in polyphenols, and the ethyl acetate extract exhibited the highest content (337.7±7.8 µg EAG/mg), followed by the n-butanol extract (298.4±5.9 µg EAG/mg).

In addition, the content of total flavonoids was determined by the aluminum trichloride method, the results are shown in Table 1, in µg quercetin equivalent per mg of extract (µg EQ/mg). The flavonoid content results showed a high value for the n-butanol extract (63.7±3.8 µg EQ/mg), which was more than ethyl acetate (31.9±0.1 µg EQ/mg).

The results also showed that the polyphenol content is higher than that of the flavonoids, and could be other polyphenols type which was confirmed by HPLC analysis.

Table 1: Content of total polyphenols and flavonoids in the *Centranthus ruber* extracts.

Extracts and Standert	Total polyphenol content [µg EAG/mg]	Total flavonoids content [µg EQ/mg]
Ethyl acetate	337.7±7.8	31.9±0.1
n-butanol	298.4±5.9	63.7±3.8

HPLC analysis

HPLC analysis chromatogram of the n-butanol extract of *Centranthus ruber* indicated that the extract contains several compounds (Figure 1). The analysis result showed the presence of polyphenolic compounds as presented in Table 2. The polyphenols in the sample were identified from the retention times and the characteristic wavelength for each compound in comparison with standards. Sixteen compounds were determined including flavonoids and phenolic acids. Twelve of them were phenolic acids, while the others were flavonoids. The main phenolic compound found in the analysis was Chlorogenic acid (646.73 mg/kg), followed by Hesperidin (341,09 mg/kg) and *P*-Coumaric acid (101.45 mg/kg). The other components showed less concentration than 100 mg/kg.

No	Phenolic Compounds	λ (nm)	RT(min)	mg/kg
1	3-Hydroxy Benzoic Acid	287	22.545	2.053
2	4- Hydroxy Benzoic Acid	278	17.647	23.592
3	Benzoic Acid	278	47.629	26.998
4	Catechin Hydrate	278	11.499	N.D.
5	Chlorogenic Acid	330	16.239	646.73
6	Caffeic Acid	330	21.476	57.848
7	Epicatechin	278	20.169	94.092
8	Gallic Acid	278	5.912	8.752
9	Hesperidin	287	65.989	341.09
10	*P*-Coumaric Acid	330	33.597	101.45
11	Quercetin	254	76.313	53.487
12	Rosmarinic Acid	330	70.655	59.185
13	Sinapic Acid	330	37.264	21.485
14	Syringic Acid	278	22.628	1.034
15	t-Cinnamic Acid	278	75.207	1.118
16	t-Ferrulic Acid	330	37.202	15.924

Table 2: The polyphenolic compounds content in n-butanol extract of *Centranthus ruber*.

Note: RT=Retention Time, **N.D**.=Not Detected.

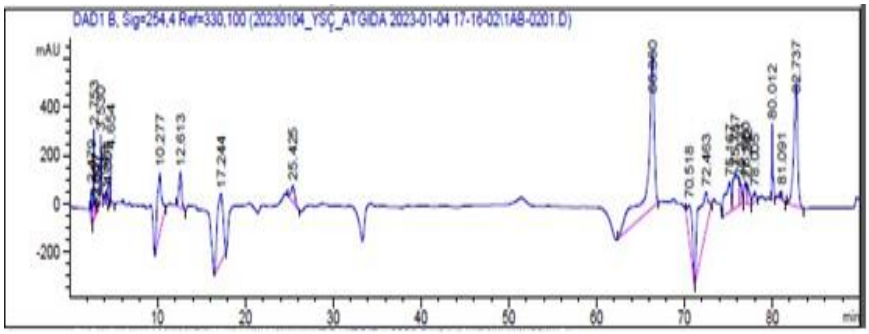

Figure 1: HPLC analysis chromatogram of the n-butanol extract of *Centranthus ruber.*

Evaluation of biological activity

Antioxidant activity

The antioxidant activity of the n-butanol and ethyl acetate extracts was evaluated using three different methods: free radical scavenging DPPH, β-carotene bleaching test, and iron reduction test (FRAP). For each method, ascorbic acid, BHA and BHT were used as references respectively, the results of antioxidant activities are shown in Table 3.

The antioxidant activity was assessed using the DPPH test where the ability of extracts and ascorbic acid to scavenge free radicals was studied.

According to the IC_{50} values obtained, the two extracts showed significant activity and the n-butanol extract exhibited the highest antioxidant activity at 0.026±0.002 mg/ml, which is very close to the ascorbic acid value at 0.02±0.01 mg/ml, followed by the extract ethyl acetate at 0.061±0.002 mg/ml.

The antioxidant activity was evaluated using the FRAP method where the ability of the extracts and the reference to reduce the ferric ion (Fe3+) to its ferrous form (Fe2+) was studied. The absorbance was measured at 700 nm for different concentrations of the extract. The EC_{50} values of BHT showed the greatest capacity at 0.06±0.02 mg/ml, while the EC_{50} of n-butanol extract showed a large reduction capacity (0.12±0.01 mg/ml) close to that of reference BHT, followed by the ethyl acetate extract (0.24±0.03 mg/ml) confirming a strong antioxidant activity of the extracts.

The results of antioxidant activity evaluated by the oxidation reaction of β-carotene of the extracts using BHA which was used as a reference exhibited the greatest antioxidant activity at 96.2 ± 2.7%, which proves its high antioxidant power. In contrast, the n-butanol extract showed higher activity than the ethyl acetate extract at 53.7±1.0% and 29.0±1.8% respectively.

It was found that a strong antioxidant activity could be attributed to a high content of polyphenols and flavonoids (Falleh et al., 2011; Al-Hadhrami and Hossain, 2016), which is in agreement with our results.

Polyphenol compounds possess strong antioxidant and biochemical activity (Kiokias et al., 2020). Chlorogenic acid is one of hydroxycinnamic acid derivatives that are abundant in plant sources (Rocha et al., 2012). Several studies have been reported that Chlorogenic acid has many health benefits such as antibacterial, antifungal, antiviral, antiphlogistic, antioxidant, chemopreventive, and other biological activities (Ayaz et al., 2008; Wang et al., 2009). Hesperidin is a flavanone type used for broad spectrum biological activities including type 2 diabetes, antioxidant, anti-inflammatory, anticancer, antiviral effects, biofilm protection, and protection against cardiovascular disorders (Pyrzynska, 2022; Syahputra et al., 2022; Shamsudin et al., 2022). The

hydroxycinnamic acid, *P*-Coumaric acid also have demonstrated several bioactivities such as antioxidant, antibacterial, anti-inflammatory, anticancer and antimelanogenic effects (Singha et al., 2022; Boo, 2019; Roy et al., 2016).

Previous studies have been reported that the antioxidant activity of the extracts associated with the presence of phenolic compounds and chlorogenic acid derivatives (Noman et al., 2017; Noman et al., 2015a).

Table 3: Antioxidant activities of *Centranthus ruber* extracts.

Extracts and Standert	**DPPH assay IC_{50} [mg/mL]**	**FRAP assay EC50 [mg/mL]**	**β-carotene bleaching assay**
Ethyl acetate	0.061±0.002	0.24±0.03	29.0±1.8%
n-butanol	0.026±0.002	0.12±0.01	53.7±1.0%
Ascorbic acid	0.02±0.01	-	-
BHT	-	0.06±0.02	-
BHA	-	-	96.2± 2.7%

Antibacterial activity

The antibacterial activity of the extracts was determined by the agar disk diffusion method, where each disk was impregnated with the solutions of the extracts at a certain concentration using DMSO as the solvent and placed in three different bacterial colonies, the results of the zones of inhibition are shown in Table 4.

Both of the tested extracts showed inhibition of bacterial growth against three strains, with the n-butanol extract showing the highest antibacterial activity among the extracts but remaining lower than that of gentamicin.

Several studies have demonstrated a strong positive correlation between the antibacterial activity of plant extracts and their content of phenolic compounds. This suggests that phenolic compounds play a significant role in the antimicrobial properties of these extracts (Noman et al., 2015a; Noman et al., 2015b).

The presence of the polyphenols besides the sixteen compounds determined in the n-butanol extract may contribute to its activity.

The increasing resistance of microorganisms to current antimicrobial drugs is a growing concern for scientists and healthcare professionals globally. Notably, treating bacterial

infections with existing medications has become increasingly challenging due to the emergence of drug-resistant strains (Koomen et al., 2002). So, the commonly used *Centranthus ruber* in traditional Italian cuisine protects against microorganisms and enhances people's health.

Table 4: Diameter of Inhibition Zone of *Centranthus ruber* extracts and standard against bacterial strains.

	Zone of inhibition (mm)		
Extracts and Controls	*Pseudomonas aeruginosa* ATCC 27853	*Bacillus cereus* ATCC 10876	*Staphylococcus aureus* ATCC 6538
Ethyl acetate	11.13±0.15	10.9±0.05	9.96±0.04
n-butanol	13±0.1	17.1±0.05	15±0.13
Positive control Gentamicin	27±0.2	29.1±0.15	25.9±0.09
Negative control (DMS)	-	-	-

Conclusion

In conclusion, the total polyphenols and flavonoids content of *Centranthus ruber* extracts correlated with its antioxidant and antibacterial properties. n-butanol extract demonstrated significant antioxidant and antibacterial activity. Phytochemical constituents of the most active extract n-butanol have been determined by HPLC, it was found that the extract is rich in phenolic acids and flavonoids. The high concentration of the Chlorogenic acid, Hesperidin and *P*-Coumaric acid may have a major role to play in the biological activities. Based on these findings, the plant *Centranthus ruber* which has traditionally been used in Italian cuisine, may serve as a potential source of antioxidant and antibacterial properties and as a safe alternative natural drug.

Conflict of interest

The authors declare that there is no conflict of interest.

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