



Comparison of Major Bioactive Components of Various Solvent Extracts from Algerian *Bunium incrassatum* Umbellules and Antioxidant Activity

Fethi TOUL^{1*} , Amina DJENDAR² 

¹ Mohammed Tahri University, Laboratory of Valorization of Plant Resources and Food Security in Semi-Arid Areas, Department of Biology, Bechar, 08000, Algeria

² Abu Bakr Belkaïd University, Department of Biology, Tlemcen, 13000, Algeria

Abstract: The present study was designed to examine the chemical composition and the antioxidant activity of *Bunium incrassatum* extracts prepared in five different solvents with increasing polarities. RP-HPLC analysis resulted in the identification of 12 phenolic compounds, including 8 phenolic acids (caffeic acid, ellagic acid, ferulic acid, gallic acid, rosmarinic acid, sinapic acid, syringic acid, and vanillic acid) and 4 flavonoids (catechin, hesperetin, luteolin, and quercetin). Two different test systems were used to assess the antioxidant activity: DPPH and β -carotene/linoleic acid assays. Methanol extract exhibited the highest DPPH scavenging potential and even low inhibitory effect against β -carotene bleaching, whereas chloroform extract showed the highest effect against β -carotene bleaching and a weak scavenging potential. As expected, methanol extract recorded the highest content of total phenolics (26.57 ± 0.15 mg GAE/g E), while chloroform extract has been found to be the richest in flavonoids.

Keywords: *Bunium incrassatum*, umbellules, antioxidant activity, phenolic compounds, RP-HPLC.

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***Corresponding author. E-mail:** toul.fethi@univ-bechar.dz

1. INTRODUCTION

For several years, numerous products in the pharmaceutical, cosmetic, and food industries have been the subject of growing concern, criticism, and even health scandals because of the harmful effects of synthetic and chemical compounds they contain (1). In addition, consumers are paying more attention to their lifestyle and environment by changing the way they consume (2). For these reasons, the quest for natural products in the plant kingdom, especially for phenolic compounds, for their physiological properties affecting human health, including antioxidant activity, is at its height (3).

Phenolics' family includes phenolic acids, flavonoids, anthocyanins, highly polymerized substances such as tannins, and other compounds. Flavonoids and phenolic acids are highly valued and used in the pharmaceutical and food industries due to their potent antimicrobial and antioxidant properties that benefit human health (4,5).

The genus *Bunium* includes seven species, four of which are endemic to the Algerian flora (6). *Bunium incrassatum* (Boiss.) Batt. & Trab., vernacularly called 'Talgouda', is widely spread and its tubers are used for the treatment of thyroid in Algeria, taken alone or in mixtures of two or more ingredients such as honey, olive oil, and goat milk (7). In addition, dried and powdered tubers are regarded as astringent, anti-diarrheic, anti-hemorrhoidal, and for bronchitis and cough treatment (8). In the collective memory of Algerian society, it is considered as a symbol of misery which recalls the famine of the years of poverty, especially during the Second World War and the period of national revolution between 1954-1962. It was consumed as bread after being powdered and mixed with wheat flour (9).

Despite the socio-economic importance and historical position of *B. incrassatum* within Algerian society, only a few studies have been conducted to investigate its biological activities: the effect of tubers, aerial parts, and seeds' extracts on biochemical, hematological, ovarian and uterine parameters (10,11), antimicrobial activity (8), antioxidant activity (12-14), and chemical composition of extracts (8,12,14) and essential oil (13,15) of tubers.

The present study was conducted to investigate, for the first time, the phenolic profile of the flowering heads of *B. incrassatum* by RP-HPLC, as well as the antioxidant activity of their extracts.

2. EXPERIMENTAL

2.1. Plant Material

The umbrella-shaped clusters (flowering heads) were collected in April 2016, from Djebala, Tlemcen, Algeria. Umbellules were separated from clusters after being identified at the laboratory of botany, Department of Biology, Faculty of Nature, Life, Earth,

and Universe Sciences, Abu Bakr Belkaid, University of Tlemcen, Algeria.



Figure 1: A flowering head of *B. incrassatum*.

2.2. Extraction

Twenty grams of freshly ground shade air-dried umbellules were sequentially extracted under reflux for 1 hour with increasing polarity solvents (chloroform, ethyl acetate, acetone, methanol, then water). The extracts were then concentrated to dryness, resolubilized in a few milliliters of their extraction solvents and stored at 4 °C for later use. The following solid residues were obtained, respectively: acetone extract (AcE): 330 mg, chloroform extract (CE): 390 mg, ethyl acetate extract (EAE): 531 mg, methanol extract (ME): 840 mg, and aqueous extract (AqE): 2.56 g.

2.3. Total Phenolic Content (TPC)

The estimation of total phenolics was carried out in triplicate according to the traditional method (16). Briefly, a 200 µL aliquot of extract was mixed with 1 mL of 10 times aqueous diluted Folin-Ciocalteu reagent and 0.8 mL of a 7.5% sodium carbonate solution. The absorbance was measured at 765 nm after 30 minutes of incubation. Gallic acid was used as a standard for the calibration curve. The total phenolic content was expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g E).

2.4. Total Flavonoid Content (TFC)

As described by Zhishen et al. (1999) (17), at room temperature, a volume of 500 µL of extract was mixed with 1500 µL of distilled water and 150 µL of 5% NaNO₂. After 5 min, 150 µL of AlCl₃ 10% were added. Six minutes later, 500 µL of NaOH (1 M) were added. After being thoroughly shaken, the absorbance of the mixture was immediately measured at 510 nm. The total flavonoid content of extracts was expressed as milligrams of catechin equivalents per gram of extract (mg CE/ g E).

2.5. Condensed Tannin Content (CTC)

Condensed tannins were analyzed by the conventional method of Julkunen-Titto. (1985) (18). An aliquot of 50 µL extract was added to 1500 µL of

a 4% (m/v) vanillin/methanol solution. After being stirred, 750 μ L of concentrated hydrochloric acid was added. The absorbance was measured at 550 nm after 20 minutes of incubation. Tannin content was expressed as milligrams of tannic acid equivalents per gram of extract (mg TAE/g E).

2.6. DPPH Radical Scavenging Assay

Fifty microliters of various concentrations of extracts were mixed with 1950 μ L of a 0.025 g/L DPPH methanolic solution. A negative control, in which the extract was replaced by methanol, was also prepared. After 30 minutes of dark incubation, the absorbance was measured at 515 nm against a methanol blank (19). The radical scavenging activity (RSA) was calculated as the percentage of DPPH discoloration using the following equation:

$$RSA(\%) = (A_C - A_S/A_C) \times 100$$

Where A_C is the control's absorbance and A_S is the absorbance of the tested extract. The 50% inhibitory concentrations (IC_{50}) were then computed using the regression equation of the $RSA(\%)=f(C)$ graph. BHA was used as the reference compound.

2.7. β -carotene Bleaching Assay

The antioxidant potential of extracts in apolar conditions was assessed using β -carotene-linoleate model system as described by Moure et al. (2000) (20). β -carotene (0.2 mg) was dissolved in 1 mL of chloroform and mixed with 20 μ L of purified linoleic acid and 200 mg of Tween 40 emulsifier. Chloroform was then evaporated, and the resulting mixture was immediately diluted in 100 mL of distilled water. Four milliliters of this reaction mixture were mixed with 200 μ L of various extract concentrations. After being stirred, the absorbance was immediately measured ($t=0$ min) at 470 nm, against a blank consisting of the emulsion without β -carotene. The capped tubes were placed in a water bath at 50 $^{\circ}$ C for 120 min. A negative control consisting of 200 μ L of the extract's solvent instead of extract was also prepared. The antioxidant activity (AA) was calculated using the following equation:

$$AA = \left[\frac{(A_{S120} - A_{C120})}{(A_{C0} - A_{C120})} \right] \times 100$$

Where A_{S120} is the sample absorbance at 120 min; A_{C120} is the negative control absorbance at 120 min; A_{C0} is the negative control absorbance at 0 min.

2.8. Hydrolysis of Extracts for HPLC Analysis

The selected extracts for HPLC analysis (the most potent ones) were hydrolyzed under reflux in a 1.2 M HCl water bath for 1 h (21). Hydrolyzed samples were then filtered through a 0.45 μ m syringe-driven filter prior to injection.

2.9. Chromatographic Identification of Phenolic Compounds by HPLC

A 20- μ L sample aliquot was analyzed using high performance liquid chromatography system (YL 9100 HPLC system, Korea). Reversed-phase chromatography was performed on a C18 column (250 \times 4.6 mm; 5 μ m). Data were monitored and analyzed using Clarity data-processing system. The mobile phase consisted of solvent A (water/formic acid 0.4%) and solvent B (acetonitrile). Solvents gradient was used as followed: 0–2 min, 1% B; 2–15 min, 7% B; 15–25 min, 20% B; 25–35 min, 40% B; 35–46 min, 100% B; 46–47 min, 100% B; 47–48 min, 1% B; 48–55 min, 1% B. The flow rate was 1.2 mL/min. UV-detection was performed at 280 nm. Phenolic compounds were identified according to their retention times as well as to their spectral matching with 18 reference standards (14).

2.10. Statistical Analysis

Except for the extraction and chromatographic analyses, all data were expressed as mean \pm standard derivation (SD) of three replicates and were statistically analyzed using Excel software.

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1. FTIR analyses of compounds 3a and 3b total phenolic, flavonoid, and tannin contents

The results of total phenolics, as shown in Table 1, of *Bunium incrassatum* umbellifera extracts estimated following the Folin-Ciocalteu method were, decreasingly, methanol (26.57 \pm 0.15 mg GAE/g E), ethyl acetate (18.08 \pm 0.21 mg GAE/g E), aqueous (17.46 \pm 0.43 mg GAE/g E), acetone (11.78 \pm 0.01 mg GAE/g E), and chloroform at the end with 11.77 \pm 0.09 mg GAE/g E. Differently, the flavonoids dosing results showed the highest content in chloroform extract (19.74 \pm 0.37 mg CE/g E), followed by ethyl acetate and methanol extracts. However, aqueous extract recorded the highest tannin content (12.18 \pm 0.02 mg TAE/g E), closely followed by methanol extract (12.03 \pm 0.02 mg TAE/g E).

3.1.2. Antioxidant activity

For the antioxidant activity assessment, two different model systems were used: the DPPH and β -carotene bleaching assays. The antioxidant potential of the different solvent extracts is inversely proportional to IC_{50} values. According to the results shown in Table 1, in the DPPH assay, methanol extract showed the highest scavenging potential (1.65 \pm 0.18 mg/mL), followed by ethyl acetate extract (1.75 \pm 0.13 mg/mL), and the remaining extracts' potentials decreased according to the following order: AqE>CE>AcE. However, the β -carotene bleaching assay indicated a totally different order as follows: CE>EAE>ME>AcE>AqE.

Table 1: Phenolic contents and antioxidant potentials of *B. incrassatum* extracts.

Extracts	Yields (%)	TPC ^B (mg GAE/g E)	TFC ^B (mg CE/g E)	CTC ^B (mg TAE/g E)	IC ₅₀ ^{B,C} DPPH (mg/mL)	IC ₅₀ ^{B,C} β-Carotene (mg/mL)
Aqueous	12.8	17.46±0.43	9.93±0.12	12.18±0.06	2.05±0.09	4.62±0.16
Methanol	4.2	26.57±0.15	13.79±0.24	12.03±0.02	1.65±0.18	3.09±0.03
Acetone	1.65	11.78±0.01	5.35±0.16	4.77±0.01	2.48±0.11	3.34±0.20
Chloroform	1.95	11.77±0.09	19.74±0.37	7.36±0.01	2.34±0.21	2.05±0.02
Ethyl acetate	2.65	18.08±0.21	16.65±0.15	3.12±0.02	1.75±0.13	2.13±0.10
BHA^A	/	/	/	/	0.09±0.00	0.24±0.02

^AReference compound; ^Bmean±SD (n=3); ^CConcentration that showed 50% of activity.

3.2. Discussion

Plant phenolics, as bioactive compounds with potent antioxidant properties and marked effects in the prevention of diseases associated with oxidative stress such as cancer, are the subject of growing interest. Due to their physicochemical properties, there is no universal extraction procedure suitable for the extraction of all plant polyphenols. It is generally known that the solubility of polyphenols depends on the chemical nature of the plant sample as well as the polarity of used solvents (22). No results treating the chemical composition or biological activities of the umbellules' extracts of *B. incrassatum* were found. All the existing ones were about tubers and aerial parts' extracts and essential oils.

The yield of chemical extraction is widely known to be dependent on the solvent's polarity, pH, extraction time, and temperature, as well as the chemical composition of the sample. The solvent and the chemical characteristics of the sample are the two most relevant parameters under the same extraction time and temperature. Previously, solvents such as methanol, ethanol, butanol, acetone, chloroform, and water were extensively utilized for polyphenols' extraction (14,23). The yield results of different solvent extracts of *B. incrassatum* showed the highest yield in aqueous extract, three and four times more than methanol and ethyl acetate extracts, respectively. These results are in accordance with those of Dehimi et al. (2020) (12), where aqueous extract of *B. incrassatum* tubers showed the highest yield (24.48%), much more than methanol and acetone extracts, and are in discordance with those of our previous study (14), in which methanol extract of *B. incrassatum* seeds showed two times more yield than aqueous and chloroform extracts. As can be seen, no relationship between yields and phenolic content was observed. An increase in extraction yield didn't lead to an increase in TPC, TFC, and/or CTC. Even with three and four times less yields, methanol and ethyl acetate extracts showed

higher contents of TPC than aqueous extract, which showed the highest CTC. In the same way, chloroform and ethyl acetate showed, respectively, the highest TFC. These results are in discordance with the previous study (14) where methanol extract of *B. incrassatum* seeds showed the highest extraction yield (7.1%), TPC (185.04±4.00 mg GAE/g E), and CTC (33.42±3.56 mg TAE/g E), and also with those reported by Dehimi et al. (2020) (12) on *B. incrassatum* tubers' extracts prepared in different polarity solvents where acetone was shown as the best solvent of total phenolics, flavonoids, and even tannins followed by methanol and water as the second and third best ones. Water frequently records the highest yields because when it is used as the extraction solvent, a large number of inorganic salts and polysaccharide impurities dissolve over and above amides, alcohols, and carboxylic acids (24). However, the variation in solvent ranking towards phenolic content (TPC, TFC, and CTC) might be attributed to the polarity of phenolic compounds in the different organs (umbellules, seeds, and tubers), as well as the environmental circumstances under which the plants grew.

The assessment of antioxidant activity through the DPPH assay showed that methanol extract was the most potent (IC₅₀=1.65±0.18 mg/mL), followed by ethyl acetate extract (1.75±0.13 mg/mL). These IC₅₀ are ten times weaker as compared to the reported results of Algerian *B. incrassatum* seeds' extracts (14) and even weaker than that of the used reference compound (BHA, 0.09±0.00 mg/mL), known as a strong antioxidant. The scavenging potential increased with the increasing concentrations in a dose-dependent manner and was much higher than those reported by Dehimi et al. (2020) (12) and even higher than four different species of *Bunium* from Turkey (22). It has been proven that the antioxidant activity of plant extracts is related to their phenolic content, suggesting a correlation between TPC and antioxidant activity (25). The chromatographic

analysis of methanol extract (Figure 2) revealed the presence of 06 phenolic compounds: four phenolic acids (gallic acid, syringic acid, ellagic acid, and sinapic acid), a flavone (luteolin), and a flavanol (quercetin). For ethyl acetate extract (Figure 4), seven compounds were identified: 06 phenolic acids (gallic acid, caffeic acid, rosmarinic acid, vanillic acid, syringic acid, and ferulic acid) and a flavanone (hesperetin). Gallic acid was identified in the two extracts but as the major absorbing peak, by far, in methanol chromatogram. Nickavar & Esbati (2012) reported that gallic acid is a strong DPPH scavenger (5.18 $\mu\text{g/mL}$) and a weak inhibitor of β -carotene bleaching (570 $\mu\text{g/mL}$) (26). It was also reported that the mass fraction solubility of gallic acid in methanol is 18 times higher than in water and 21 times higher than in ethyl acetate at 25°C, and increases smoothly with temperature (27). However, gallic acid is practically insoluble in chloroform. Ellagic acid and quercetin have been shown to be more effective DPPH scavengers than gallic acid (28). Koleva et al. (2003) have found that if rosmarinic acid and caffeic acid, which are strong antioxidants in polar conditions, were tested only by the β -carotene bleaching assay, they would be considered as weak antioxidants (29). In Figure 4, syringic acid, represented as the strongest absorbing compound's peak, showed in a previous study a strong radical scavenging activity, 1.53 times higher than that of ascorbic acid, in polar environments and a weak activity in lipidic medium (30). Otherwise, chloroform

extract, after being the second weaker extract in the methanolic medium of DPPH, exhibited the highest inhibitory potential against β -carotene bleaching, followed by ethyl acetate extract, which can be due to the low polarity of lipophilic compounds soluble in chloroform and highly effective in β -carotene-linoleic acid emulsion system than in methanolic DPPH medium. The chromatogram of chloroform extract (Figure 3) shows the presence of 06 phenolic compounds: three phenolic acids (gallic acid, caffeic acid, and sinapic acid), a flavone (luteolin), a flavanol (catechin), and a flavanone (hesperetin). Two studies reported that chloroform was useful for the extraction of flavonoids, flavonols and phenolic acids (31,32). As for other species, luteolin, quercetin, and syringic acid had been identified in the aerial parts (including flowers) of *Bunium brachyactis*, *B. microcarpum*, and *B. pinnatifolium*, respectively (33). Luteolin, as the main absorbing compound's peak in chloroform extract, is known to be soluble in organic solvents and sparingly soluble in polar ones (34). Von Gadow et al. (1997) reported that in equimolar concentrations, luteolin was slightly more potent than BHA, 33% more potent than vanillic acid, 37% more potent than ferulic acid, and 61% more potent than syringic acid in the β -carotene-linoleic acid emulsion system (35). Sinapic acid has also been shown to have a strong inhibitory effect against β -carotene bleaching and a low DPPH scavenging potential (28).

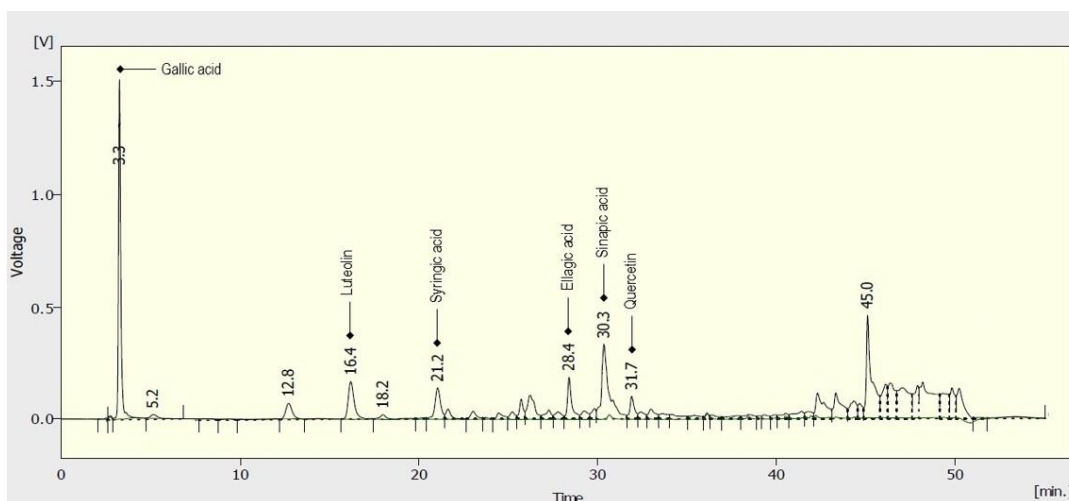


Figure 2: HPLC chromatogram of methanol extract.

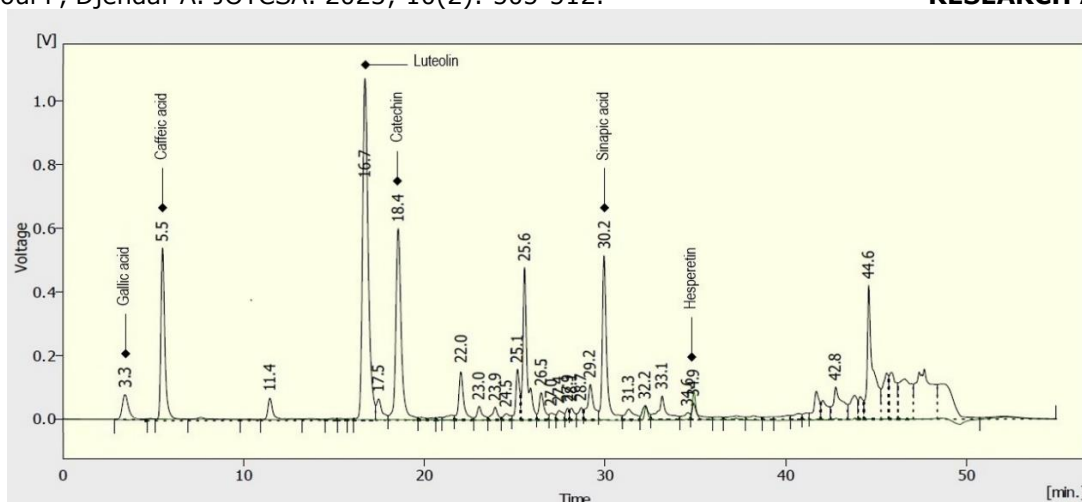


Figure 3: HPLC chromatogram of chloroform extract.

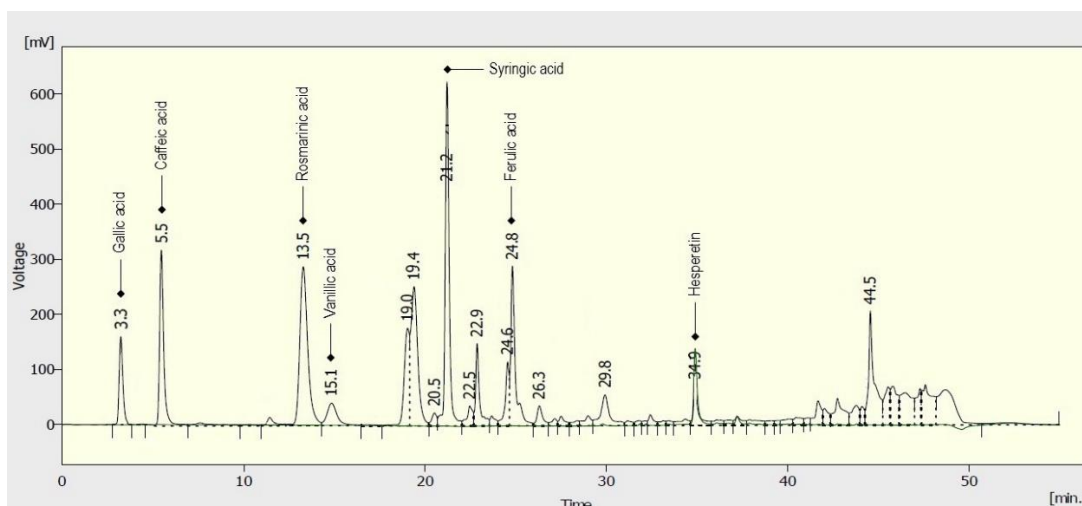


Figure 4: HPLC chromatogram of ethyl acetate extract.

4. CONCLUSION

The present study reports for the first time the quantitative profiles of different solvent extracts of *B. incrassatum* umbellules: TPC, TFC, CTC and the qualitative profiles of phenolics contained in methanol, chloroform, and ethyl acetate extracts due to their high antioxidant potentials. These results support the previous studies showing that the genus *Bunium* is considered as a good source of bioactive compounds that requires further investigation.

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