JOTCS A
Journal of the Turkish Chemical Society
Section: A
Chemistry
TURKISH CHEMICAL SOCIETY

Algerian *Bunium incrassatum* Seeds: Effects of Extraction Solvent Polarity on Phenolic Profile and Antioxidant Activity

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Abstract: *Bunium incrassatum*, commonly called 'Talghouda', is one of the most important plant species in the traditional Algerian pharmacopoeia, used for medicinal and culinary purposes. Despite its benefits, it still remains a scientifically neglected species, particularly in terms of its phenolic profile. The current study sought to evaluate the influence of the variation in solvents' polarities on the phenolic profile of *B.incrassatum* seeds by conventional spectrophotometric techniques and also by high-performance liquid chromatography, as well as their effects on the antioxidant activity of extracts using DPPH and β-carotene bleaching assays. Methanol extract showed the highest DPPH scavenging ability and also the highest inhibitory potential against the bleaching of β-carotene (IC_{50}=0.15±0.02 and 0.41±0.03 mg/mL, respectively). Thirteen phenolic compounds were identified, a flavanol (catechin), two flavonols (kaempferol, quercetin), two flavanones (hesperetin, naringenin) and eight phenolic acids: caffeic acid, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, p-coumaric acid, sinapic acid, and syringic acid. These results support the few previous studies showing that *Bunium incrassatum*, as an endemic species, is a valuable source of bioactive compounds that requires further investigations.

Keywords: *Bunium incrassatum*, seeds, phenolic compounds, antioxidant potential, RP-HPLC.

Submitted: January 15, 2022. **Accepted:** March 03, 2022 .

Cite this: Toul F, Djendar A, Seladji M, Berroukeche F. Algerian Bunium incrassatum Seeds: Effects of Extraction Solvent Polarity on Phenolic Profile and Antioxidant Activity. JOTCSA. 2022;9(2):415–22.

DOI: https://doi.org/10.18596/jotcsa.1058060.

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INTRODUCTION

The food industry, like many other industries, relies on synthetic additives, which are now one of the most widespread means of food preservation due to their availability and price (1). Recently, there has been an increasing interest in plants' natural products from the public and food manufacturers, especially phenolic compounds, for their physiological properties affecting human health, including antioxidant activity (2).

Algerian flora appears to be extremely rich in medicinal and aromatic plants, providing an intriguing field for supplementary ethno-medicinal and phytochemical research (3). *Bunium incrassatum* (Boiss.) Batt. & Trab., vernacularly called 'Talghouda', is an endemic species of northern Algeria belonging to the Apiaceae family (4), frequently mentioned in the Algerian traditional pharmacopoeia. The tubers are used for the treatment of thyroid problems, either taken alone or in mixtures of two or more ingredients, such as honey, olive oil, and goat milk (5). Sharif

Al-Idrissi (1100-1175 AD) mentioned that Berbers took 10 g of its powdered tubers in a mixture with the water of cooked *Tribulus terrestris* on an empty stomach to disintegrate kidney stones and also as anthelmintic (6). In addition, dried and powdered tubers are regarded as astringent, anti-diarrheic, anti-hemorrhoidal, for bronchitis, and cough treatment (7). In the collective memory of Algerian society, it is considered as the 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, when it was consumed as bread after being powdered and mixed with wheat flour (8).

Despite the importance of *B.incrassatum* in folk medicine and its historical position within Algerian society, only a few studies have been conducted, only on tubers, to investigate its biological activities: the effect of tubers' extracts on biochemical, hematological, ovarian, and uterine parameters (3, 9); antimicrobial activity (7); antioxidant activity (10, 11); chemical composition of extracts (7, 11) and essential oil (10, 12).

The current study attempted to evaluate, for the first time to our knowledge, the effects of solvents' polarities on the chemical composition, especially the phenolic profile, of extracts derived from the seeds of *Bunium incrassatum*, as well as their impacts on the antioxidant activity.

EXPERIMENTAL SECTION

Plant material

The seeds of *Bunium incrassatum* were collected in June 2016, in Djebala, Tlemcen, Algeria. Species identification was carried out at the laboratory of botany, Department of Biology, Faculty of Nature and Life Sciences, Earth and Universe Sciences, Abu Bakr Belkaid University of Tlemcen, Algeria.

Preparation of extracts

Twenty grams of shade air-dried seeds, freshly ground, were sequentially extracted under reflux for 1 h with increasing polarity solvents (Petroleum ether, Chloroform, Ethyl acetate, Methanol, and Water). The extracts were then concentrated to dryness.

Total Phenolic Content

The amounts of total phenolic compounds in methanolic extracts were determined by Folin-Ciocalteu reagent assay (13). Gallic acid was used as standard for the calibration curve. The total phenolic content (TPC) was expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g E).

Total Flavonoid Content

As described by Zhishen et al. (1999)(14), the total flavonoid contents (TFC) of extracts were expressed as milligrams of catechin equivalents per gram of extract (mg CE/g E).

Condensed Tannin Content

Using vanillin assay method (15), the concentrations of tannins were expressed as milligrams of tannic acid equivalents per gram of extract (mg CE/g E) from a calibration curve.

DPPH radical scavenging assay

Fifty microliters of various concentrations of extracts were mixed with 1950 µL of a 0.025 g/L methanolic DPPH solution. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance was measured at 515 nm (16). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the following equation:

Where A_{blank} is the absorbance negative control and A_{sample} is the absorbance of the tested compound. The concentration providing 50% of inhibition (IC50) was calculated from the graph plotted of inhibition percentages against extract concentrations. BHA was used as the reference compound.

β-carotene bleaching assay

The antioxidant activity of extracts was evaluated using β-carotene-linoleate model system, as described by Moure et al. (2000) (17). β-carotene (0.2 mg) was dissolved in 1 mL of chloroform then mixed with 20 µL of purified linoleic acid and 200 mg of Tween 40 emulsifier. Chloroform was then evaporated under vacuum evaporator and the resulting mixture was immediately diluted in 100 mL of distilled water. 4 mL of this reaction mixture were mixed with 0.2 mL of various extracts concentrations or positive control (BHA). After being homogenized, the absorbance at 470 nm was immediately recorded at t=0 min, against a blank consisting of the emulsion without β-carotene. The capped tubes were placed in a water bath at 50 °C for 120 min. A negative control consisted of 0.2 ml distilled water or solvent instead of extract. The antioxidant activity (AA) was calculated to the following equation:

Where $A_{A(120)}$ is the absorbance of the antioxidant at 120 min; $A_{C(120)}$ is the absorbance of the negative control at 120 min; $A_{C(0)}$ is the absorbance of the negative control at 0 min.

Hydrolysis for HPLC

For HPLC analysis, the selected extracts were hydrolyzed under reflux in a 1.2 M HCl water bath for 1 h. Hydrolyzed samples were then filtered through a 0.45 µm syringe-driven filter before injection (18).

Chromatographic identification of phenolic compounds by RP-HPLC

A 20 µL of an aliquot of sample solution was separated using high performance liquid chromatography system (YL 9100 HPLC system, Korea). Reversed-phase chromatography was performed on a C18 column (250 × 4.6mm; 5µm). 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.

Statistical analysis

Except yields, all data were expressed as mean ± standard derivation (SD) of three replicates and were statistically analyzed using one-way ANOVA followed by Tukey test ($p<0.05$) on a SigmaPlot 12.2 software.

RESULTS

Total phenolic, flavonoid, and tannin contents

The highest content of TPC, as shown in Table 1, was recorded in methanol extract (185.04±4.00 mg GAE/g E) followed by petroleum ether and aqueous extracts (141.65±1.92 and 101.20±3.64 mg GAE/g E, respectively). Ethyl acetate extract showed the highest amount of TFC (89.26±3.13 mg CE/g E) followed by methanol and chloroform extracts (72.07±3.80 and 56.65±1.72 mg CE/g E). Methanol extract and among all extracts exhibited the highest content in CTC (33.42±3.56 mg TAE/g E).

Antioxidant activity

The antioxidant potential of extracts was assessed following two different assays in two different media. In the DPPH assay, all extracts exhibited significant radical scavenging abilities in comparison to the reference compound (BHA, 0.09±0.00 mg/mL), where methanol extract showed, as the most potent, an IC_{50} equal to 0.15±0.02 mg/mL, followed by petroleum ether extract with 0.21±0.03 mg/mL. The remaining extracts were between aqueous and chloroform extracts (0.29±0.02 and 0.48±0.03 mg/mL, respectively).

The methanolic extract also exhibited the highest inhibitory effect against the bleaching of β-carotene (0.41±0.03 mg/mL) which was 80% more potent than ethyl acetate extract as the weakest one (1.60±0.09 mg/mL). Even stronger, methanol extract remains 70% less potent as compared to the reference compound (BHA, 0.24±0.00 mg/mL).

Chromatographic identification of phenolic compounds by RP-HPLC

Based on three parameters (extraction yields, phenolic content, and antioxidant potential), two extracts have been selected for the qualitative profiling of phenolic compounds by RP-HPLC (methanol and petroleum ether extracts). These two extracts have been hydrolyzed in an acidic medium before being injected to free the bound phenolic compounds. The obtained chromatograms, represented in Figures 1-2, compared to those of 18 reference standards, showed several main peaks with intense absorption at the selected wavelength (280 nm). Analyzing these chromatograms, thirteen phenolic compounds were identified: six in methanol extract (five phenolic acids and a flavanone) and seven in petroleum ether (three phenolic acids, two flavonols, a flavanol and a flavanone).

Table 1: Phenolic contents and antioxidant activities of *Bunium incrassatum* seeds' extracts.

Extracts	**Yields (%)**	**TPC[B] (mg GAE/g E)**	**TFC[B] (mg CE/g E)**	**CTC[B] (mg TAE/g E)**	**IC50[B,C] DPPH (mg/mL)**	**IC50[B,C] β-Carotene (mg/mL)**
Aqueous extract	3.0	101.20±3.64[a]	46.61±0.91[a]	30.24±1.83[a]	0.29±0.02[a]	0.87±0.01[a]
Methanol extract	7.1	185.04±4.00[b]	72.07±3.80[b]	33.42±3.56[b]	0.15±0.02[b]	0.41±0.03[b]
Chloroform extract	2.9	71.25±2.01[c]	56.65±1.72[b]	16.34±0.66[c]	0.48±0.03[c]	1.13±0.22[c]
Ethyl acetate extract	2.2	95.51±4.34[d]	89.26±3.13[c]	5.28±0.09[d]	0.34±0.01[d]	1.60±0.09[d]
Petroleum ether extract	8.5	141.65±1.92[e]	55.33±2.28[d]	8.16±00.37[e]	0.21±0.03[e]	0.54±0.01[d]
BHA[A]	/	/	/	/	0.09±0.00	0.24±0.02

[A]Reference compound; [B]mean±SD (n=3) by one-way ANOVA (Tukey test, $p<0.05$); [C]Concentration that showed 50% activity. TPC, total phenolic content; TFC, total flavonoid content; CTC, condensed tannin content; BHA, butylated hydroxyanisole.

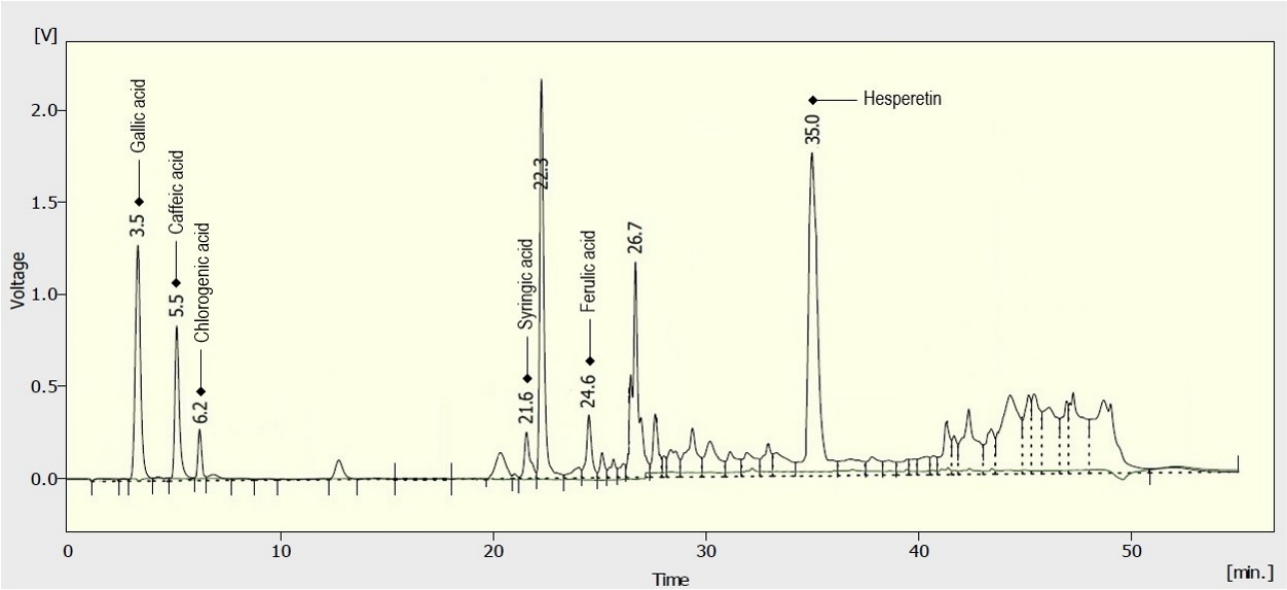

Figure 1: HPLC chromatogram of methanol extract of *Bunium incrassatum* seeds'.

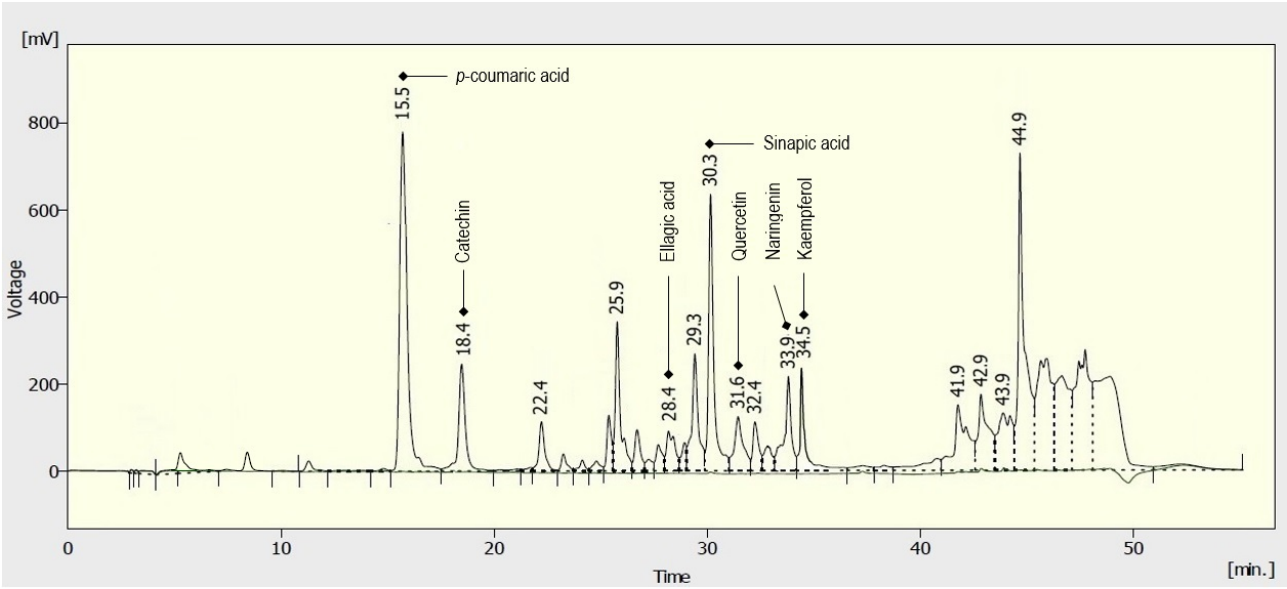

Figure 2: HPLC chromatogram of petroleum ether extract of *Bunium incrassatum* seeds'.

DISCUSSION

Phenolics are undoubtedly the most abundant secondary metabolites of plants. Even abundant, their extraction turns out to be difficult, due to their large chemical variability. There is no universal extraction procedure suitable for the extraction of all plant phenolics (19). It is generally known that the solubility of polyphenols depends on the chemical nature of the plant sample, as well as on the polarity of used solvents (20, 21) and mostly on the extraction protocol conditions' (extraction time, temperature, and pH)(22). All previous studies have reported only on the tubers' chemical composition and the antioxidant activity of their extracts. As far as we know, the present study is the first to investigate the phenolic profile and antioxidant activity of different solvent extracts of *Bunium incrassatum* seeds.

The results of yields and phenolic contents showed a large variability between extracts. Firstly, petroleum ether extract, followed by methanol, had the highest extraction yields, respectively. Secondly, methanol showed the highest TPC content, followed by petroleum ether. Thirdly,

ethyl acetate was the best solvent, recording the highest TFC content, and finally, methanol showed the highest CTC content, followed by the aqueous extract. These results strongly disagree with those reported by Adelifar et al. (2021) (23) where methanol extracts of different organs, including seeds prepared from four different *Bunium* species (*B.cylindricum, B.paucifolium, B.persicum, and B.wolffii*) showed much lower contents of TPC, TFC, and TTC (total tannin content). Dehimi et al. (2020) (11) reported even lower phenolic contents in different solvent extracts of *B.incrassatum* tubers. These noted differences may be due to the variability in phenolic compounds between species of the same genus or organs of the same species, and even due to environmental conditions in which the plants have grown (24).

Regarding antioxidant activity, it is well known that the measurement of antioxidant potentials of plants' extracts must be conducted using tests based on different mechanisms since each assay can reflect only the chemical reactivity under the specific conditions applied to it. In both selected assays, DPPH scavenging potential and β-carotene bleaching assay, all tested extracts, compared to the reference compound, showed good to moderate potentials, which increased with the increasing concentrations of phenolics in a dose-dependent manner and were much higher than those reported by Dehimi et al. (2020) (11) and even higher than those reported by Zengin et al. (2019) (25) and Adelifar et al. (2021) (23), in which four different species of *Bunium* from Turkey showed less potent effects. The highest antioxidant activity shown by methanol and petroleum ether extracts may be due to a synergistic effect of the compounds they contain. It has also been proven that the antioxidant activity of plant extracts is related to their phenolic content, suggesting a correlation between TPC and antioxidant activity (26).

With regards to the individual phytocompounds, 13 phenolic compounds were identified in both extracts. On the one hand, gallic acid, caffeic acid, chlorogenic acid, syringic acid, ferulic acid, and hesperetin were present in methanol extract. All these compounds were previously found to possess antioxidant properties. Hajimehdipoor et al. (2014) highlighted the synergistic antioxidant effect of several phenolic acids' combinations, where the binary combination of gallic acid and caffeic acid enhanced their antioxidant potential (137%), followed by gallic acid and chlorogenic acid (28%); while the ternary combination of gallic acid, caffeic acid, and chlorogenic acid showed a lower synergistic effect (25.7%)(27). Another study reported that antioxidant activity showed an extremely significant positive correlation with TPC, TFC, ferulic acid, caffeic acid, and chlorogenic acid (28). In addition to the intense absorption of the main peaks as recorded for Hesperetin, which is known as a strongly antioxidant flavonoid (29), synergy may explain why methanol extract was the most potent one. On the other hand, the chemical analysis of petroleum ether extract, as the second potent extract, showed the presence of ellagic acid, *p*-coumaric acid, sinapic acid, catechin, quercetin, kaempferol, and naringenin. The presence of these phenolic compounds in such nonpolar solvent can be explained by the fact that they are sparingly soluble in high polarity solvents such as water and highly soluble in medium and low polarity solvents (30). In addition, it was reported that these compounds possess various biological and pharmacological properties, such as anticarcinogenic effects, superoxide scavenging, metal chelating, and antioxidant activities, and could be used for several applications in pharmacy (31, 32). *P*-coumaric acid is a hydroxycinnamic acid found in fruits. Its presence correlated significantly with antioxidant activity, unlike sinapic acid, which showed a negative correlation (28). Ackland et al. (2005) and Campbell et al. (2006) reported, respectively, that the binary combination of quercetin and kaempferol as well as the ternary combination of quercetin, kaempferol, and naringenin inhibited cancer cell proliferation in a dose dependent manner without cytotoxicity (33, 34). Another study reported that the combination of ellagic acid and quercetin synergistically induces cell apoptosis and suppresses proliferation in leukemia cells better than each separately (35). This may explain the high antioxidant efficiency of bioactive compounds that petroleum ether extract contains as compared to the reference compound (BHA), which is a strong synthetic antioxidant. But as usual for synthetic additives, BHA has been accepted as possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC) due to the toxicological data on its mechanism of action (36). It should also be noted that all identified compounds have been reported previously in several studies performed on different species of *Bunium* (25, 37, 38), but none of them was about the seeds of *B.incrassatum*.

CONCLUSION

The present study provides novel information about the phytochemical composition of *B.incrassatum* seeds as well as the interesting antioxidant activity of their extracts, which has never been investigated before. The obtained results suggest that the antioxidant activity of the studied extracts can be associated with the presence and synergistic effects of combinations of phenolic compounds, especially phenolic acids and flavonoids. Thus, this plant could be considered as an excellent source of bioactive compounds, especially polyphenols with considerable

antioxidant activity, which could represent an important option for food, cosmetics, and pharmaceutical industries to be supplied with natural antioxidants as an alternative to synthetic additives.

CONFLICT OF INTEREST

Authors state no conflict of interest.

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