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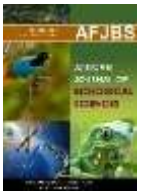

Bunium mauritanicum tubers: Phytochemical Analysis, Anti-microbial and proteinase K inhibitors properties

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Abstract

This study explores the phytochemical composition and therapeutic potential of *Bunium mauritanicum* tubers, with a focus on their Antimicrobial and proteinase K inhibitors properties. Quantitative analysis confirmed higher polyphenol and flavonoid contents in methanolic extract (0.509 ± 0.141 mg of GAEq/g of extract and 0.097 ± 0.030 mg QEq/g of extract) compared to aqueous extract (0.352 ± 0.112 mg of GAEq/g of extract and 0.078 ± 0.023 mg QEq/g of extract).In antimicrobial activity, methanolic extract demonstrated bactericidal effects against *E. coli*, *K. pneumoniae*, and *S. aureus*, with MBC/MIC ratios < 4. However, it displayed moderate activity against *Candida albicans* (MBC/MIC ratio= 1). The proteinase K inhibitor assay showed a significant decrease in enzymatic activity, potentially reducing the risk of heart disease, stroke, and cancer.This study highlights the multifaceted therapeutic potential of *B. mauritanicum* tubers as a source of bioactive compounds with promising health implications.

Keywords: *Bunium mauritanicum*, phytochemicals, Antimicrobial activity, proteinase K inhibitors.

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INTRODUCTION

Medicinal plants have been utilized for their curative properties since ancient times (Siddiqui et al, 2020). Nowadays, many people are oriented towards "green therapy" and the use of natural compounds from plants that are found to be free from side effects and less toxic than commercial drugs (Barteková et al, 2021). According to estimates provided by the World Health Organization, more than 80% of people worldwide currently use herbal medicines in some capacity to provide primary medical care (Ciampi et al, 2020). The beneficial effects of medicinal plants have mainly been attributed to several compounds, such as flavonoids and phenolic acids (Hemmami et al, 2023). These compounds have a variety of beneficial effects on human health (Siddiqui et al, 2020). Previous studies have reported that the consumption of plants, particularly phenolic compounds, has been used for the treatment and prevention of a wide spectrum of chronic diseases, including hypertension and hyperglycemia (El Hachlafi et al, 2020). For this reason, several scientists have conducted extensive studies using different plant extracts to evaluate the antioxidant, antibacterial, anti-inflammatory, and many other medicinal properties of these extracts (Zekeya et al, 2022).

The mid-sized genus Bunium L. from the family Apiaceae, which includes 53 species, grows in dry or semi-dry environments in Asia, Europe, and North Africa. Bunium plants are a type of geophyte with tubers, divided leaves, and white flowers with bent tips. They have calyces without teeth and white petals with inflexed terminal lobes. Their fruits are not significantly compressed dorsally or laterally, featuring keeled or filiform ribs, no special lignified elements in the mesocarp, and flattened endosperm on the commissural side. The genus Bunium is relatively uniform in several morphological characters (Karouche et al, 2022; Nouir et al, 2023).

One such species, *Bunium mauritanicum*, is native to the Mediterranean region, including Turkey and North Africa (Yabesh et al, 2014). Locally, it is known as "Talghouda" or "Terghouda" in Algeria and "Zraq Er'rar" in Morocco. The medicinal properties of *B. mauritanicum* tubers have been esteemed for centuries in medicine due to their effectiveness in treating conditions such as diabetes, inflammation, and respiratory infections (Yabesh et al, 2014). Despite its widespread use in traditional medicine, there is a scarcity of scientific information on the phytochemical composition and therapeutic potential of *B. mauritanicum* tubers.

In vitro assessment of various biological properties of aqueous and methanolic extracts from the Algerian variety of *B. mauritanicum* has not been documented. Furthermore, the existing literature provides limited information on the distribution of bioactive compounds within its tubers' aqueous and methanolic extracts. Consequently, this study aimed to evaluate the biological potential of plants growing in Algeria through in vitro assays. The study could also help to validate the traditional uses of *B. mauritanicum* and promote its use as a safe and effective herbal medicine.

MATERIALS AND METHODS

Chemicals

Prolabo (USA) supplied aluminum chloride ($AlCl_3$), sodium carbonate (Na_2CO_3), Biochemchemopharma Co. (Cosne-Courssur-Loire, France) supplied Folin–Ciocalteu reagent. Methanol (99.7% GC) and organic solvents were purchased from Sigma-Aldrich (Burlington, MA, USA), Casein agar (HiMedia, India), Proteinase K (Servicebio®, China). All the medium of microbiology assay such as Muller-Hinton broth, Muller Hinton agar, Sabouraud dextrose broth, and Sabouraud dextrose agar from Institute of Pastor, Alger's, Algeria.

Plant material collection

The tubers of *Bunium mauritanicum*were collected from the Batna region (East Algerian) between November 2022 and January 2023. The tubers were dried under shade and ground into a fine powder using a laboratory mill.The plant was washed well and parched at roomtemperature for 20 days, in conditions away from moisture, light dust, and dirt, with adequate ventilation. After drying, it wascrushed, and the powder was stored in a Dark colored glass bowlcontainer.

Sources of microorganism strains

The antimicrobial activity of *B. mauritanicum* tubers was evaluated using laboratory reference strains (American Type Culture Collection ''ATCC'' for bacteria and *Candida albicans,* National Museum of Natural History ''NMHN'' for filamentous fungi), obtained from Institute of Pastor Algeria, Alger's: Gram-positive bacteria: *Staphylococcus aureus* ATCC 25932, Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Klebsiellapneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231.

Preparation of the extracts of plant material

The phytochemical study and biological tests were conducted using the methanolic and aqueous extracts. The methanolic extraction process involved immersing 50 g of *B. mauritanicum* tubers in 500 mL of methanol for 24 hours. For the aqueous extract, 50 g of dried tubers was placed in 500 mL of water for 24 hours. Subsequently, all the extracts underwent additional filtration through Whatman filter paper grade 1. The filtered extract was then concentrated using a rotavapor (Buchi R-200, Switzerland) at a temperature of 55°C (Chouikh et al, 2015).

The yield of the extracts was determined using the following formula (Ben Ali et al, 2023):

$$\text{Yield (\%)} = (W_1 / W_2) \times 100.$$

where:

W_1 = weight of the extract dried (g).

W_2 = weight of the plant starting material (g).

Phytochemical Study

Determination of total polyphenols (TPC)

For TPC determination, a method employing Folin–Ciocalteu reagent (FC) was utilized (Singleton and Rossi, 1965) with some modifications. A 0.2 mL of the sample was combined with 1 mL of 10% Folin-Ciocalteau reagent and allowed to incubate for 5 min. Subsequently, 0.8 mL of a 7.5% Na_2CO_3 solution was introduced into the mixture and left to incubate for a span of 40 min in the obscurity of ambient temperature. The measurement of the absorbance of the mixture was done at 765 nm, and the quantification of total phenolic content was determined in terms of the comparable measure of gallic acid, expressed in micrograms per milligram of extract.

Determination of total flavonoids (TFC)

To determine TFC, the aluminum chloride colorimetric method with some modifications (Chouikh et al, 2020), In this approach, 0.5 mL of the extracts was mixed with 0.5 mL of 2% aluminum chloride ($AlCl_3$), and the mixture was incubated at room temperature for 5 minutes. The absorbance was measured at 420nm using spectrophotometry. The quantification total flavonoid content (TFC) was expressed in micrograms of quercetin equivalents per milligram of dry extract of *B. mauritanicum*.

Antimicrobial activity

The broth microdilution assay is a standardized method for determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts against a variety of bacteria and yeast strains. The assay is performed in accordance with the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI) (PA, 2002; Qaiyumi, 2007; Wayne, 2011).

First, the bacterial and yeast suspensions are prepared. For bacteria, the strains are cultured on Mueller-Hinton agar (MHA) and then inoculated into cation-adjusted Mueller-Hinton broth (MHB). The cultures are incubated until visibly turbid and then diluted to a turbidity corresponding to 0.5 McFarland (1.5×10^8 CFU/mL) using BioMerieux *DensiCHEK Plus* for *VITEK* 2 Systems. For yeast, the strain is cultured on Sabouraud dextrose broth (SDB) and then diluted to a concentration of 10^6 CFU/mL.

Next, the plant extract solution is prepared by dissolving the extract in dimethyl sulfoxide (DMSO) to a concentration of 80 mg/mL. The solution is then homogenized by vortexing for 1 minute.

The microtiter plate is set up by adding 50 µL of the plant extract solution to each well, the highest concentration incorporated into the plate is 40 mg/ml and the lowest achieved through double serial dilution is 1.25 mg/ml. Then, 50 µL of the bacterial or yeast suspension is added to each well. A growth control (no antibiotic, no xenobiotic) and a sterile control (MHB only) are included for all isolates(Schwalbe et al, 2007).

The microtiter plate is incubated at 37°C for 18-24 hours for bacteria and 48 hours for yeast. After incubation, the MIC is determined as the lowest concentration of plant extract that inhibits the growth of the bacteria or yeast. The MBC is determined by plating 10 µL from each well displaying no visible bacterial growth onto Sabouraud dextrose agar for yeast and MHA for bacteria. The extract with the lowest concentration that exhibits no bacterial growth (with 99% precision) is reported as the MBC concentration (Wayne, 2011).

After incubation for 24 h at 37 °C, resazurin (0.020 %) was added to all wells (20 µl per well), and further incubated for 72 min for the observation of colour change. On completion of the incubation, columns with no colour change (blue/purple) were scored as above the MIC value of plant extract that inhibits the growth of the bacteria or yeast (Cirillo, 1962) with some modification. The MBC is determined by plating 10 µL from each well displaying no visible

bacterial growth onto Sabouraud dextrose agar for yeast and MHA for bacteria. The extract with the lowest concentration that exhibits no bacterial growth (with 99% precision) is reported as the MBC concentration (Wayne, 2011).

Casein plate method for a quantitative analysis of Proteinase K activity

This study evaluates the effect of plant extracts on Proteinase K activity using a casein hydrolysis assay. A HiMedia® M763-500G casein plate was prepared by drilling three parallel holes. The first hole received 5µL of Proteinase K solution (Servicebio®, final concentration 1000 IU/mL) diluted 50% (v/v) in DMSO. The second and third holes received 50% (v/v) mixtures of Proteinase K and the 20 mg/mL aqueous extract and methanolic extract of plant extracts. After a 10-minute, the casein plate was subjected to 18 hours of incubation at 37 °C, the diameter of the hydrolysis ring surrounding each hole was measured on three replicate plates. The obtained values hydrolysis area (πr^2) was calculated then compared with the control (Proteinase K only) to confirm the presence of a stimulatory or inhibitory effect of the tested extracts on the proteinase K enzyme (Zhang et al, 2021).

Statistical analysis

All assays were performed in triplicate and results are expressed as mean ± SEM. *In vitro* assay data were analyzed using GraphPad Prism 8.0.2 software. The significance level was set at $\alpha = 0.05$ and the threshold for significance was defined as $p < 0.05$. P-values are displayed in figures as follows: ***, $p < 0.001$; **, $p < 0.01$; and ns, $p > 0.05$.

RESULTS AND DISCUSSION

Phytochemical Study

Total polyphenol and flavonoids contents

The table 1 shows the yield and polyphenol and flavonoids contents of aqueous and methanolic extracts of *B. mauritanicum* tubers. The yield percentage indicates the amount of extract obtained from the plant material. In this case, aqueous extract has a slightly higher yield (15.08%) compared to the methanolic extract (12.23%) , suggesting that methanol is more efficient in extracting compounds from the tubers because it was the richest one in polyphenols and flavonoids.

Moving on to the content of polyphenols and flavonoids, the results (Table 1) indicate that the methanolic extract has a higher concentration of both polyphenols (0.509 ± 0.141 mg of GAEq/g of extract) and flavonoids (0.097 ± 0.030 mg QEq/g of extract) compared to the aqueous extract (polyphenols: 0.352 ± 0.112 mg of GAEq/g of extract; flavonoids: 0.078 ± 0.023 mg QEq/g of extract). Moreover, (Karouche et al, 2020) observed pronounced variations in the polyphenols, flavonoids, contents in *B. mauritanicum* tubers, reported even higher concentrations of total phenols and flavonoids in the methanolic extract, emphasizing the efficiency of methanol as a solvent for extracting these compounds. The concentration of phenolic compounds in both extracts in the current study appears to be relatively high, in the following order: aqueous extract < methanolic extract. The further intention of determining TPC and TFC is to investigate the correlation between these secondary metabolites and possible biological activities, and to evaluate *B. mauritanicum* as a potential source of natural bioactive chemicals, and these results suggesting that *B. mauritanicum* tubers could be a promising source of phenols and flavonoids with potential health benefits.

Table 1. Yield, Polyphenols and flavonoids content in *B. mauritanicum* tubers extracts

Extract	**Yield (%)**	**Polyphenols** (mg of GAEq/g of extract)	**Flavonoïds** (mg QEq/g of extract)
Aqueous extract	15.08%	0.352 ± 0.112	0.078 ± 0.023
Methanolic extract	12.23%	0.509 ± 0.141	0.097 ± 0.030

Antimicrobial activity

Comparative analysis of the antimicrobial activity of aqueous and methanolic extracts of *B. mauritanicum* tubers against different bacterial and fungal strains using precise scientific terminology and texts quoted from previous studies to interpret the results of the (Table 2 and Figure 1, 2):

themethanolic extract of *B. mauritanicum* tubers had higher antimicrobial activity than the aqueous extract against all bacterial and fungal strains tested. The MIC and MBC values of the methanolic extract were lower than those of the aqueous extract for all the strains tested, except for *Candida albicans* (Table 2).

An MBC/MIC ratio of ≥4 is generally considered to indicate a bactericidal compound, whereas an MBC/MIC ratio of >4 is generally considered to indicate a bacteriostatic compound (Lushniak, 2014; Suzuki et al, 1953). Based on MBC/MIC ratios, the methanolic extract of *B. mauritanicum* tubers showed bactericidal activity against *Escherichia coli*, *Klebsiellapneumoniae*, *Staphylococcus aureus*, and *Candida albicans*. However, the MBC/MIC ratio of the methanolic extract against *Pseudomonas aeruginosa* was not available, so it is not possible to determine whether it is bactericidal or bactericidal against this strain since the antibacterial effect of the extract requires a very high concentration exceeding 80 mg/ml.

The results of this study are consistent with those of previous studies showing that the methanolic extract of *B. mauritanicum* has antimicrobial activity against a variety of bacterial strains. For example, a study by Zengin et al. found that methanolic extracts of four species of *Bunium*, including *B. sayai* and *B. pinnatifolium* and *B. brachyactis* and *B. macrocarpum*, had high antimicrobial activity against *P. mirabilis* and *E. coli*, with MIC values less than 1 mg. ml^{-1} (Zengin et al, 2019).

However, the results of this study differ from those of a previous study that evaluated the antibacterial activity of a methanolic extract of *B. mauritanicum* tubers using the disc diffusion method. The previous study found that the extract had moderate activity against *S. aureus*and *E. coli*, with inhibition diameters of 13.64 mm, 11.84 mm, and 9.09 mm for *S. aureus* and 10.86 mm for *E. coli* (Fadia and Khawla, 2023). The difference in the results between these two studies may be due to the different methods used to evaluate antimicrobial activity. The broth microdilution test used in this study is more sensitive than the disk diffusion method used in a previous study. Overall, the results of this study indicate that the methanolic extract of *B. mauritanicum* tubers exhibits antimicrobial activity against a variety of bacterial and fungal strains.These results support earlier studies and show how effective biologically generated *B. mauritanicum*are against a variety of pathogenic bacteria.

Table 2. MIC, MBC and MBC/MIC ratio of tubers extracts of *B. mauritanicum.*

Bacteria strains (n = 3)	**Aqueous extract**			**Methanolic extract**		
	MIC value (mg/ml)	MBC value (mg/ml)	MBC/MIC ratio	MIC value (mg/ml)	MBC value (mg/ml)	MBC/MIC ratio
Escherichia coli	10	20	2	2.5	10	4

***Klebsiellapneumoniae*ATCC**	10	20	2	2.5	10	4
Pseudomonas aeruginosa	V.H.C	V.H.C	NA	20	20	1
Staphylococcus aureus	5	10	2	5	10	2
Candida albicans	10	10	1	5	5	1

NA; not available, V.H.C; very high concentrations.

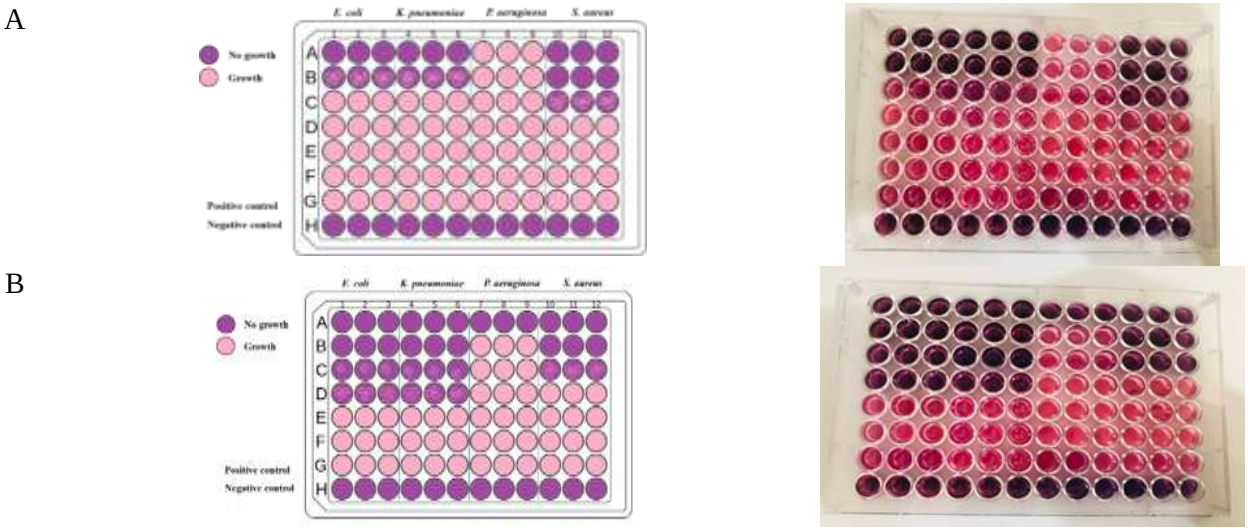

Figure 1. Tuber broth microdilution assay of aqueous extracts of *B. mauritanicum* (**A**), and methanol extracts (**B**), against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* , and *Staphylococcus aureus*. Live-activated cells cause resazurin reduction (purple -blue) to resorufin (pink and red).

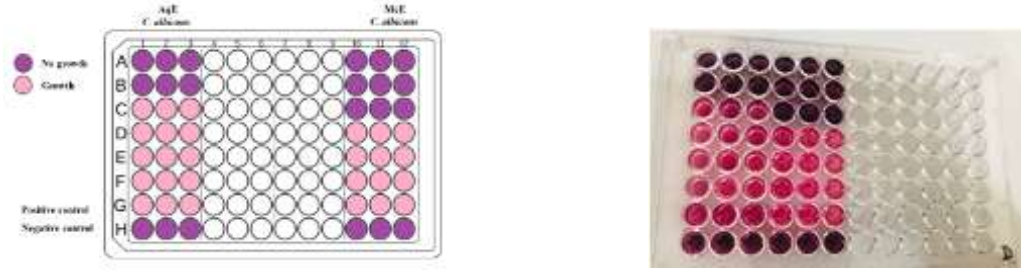

Figure 2. Broth resazurin, yeast microdilution assay of aqueous extracts (on the right) and methanol extracts (on the left) of *B. mauritanicum*, against *Candida albicans.*

Proteinase K inhibitors assay

The table 3 presents the percentage of Proteinase K (ProK) activity after the application of tuber extracts of *B. mauritanicum* (Figure 3).

Table 3. Percentage of ProK activity after the effect of of tubers extracts of *B. mauritanicum.*

	ProK	**Aqueous extract**	**Methanol extract**
% of *ProK* activity	100.0	88.33± 0.33	101.85 ± 1.20

The aqueous extract resulted in a notable decrease in ProK activity, reducing it to 88.33% compared to the control group, where ProK activity was 100%. This decrease suggests that the aqueous extract of *B. mauritanicum* effectively inhibited the enzymatic activity of ProK, specifically its ability to hydrolyze casein. ProKis known for its role in casein digestion, and the extract's impact on reducing ProK activity indicates its potential as a protease inhibitor. Inhibiting ProK activity can have implications for nutrient absorption and metabolism, underscoring the importance of this finding. Additionally, inhibiting ProK activity could have a number of health benefits, such as reducing the risk of heart disease, stroke, and cancer (Bajorath et al, 1988). This demonstrates the potential of *B. mauritanicum* extracts to influence protein digestion processes and their broader metabolic consequences (Miyamoto et al, 2017).

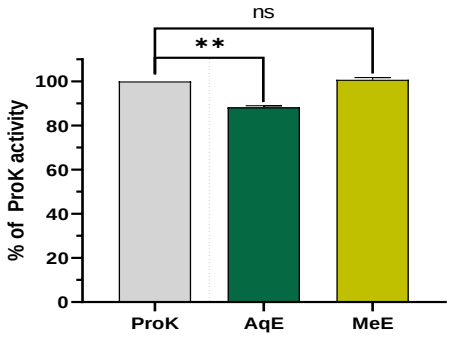

Figure 3. Percentage of ProKactivity after the effect of tubers extracts of *B. mauritanicum.*

CONCLUSION

This study represents a significant contribution to the understanding of *B. mauritanicum* tubers' therapeutic potential. The quantitative assessments further underscored the potency of methanol extracts in terms of polyphenol and flavonoids contents. The antimicrobial testing unveiled the bactericidal effects of methanol extracts against various bacterial strains, further emphasizing its potential as a natural antimicrobial agent. Interestingly, the study revealed the plant's moderate activity against *Candida albicans*, indicating a selective antimicrobial effect. Finally, the

inhibition of proteinase K activity by the aqueous extract presents an intriguing avenue for potential health benefits, including reduced risks of heart disease, stroke, and cancer. These promising and multifaceted findings open new horizons for further research and potential applications of *B. mauritanicum* tubers in pharmaceutical, nutraceutical, and functional food industries.

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Conflict of interest

There is no actual or potential conflict of interest in relation to this article.

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