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Original Paper

Thymus numidicus: phenolic constituents, antibacterial, and antioxidant activities of butanolic extract

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Abstract

Thymus numidicus Poir. (Lamiaceae) is a native plant of northern Algeria and Tunisia. It is used in traditional medicine in Algeria to treat respiratory and digestive diseases, and as a spice in food preparations. This study aimed to characterize the chemical constituents and to evaluate antibacterial and antioxidant activities of 1-butanol (BuOH) extract of aerial parts of *T. numidicus*. A total of 15 phenolic compounds were identified using HPLC-TOF/MS analysis, among them eight phenolic acids, five flavonoids and a stilbenoid glycoside named polydatin, which were reported for the first time from the species. Antibacterial effects of BuOH extract were determined using the disc diffusion method against four bacteria strains, where it has displayed a weak activity against three of them. In addition, BuOH extract showed significant antioxidant activity, which was measured using 1,1-diphenyl-2-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assays for free radical scavenging activity, and cupric ion reducing antioxidant capacity assay. Our results suggest the use of *T. numidicus* as a natural antioxidant in food and pharmaceutical industries.

Key words: antibacterial activity, antioxidant activity, phenolic compounds, *Thymus numidicus*.
Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AcOEt, ethyl acetate; BuOH, 1-butanol; CUPRAC, cupric ion reducing antioxidant capacity assay; DMSO, dimethyl sulphoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

Introduction

Thymus is one of the largest genera of the Lamiaceae family; it contains 215 species, mainly in the West Mediterranean area (Morales 2002). *Thymus* species are generally popular plants in the traditional medicine of various countries and are used as drugs, herbal drinks, spicy agents, and additives in food preparation and conservation. Especially, decoctions of aerial parts of *Thymus* species are used as stomachic, antispasmodic, whooping cough, antiseptic, analgesic, antitussive, cardiotonic, carminative, depurative, diuretic, emmenagogue, expectorant, fungicide, hypotensive, and respiratonic drugs (Boulous 1983; Duke 1989).

The endemic species *Thymus numidicus* Poir., which grows wild in the region between north-eastern Algeria and north-western Tunisia (Quezel, Santa 1963), is used particularly to treat respiratory and digestive diseases as many other *Thymus* species and has attracted the attention of researchers for its essential oil. Studies have indicated that *T. numidicus* essential oil is a thymol chemotype (Kabouche et al. 2005a; Kabouche et al. 2005b; Hadef et al. 2007; Giordani et al. 2008; Saidj et al. 2008; Zeghib et al. 2013; Benayache et al. 2014; Ghorab et al. 2014; Kouch et al. 2014; Zellagui et al. 2014; Messara et al. 2017; Boughendijoua, Djeddi 2017). The other main components are carvacrol, *p*-cymene, γ-terpinene, thymol methyl ether and linalool. Essential oils of *T. numidicus* showed strong insecticidal activity (Saidj et al. 2008), antifungal activity (Giordani et al. 2008; Messara et al. 2017), antibacterial, antioxidant, and allelopathic activity (Ben El Hadj Ali et al. 2014; Kabouche et al. 2005a; Kouch et al. 2014; Messara et al. 2017; Zeghib et al. 2013).

The highest contents of polyphenol, flavonoids, flavonols, and proanthocyanines were found in leaves of the species collected from Tunisia (Ben El Hadj Ali et al. 2014). Also, it is known that *Thymus* species are rich in flavonoids and diterpenes (Xiao et al. 2019). Few studies on flavonoids of *T. numidicus* have been undertaken, where ten aglycones and only two flavonoid glycosides were identified but no phenolic acids (Benkiniouar et al. 2010; Benayache et al.

2014; Georgiou et al. 2015).

The aim of the present study was to analyze the content of phenolic acids and flavonoids of butanol extract of *T. numidicus* and evaluate antibacterial and antioxidant properties of this extract.

Materials and methods

Plant material

Aerial parts of *T. numidicus* were collected around El-Kala City (East of Algeria) during the flowering period. The plant identification was performed by one of the authors (Dr. R. Benkiniouar), a specimen (N° Th.nu.05/2016) was deposited in the herbarium of Biology Department (Mentouri-Constantine-1 University). The aerial parts of the plant were dried at room temperature away from light before use.

Extraction procedure

Dried aerial parts of *T. numidicus* (500 g) were powdered and soaked in a methanol/water (7:3) solution for two days, and then the solution was filtered, and evaporated under reduced pressure to dryness. The operation was repeated three times and the extracts were combined. The obtained extract was diluted in hot water, kept cold over night, and then filtered to eliminate chlorophyll and waxes. The aqueous solution (filtrate) was extracted successively with ethyl acetate (AcOEt) and 1-butanol (BuOH) three times for each. The AcOEt and BuOH extracts were evaporated to dryness under vacuum pressure to obtain 5.2 and 20.6 g, respectively. Chromatographic analysis by thin layer chromatography on cellulose for the two obtained extracts using water:acetic acid, (85:15) and 1-butanol-acetic acid-water, (4:1:5, top layer) systems has shown that AcOEt and BuOH extracts are rich in flavonoid aglycones and polar phenolic compounds, respectively (Mabry et al. 1970).

HPLC-TOF/MS analysis

The BuOH extract of *T. numidicus* was analyzed by a HPLC-TOF/MS method, which was developed and validated by Abay et al. (2015) to analyse flavonoids and phenolic acids in plant extracts. The analysis was carried out on an Agilent Technology 1260 Infinity HPLC System coupled with a 6210 TOF LC/MS detector. The separation of compounds was performed on a Zorbax SB-C18 (4.6 × 100 mm, 3.5 µm) column. Ultra-pure water with 0.1% formic acid (solvent A) and acetonitril (solvent B) were used as mobile phases. The flow rate was 0.6 mL min^{-1} and the column temperature was maintained at 35 °C. Injection volume was 10 µL. The total run time for separation was 30 min according to the below programme: 0 to 1 min 10% B, 1 to 20 min 50% B, 20 to 23 min 80% B, 23 to 25 min 10% B, and 25 to 30 min 10% B. Negative ionization mode was used on the TOF/MS instrument. Nitrogen was supplied at a flow of 10 L min^{-1} at 325 °C. Nebulizer pressure was 40 psi, capillary voltage was 4 kV and the fragmentation voltage was 175 V. Before analysis, the dried extract sample (200 ppm) was dissolved in 1 mL methanol and then filtered with a PTFE filter (0.45 µm) to eliminate particulates. The identification of phenolic compounds was carried out by comparing retention times and spectral data with those of standards.

Antibacterial activity

Four bacterial strains, two Gram-negative (*Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853), and two Gram-positive bacteria (*Staphylococcus aureus* ATCC25923 and *Enterococcus faecalis* ATCC29212) were used in the evaluation of antibacterial activity of BuOH extract of *T. numidicus*. The tested bacterial strains were obtained from the Biology Laboratory of Abdelhafid Boussouf University Center (Mila, Algeria). The tested bacteria strains were inoculated on nutrient broth at 37 °C for 24 h. The bacterial suspension was then adjusted to 108 CFU mL^{-1} with sterile saline (SAÉN 2011) and inoculated on the surface of Mueller-Hinton agar plates. BuOH extract was dissolved in dimethyl sulphoxide (DMSO) and tested using the agar diffusion method at concentrations 500 µg mL^{-1} and 2000 µg mL^{-1}. Aliquots of 20 µL were impregnated onto sterile 6 mm diameter filter paper discs (Whatman N°3). Ciprofloxacin was used as a positive control, while DMSO was used as a negative control. Growth inhibition activity was evaluated after an incubation period of one night at 37 °C. All experiments were performed in duplicate and the average zones of inhibition were measured.

DPPH free radical scavenging assay

The scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined using the method described by Blois (1958). The effect of antioxidants on DPPH radical is thought to be due to their hydrogen donating ability. In a 96-well microplate, 160 µL of DPPH (1 mM) was mixed with 40 µL of the tested extract at a final concentration range 3.125 to 200 µg mL^{-1}. The absorbance was read at 517 nm after 30 min using a microplate reader (Perkin Elmer En Spire Alpha Plate Reader) multimode. Butyl hydroxylanisole was used as a standard. All experiments were performed in triplicate and the inhibition percentage was calculated using the following formula:

$$\text{DPPH scavenging effect (\%)} = [(Ac - As) / Ac] \times 100,$$

where *Ac* and *As* are absorbances of the control and the sample respectively.

ABTS assay

The scavenging capacity of the BuOH extract was determined according to the method described by Re et al. (1999). Briefly, 160 µL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) solution (7 mM ABTS in water and 2.45 mM potassium persulfate, stored in dark at room temperature for 16 h, absorbance

0.70 ± 0.02 at 734 nm) was added to 40 µL of the tested extract at a final concentration range 3.125 to 200 µg mL^{-1}. After 10 min the absorbance was measured at 734 nm. Butyl hydroxylanisole was used as standard. All experiments were performed in triplicate. The free radical scavenging activity was calculated as percentage of inhibition using the same equation mentioned above.

CUPRAC assay

Cupric ion reducing antioxidant capacity (CUPRAC) of the BuOH extract was determined according to the method described by Apak et al. (2004). 50 µL of Cu (II) solution (10 mM) was mixed with 50 µL of neocuproine solution (7.5 mM), 60 µL of NH_4Ac buffer (1 M, pH 7.0) solution and 40 µL of the tested extract at a final concentration range 3.125 to 200 µg mL^{-1}. The absorbance was measured after 1 h at 450 nm. Butyl hydroxylanisole was used as standard. All experiments were performed in triplicate. The results were given as $A_{0.5}$ (µg mL^{-1}) corresponding to the concentration indicating half absorbance intensity.

Statistical analysis

All results are expressed as means ± SD obtained from three separate experiments. Significant differences were determined using one-way ANOVA test followed by Newman-Keuls multiple comparison tests ($p < 0.05$). The analysis was performed using Graph Pad Prism 5 software, Inc., San Diego, CA, USA.

Results

The HPLC-TOF/MS analysis of BuOH extract of *T. numidicus* revealed the presence of 15 phenolic compounds (Table 1 and Fig. 1), among them, eight phenolic acids, six flavonoids, and a stilbenoid glycoside named polydatin. The major phenolic acid was *p*-hydroxybenzoic acid, while the major flavonoid was diosmin (diosmetin-7-*O*-rutinoside). To our knowledge, all these compounds except apigenin are reported for the first time from *T. numidicus*. The antibacterial properties of the BuOH extract of *T. numidicus* was evaluated using agar diffusion method against two Gram-negative bacteria (*E. coli* ATCC25922 and *P. aeruginosa* ATCC27853) and two Gram-positive bacteria (*S. aureus* ATCC25923 and *E. faecalis* ATCC29212). The zones of inhibition of tested extract against tested organisms ranged between 7.0 and 9.5 mm with the highest values for *S. aureus* ATCC25923. The tested extract was inactive against *P. aeruginosa*. It is interesting to note that *E. coli* ATCC25922 and *E. faecalis* ATCC29212 exhibited a narrow zone of inhibition (Table 2). In addition, the antioxidant activity of BuOH extract of *T. numidicus* was evaluated using three assays (DPPH, ABTS, and CUPRAC) using butylated hydroxyanisole as a positive standard (Table 3). The obtained values showed that BuOH extract has significant antioxidant activity.

Table 1. Phenolic constituents of BuOH extract of *T. numidicus*

No.	Compound	RT	Final concentration (ng mL^{-1})
1	Fumaric acid	2.390	20.3
2	Gentisic acid	4.525	126.9
3	Chlorogenic acid	5.519	47.0
4	Caffeic acid	7.653	88.1
5	*p*-Hydroxybenzoic acid	6.691	369.2
6	Protocatechuic acid	7.284	78.1
7	Syringic acid	7.236	90.7
8	Vanillic acid	8.151	30.2
9	Polydatine	9.611	6.6
10	Catechin	5.247	42.8
11	Naringin	10.397	23.0
12	Diosmin	10.558	171.1
13	Hesperidin / neohesperidin	10.959	4.4
14	Morin	13.911	53.9
15	Apigenin	15.499	10.9

Discussion

Herbal medicines have received much attention as new antibacterial agents and are considered as more safe for humans and the environment (Fazly-Bazzaz et al. 2005). *T. numidicus* is used in Algerian folk medicine as a hot herbal tea for the treatment of digestive and respiratory disorders. Herbal tea can contain essential oil and water-soluble compounds like phenolic acids and flavonoids. These two classes display many biological activities, but their principal property is their antioxidant activity by scavenging free radicals and chelating metals.

In this study eight phenolic acids were identified for the first time from the species. To our knowledge, nine phenolic acids occur in the genus *Thymus*, of which fumaric acid has not been reported. Thus, this is the first report of the presence of fumaric acid in *Thymus* genus (Vila 2002; Xiao et al. 2019). All previous studies about the flavonoids

Table 2. Antibacterial activity (measured as inhibition zone in mm) of the BuOH extract of *T. numidicus*. Negative control (DMSO) did not show any activity

Bacteria	Ciprofloxacin (5 µg $disc^{-1}$)	Concentration of extract (µg mL^{-1})	Inhibition zone (mm)
S. aureus	32.0 ± 1.41	500	8.25 ± 0.35
		2000	9.50 ± 0.70
E. faecalis	34.0 ± 0.70	500	7.50 ± 0.70
		2000	8.75 ± 0.35
E. coli	30.5 ± 0.70	500	7.00 ± 0.00
		2000	8.00 ± 0.00
P. aeruginosa	29.5 ± 0.70	500	0

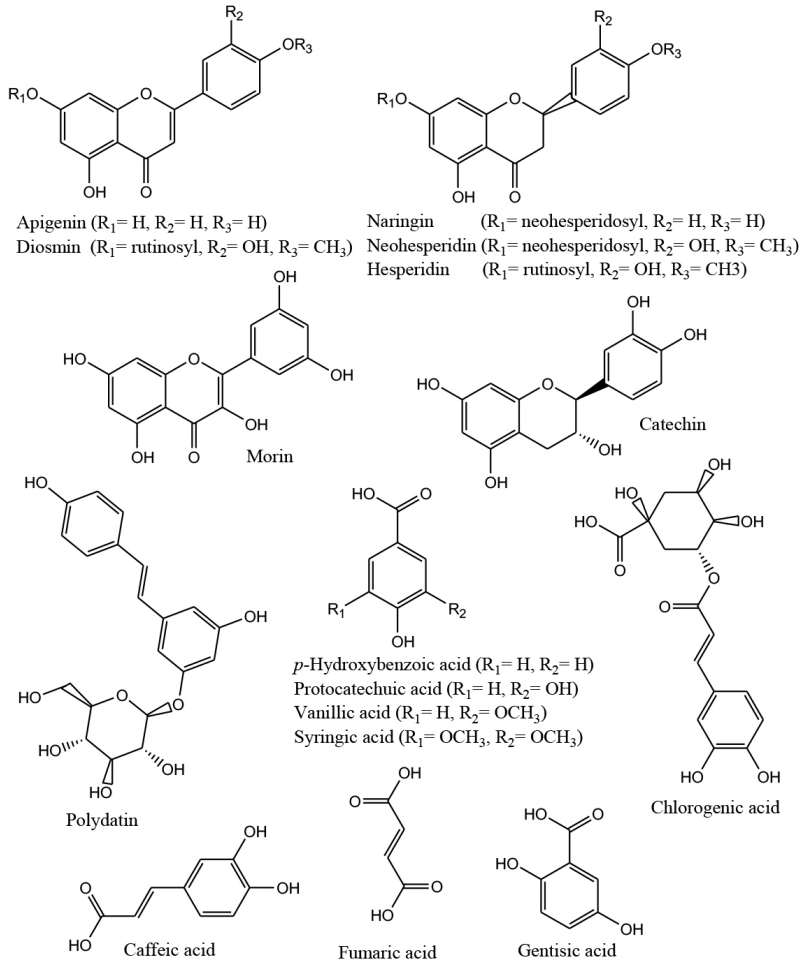

Fig. 1. Chemical structures of identified phenolic compounds.

of *Thymus* species were focused on flavonoid aglycones and there are fewer studies about flavonoid glycosides (Xiao et al. 2019). In this study, three flavonoid aglycones were reported, of which the compound morin was identified for the first time from *Thymus* genus, while apigenin and catechin were known to be common aglycones in *Thymus* species. In addition, three flavonoid glycosides were identified, with diosmin a flavone glycoside as the major flavonoid, which was reported also for the first time from *Thymus* genus, and two flavanone glycosides (naringin and hesperidin) which have beene frequently found in *Thymus* species (Xiao et al. 2019).

Finally, this study reported the identification of polydatin (resveratrol-3-*O*-β-glucoside), detected for the first time from Lamiaceae family. This compound has shown similar biological activities to resveratrol, including anti-inflammatory, antioxidant, anticancer, hepatoprotective and immunostimulatory effects (Şöhretoğlu et al. 2018). Furthermore, our results showed that the tested extract displayed weak activity on three bacterial strains and was not active against *P. aeruginosa* ATCC27853. It is known that only a few medicinal plants are effective against Gram-negative bacteria, since it has a lipopolysaccharide external membrane that provides resistance compared to Gram-positive bacteria (Wei et al. 2015). Our results are in accordance with other study about antibacterial activity of extracts of *Thymus* species (Qaralleh et al. 2009, Majob et al. 2008). Methanolic extract of *Thymus daenensis* collected from Iran was evaluated for a potential inhibitory effect on Gram-positive and Gram-negative bacteria. Significant antibacterial activity against Gram-positive bacteria was found. However, the tested extract was not active

Table 3. Antioxidant activity of BuOH extract of *T. numidicus*

	DPPH IC50 (μg mL^{-1})	ABTS IC50 (μg mL^{-1})	CUPRAC A0.50 (μg mL^{-1})
BuOH extract	42.76 ± 1.40	13.55 ± 1.53	8.76 ± 1.13
Butyl hydroxylanisole	3.54 ± 0.65	0.67 ± 2.06	3.42 ± 0.26

against Gram-negative bacteria. The presence of different phytochemical substances such as phenolic compounds, flavonoids, terpenoids and tannins might be responsible of the observed activity, according to the authors (Majob et al. 2008). In contrast, leaf ethanolic extract of *Thymus capitatus* collected from Jordan was most active on the growth of *E. coli* and *P. aeruginosa* (Quaralleh 2009). The tested extract was not active against *E. aeregenes* and *S. aureus*. The authors compared the antibacterial activity of leaf ethanolic extract of *Thymus capitatus* with essential oil leaves extract. In addition, dichloromethane and hexane extracts were tested. Ethanolic extract was the most active extract with an almost 19.5 mm inhibition zone against *P. aeruginosa*, while, dichloromethane and hexane extracts displayed 15 and 13 mm of inhibition zone. Further studies with other extracts from this species are needed to confirm whether the absence of antimicrobial activity is due to the used solvent, or the tested plant did not possess antibacterial activity.

In addition, the antioxidant activity of BuOH extract of *T. numidicus* was evaluated using three assays (DPPH, ABTS, and CUPRAC). Our results demonstrated that the extract had significant antioxidant activity with all tests used (Table 3). Our results are in accordance with a previous study on *T. numidicus* (Benayache et al. 2014; Ben El Hadj Ali et al. 2014). A moderate antioxidant activity for BuOH extract of *T. numidicus* collected from Algeria using DPPH assay was reported (Benayache et al. 2014). In addition, the antioxidant activity of methanol extract of *T. numidicus* collected from Tunisia was evaluated (Ben El Hadj Ali et al. 2014). The reported values were higher than values reported in the present study. The antioxidant activity of BuOH might be due to the presence of phenolic compounds and flavonoids reported in the present study.

Conclusion

T. numidicus is a medicinal plant largely used in Algerian folk medicine; most previous studies had focused on essential oils and flavonoid aglycones. In the present study, the HPLC-TOF/MS analysis showed the richness of the BuOH extract of *T. numidicus* on phenolic acids and flavonoids. Diosmin was the major flavonoid while *p*-hydroxybenzoic acid was the major phenolic acid. Furthermore, this study has showed that the BuOH extract exhibited a significant antioxidant activity and a weak antibacterial activity. Finally, we suggest that additional phytochemical and biological studies are necessary to discover the medicinal potential of this species.

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