https://doi.org/10.48047/AFJBS.6.15.2024.13669-13681

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Phenological variation of phenolic profiling and *in vitro* biological activities of *Pituranthos scoparius* (Coss. & Dur.) Benth. & Hook

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Volume 6, Issue 15, Oct 2024

Received: 15 Aug 2024

Accepted: 25 Sep 2024

Published: 21 Oct 2024

doi: 10.48047/AFJBS.6.15.2024.13669-13681

Abstract

Pituranthos scoparius is a plant that grows annually across various areas in Algeria, and has traditionally been used in folk medicine. This study aims at determining the phenolic profile, antioxidant, and enzyme activities of ethyl acetate extractsfrom the aerial parts of *Pituranthos scoparius* collected at three different phenological stages(vegetative, flowering, and fruiting). Antioxidant activity was assessed using β-carotene–linoleic acid, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, CUPRAC, and metal-chelating assays. For the first time, the enzyme inhibitory potential of the tested extracts was evaluated against key enzymes implicated in neurodegenerative disorders, specifically acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), as well as urease. Additionally, the inhibitory activity of the extracts was assessed against tyrosinase. The results showed that phenological stages affected significantly *P.scoparius* activities. Furthermore, ethyl acetate extract at flowering stage exhibited the greatest activities.

Keywords: *Pituranthos scoparius*, phenolic profile, phenological stages, antioxidant, enzymes activity.

Introduction

Algeria is characterized by a rich and diverse vegetation, including a wide array of spontaneous aromatic plant species. In recent years, there has been a notable increase in scientific interest in these species, owing to their pharmacological properties. Among these is *Pituranthos scoparius*, locally known as "Guezzah," an endemic species to North Africa. This plant is less prevalent in the Central Sahara but is frequently found in the Tassilin'Ajjer plateau and the Hoggar region [1]. *Pituranthos scoparius* belongs to the Apiaceae family, it is a perennial, leafless, ephedroid plant with often highly branched stems. The cauline leaves are 2-3 cm wide. Its fruits are longer than they are wide, covered with erect hairs. The flowers are white, with white petals and narrow veins, grouped in lateral umbels [2]. This plant was traditionally used in the treatment of numerous diseases such as fevers, asthma, hepatitis and rheumatism. Additionally, it has been used to treat spasms, diabetes-related discomfort, urinary infections, and digestive disorders [3]. The medicinal properties of this species are largely attributed to the presence of phytochemicals such as alkylated isocoumarins [4]**.** In this paper, the researchers have attempted to investigate the phenolic profile and biological properties, mainly focusing on their antioxidant, anticholinesterase, anti-tyrosinase and anti-urease activities of ethyl acetate extracts from aerial parts of *Pituranthos scoparius* at various stages of development.

Material and methods

Plant material and extraction

The aerial parts of *Pituranthos scoparius* were collected during three different phenological stages (vegetative, flowering, and fruiting) in 2018, in Oum El Bouaghi, Algeria. Upon harvesting, the plant material was air-dried at room temperature under dark conditions, then coarsely ground and directly used for extractions. The aerial parts (100 g) of the plant were air-dried, grinded, and macerated with 80% aqueous methanol at room temperature. After filtration, the liquid phase was evaporated under reduced pressure using a rotary evaporator to obtain a solid residue. The obtained material was further dissolved in water and re-extracted using n-hexane, chloroform and ethyl acetate and successively evaporated to dryness under reduced pressure. Before usage, the extracts were kept at 4°C.

HPLC-DAD Analyses

RP-HPLC (reversed-phase) containing a diode-array-detector (DAD) was used to detect phenolics in the extracts[5]. A Shimadzu high-performance liquid chromatography (Shimadzu Cooperation, Japan) system was used. The following were the ODS-3 column properties: ODS-3 column Inertsil, 150 mm × 4.0 mm i.d, 4 µm film thickness. A total of 27 phytochemicals was used as reference standards for qualitative identification. The elution system was as follows: The mobile phase included 0.5% acetic acid in water (A) and methanol (B). The different extracts were prepared in methanol (8 mg/ml) and filtered through a 0.45 m poresize filter. A constant

injection volume of 20.0 µL was employed for extract introduction. The detected compounds are reported as micrograms per gram of dry plant weight.

Screening of antioxidant activity

Free radical scavenging activity

Essentially, 160 µL of DPPH solution (60 µM) was mixed with 40 µL of extracts at different concentrations. After 30 min of incubation in darkness, the prepared mixture was tested for their absorbance at 517 nm. The results of DPPH scavenging effect were represented as 50% inhibition concentration (IC_{50}) and contrasted with standards [6].

ABTS cation radical decolorization

The $ABTS^{\bullet+}$ scavenging activity was determined using the method of Re et al.[7] with slight modification [8].In short, $ABTS^{\bullet+}$ solution was prepared by reacting the ABTS (7 mm), water, and potassium persulfate (2.45 mm). Then, the mixturewas stored for 12 hours in the dark. The absorbance of the ABTS solution was adjusted to 0.70 ± 0.02 (734 nm). Afterward, 40 µL of the solution was mixed with 160 µL of the diluated $ABTS^{\bullet+}$ solution. After 10 min of incubation, absorbance was measured at 734 nm. The results were established as a 50% inhibition concentration (IC_{50}= µg/mL).

Metal chelating activity assay

The ferrous ion chelating potential of extracts was measured using the following standard procedure with slight modifications [6]. To each well containing 40 µL of the extract in methanol at various concentrations. A volume of 40µL of $FeCl_2$ (0.2 mM) and 40 µL of ethanol were added. Then 80 µL of ferrene (0.5 mM) was added to initiate the reaction. After incubation at room temperature for 10 minutes, the absorbance was measured at 593 nm. The EDTA was used as a chelating standard.

Cupric ion-reducing antioxidant capacity (CUPRAC)

The CUPRAC assay was conducted using the approach described by Apak et al. [9] with slight modification[10]. Briefly, a mixture of 50 µL of copper (II) chloride (10 mM), 50 µL of neocuprine (7.5 mM), and 60 µL of ammonium acetate buffer solution (1M, pH: 7.0) was used. Subsequently, plant extract was added to the initial mixture at a volume of 40 µL. After 60 min of incubation, the absorbance was measured at 450 nm, and the findings were represented as $A_{0.5}$ (µg/mL).

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE)

Inhibitory capacities ofextracts were evaluated for their possible inhibitory effect against AchE and BChE using the Ellman method [11]. The galantamine was used as a reference standard. The inhibition percentages of

AChE or BChE were calculated by comparing sample reaction rates to a blank sample using the formula: % inhibition = [(E－S)/E]/100

E: Enzyme activity without extract. S: Activity of the enzyme in the presence of the extract.

Urease inhibitory activity

Indophenol assay was used to evaluate urease inhibition activity [12,13]**.** Thiourea was used as standard. Thefollowing equation was used to quantify the urease inhibition activity:

Urease inhibition % = [($A_{control}$ － A_{sample}) / $A_{control}$] ×100where:

$A_{Control}$: the absorbance of the reaction medium, in theabsence of the tested sample; A_{Sample}: the absorbance of the reaction medium, in the presence of the tested sample

Tyrosinase inhibitory activity

The tyrosinase enzyme inhibitory was assessed by spectrophotometric method and using L-DOPA as a substrate [14]**.** Kojic acid was used as a standard. The inhibition percentage was calculated using the same approach as described in the urease inhibition assay.

Statistical analysis

For data analysis, Graph Pad Prism software 8.3.1 was used. One-way ANOVA followed by Tukey's multiple comparisons test was performed. The results were considered statistically significant at $p< 0.05$.

Results and discussion

Phenolic composition

HPLC-DAD analyses were performed and the results are illustrated in Figure 1 and Table 1. From the 24 standard compounds quantified, 8compounds were detected in the extracts at the flowering and vegetative stages, while only 4 compounds were detected at the fruiting stage. Several additional chromatographic peaks were observed but remained unidentified due to the unavailability of corresponding reference standards. Overall, phenolic acids were the most abundant compounds detected in all samples. The chemical composition of extracts from both the flowering and vegetative stages were largely similar, though the concentrations of key compounds showed variable amounts between the stages. Interestingly, vanillic acid, ferulic acid, and 4-hydroxy benzoic acid were detected in all studied extracts. Whereas, 2-(4-hydroxyphenyl)ethanol was only detected at the vegetative stage. On the other hand, rosmarinic acid and chlorojenic acid were only detected at the flowering stage. Indeed, during vegetative and fruiting stages, *p*-coumaric acid appeared to be the

predominant constituent, while, ferulic acid was detected as the main constituent at the flowering stage. In accordance with our results, the abundance of phenolic acids was observed in the aqueous extract of *Pituranthos scoparius* [15]**.** Indeed, the contents of chlorogenic acid of the present work are much lower than those reported in another studies [16,17]. It is worth noting that some identified metabolites such as cinnamic acids (5-*O*-caffeoyl quinic acid and 5-feruloyl quinic acid) and some known flavonoids (vicenin-2, six quercetin and six isorhamnetin *O*-glycosylated derivatives) were detected in methanolic extract of *Pituranthos scoparius* at the flowering stage [17].

Phenolic compounds are one of the main groups of secondary metabolites, it can be used for their strong antioxidant and enzyme inhibition potentials. In reference to the phenological stages outlined in this study, distinct phenolic profiles were observed across the different growth stages, with a slight decline in phenolic content from the vegetative and flowering phases to the fruiting. The biosynthesis and accumulation of these secondary metabolites depend on endogenous factors such as tissue differentiation, development stage, competition, nutritional status, and also on numerous extrinsic factors, including climate, soil, irrigation, temperature range, exposure to diseases and pests, cultural practices, harvest season, drying methods, handling, and storage factors [18]. These factors have been shown to impact the metabolite profile in plants [19],and the phenolic content during the plant growth cycle. In the same vein, the plant extract composition can be changed with maturity [20]. Moreover, the major components in early growth stage are different from fruiting one, potentially due to variations in cellular division processes. Furthermore, the decrease in phenolic content with age is probably due to their dilution with growth [21, 22]. In the other hand, it was suggested that the high level of biosynthesis of secondary metabolites, at flowering stages could be needed for their fruit coloration, ultra-violet protection, pigmentation, and aroma in flower production [23]. Also, it serves as a defense mechanism against pests that may attack the flowers [24]. Additionally, it is an effective way of attracting potential pollinators [25].

Table 1. Phenolic profile of *P. scoparius* extracts by HPLC-DAD (µg/mg) at different stages.

Phenolic compounds	RT(min)	Vegetative	Flowering	Fruiting
3,4-dihydroxybenzoicacid	14.25	tr	tr	-
2-(4-hydroxyphenyl)ethanol	18.4	tr	-	-
4-hydroxy benzoicacid	19.35	0,6	0,31	0,59
Vanilic acid	22.24	0,92	0,49	1,39
Caffeic acid	23.07	0,22	0,29	-
Chlorojenic acid	27.48	-	tr	-
p-coumaric acid	28.25	14,01	-	5,71
Ferulic acid	30.19	1,18	1,46	1,66
Trans-2-hydroxycinnamic acid	33.83	0,06	tr	-
Rosmarinic acid	44.59	-	tr	-

Note: RT: Retention time of the compound in minutes; –: not detected; tr: trace.

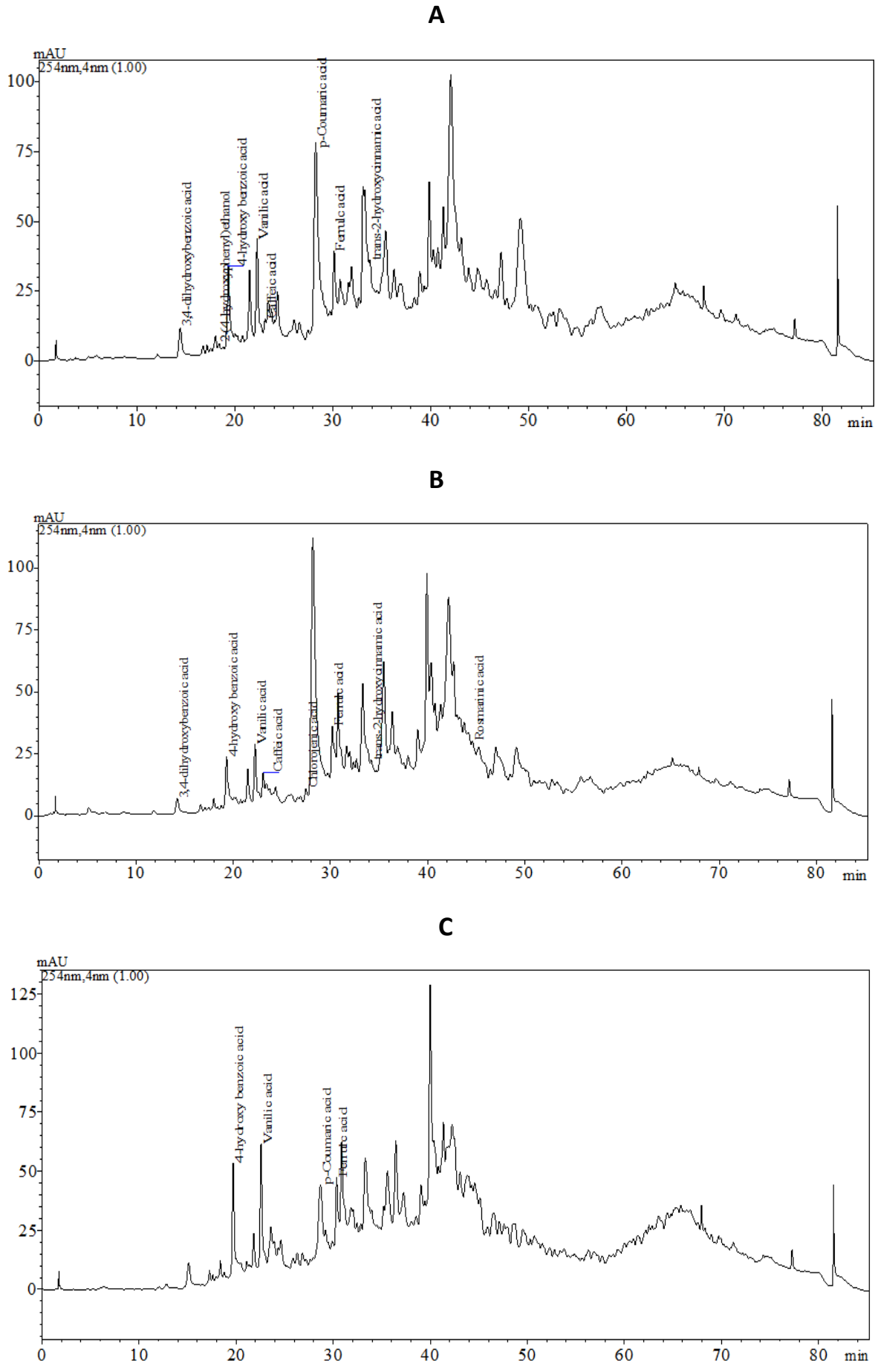

Figure1. HPLC chromatograms of *Pituranthos scoparius* extracts; A) vegetative stage; B) Flowering stage C) Fruiting stage.

Antioxidant activities

The antioxidant activity was evaluated through β-carotene-linoleic acid, $ABTS^{\bullet+}$, $DPPH^{\bullet}$, CUPRAC and metal chelating assays and reported in Table 2. All the tested extracts exhibited antioxidant activities in a dose-dependent manner. The ethyl acetate extract in full flowering stages showed a potential antioxidant activity with better IC_{50} values than the other extracts. In the $DPPH^{\bullet}$ assay, the IC_{50} values were 33.86 ±0.77µg/mL compared to 12.26±0.07µg/mL for α-tocopherol and 45.37±0.47µg/mL for BHT. Whereas, IC_{50}values were 16.81±2.95µg /mL, 4.31±0.10µg/mL (*α*-tocopherol) and 4.10±0.06 µg/mL(BHT) in the $ABTS^{\bullet+}$assay. In the *β*-carotene-linoleic acid assay, the activity of the extract was moderate(IC_{50}= 42.99 ±2.59 µg/mL) compared to those of standards *α*-tocopherol (IC_{50}=2.10±0.09µg/mL) and BHT (IC_{50}=1.34±0.04µg/mL). The metal chelating activity of the plant extracts showed identical results revealing the weakest activity (IC_{50} values were >200), compared to standards EDTA (IC_{50}= 5.50±0.35 µg/mL). According to the literature, the antioxidant activities of *P. scoparius* extracts, assessed using various methodologies in the present study, the results were lower than those reported in earlier studies.In the work of Bouaziz et al.[26] the antioxidant properties of the ethyl acetate extract of studied plant were tested using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$assays. The extract had a potent activity (IC_{50}=2,24 ±0.38µg/mL). Furthermore, Belyagoubi-Benhammou et al.[27] found that the ethyl acetate extract displayed strong antioxidant activity through two screening methods $DPPH^{\bullet}$ and reducing power test, with a values of 0.91 ± 0.02 µg/mL and 1.19 ± 0.00 µg/mL, respectively. Conversely, in a study reported by Adida et al. [28] the ethyl acetate extract of *P. chloranthus* appeared very low inhibitory activity comparing to our finding, with an IC_{50}value of 78,5± 0,12 $\mu g.mL^{-1}$ against $DPPH^{\bullet}$ radicals.

Table 2. Antioxidant activities of *P. scoparius* extracts

Ethyl acetate extracts	***β*-carotene-linoleic acid assay**	**$DPPH^{\bullet}$ assay**	**$ABTS^{\bullet+}$assay**	**CUPRAC assay**	**Metal chelating assay**
	IC_{50} (µg /mL) *	IC_{50} (µg /mL) *	IC_{50} (µg /mL) *	$A_{0.5}$ (µg/mL) **	IC_{50} (µg /mL) *
Vegetative	163.76±2.57^{a}	46.93±2.92^{a}	20.33±1.45^{a}	42.67±2.42^{b}	>200^{a}
Flowering	42.99 ±2.59^{b}	33.86 ±0.77^{b}	16.81±2.95^{a}	31.21±1.47^{c}	>200^{a}
Fruiting	>200	63.32 ±1.20^{c}	34.76 ±1.83^{b}	46.98±1.28^{a}	>200^{a}
***α*-Tocopherol**	2.10 ± 0.09^{c}	12.26 ± 0.07^{d}	4.31±0.10^{C}	10.20 ± 0.01^{d}	NT
BHT	1.34±0.04^{c}	45.37±0.47^{a}	4.10±0.06^{C}	3.80 ± 0.00^{e}	NT
EDTA	NT	NT	NT	NT	5.52±0.35^{b}

Note: The values (IC_{50} and $A_{0.50}$) presented represent means ± SD, followed by the different script letters within the same column indicates significant difference statistically using Tukey test at $p<0.05$. *IC_{50} values correspond to the µg/mL concentration of 50% inhibition while **$A_{0.50}$ values correspond to the µg/mLconcentration of 0.500 absorbance. NT: not tested.

Enzyme Inhibitory Activities

In addition to the antioxidant ability, we aimed to evaluate the enzyme inhibitory activities of *Pituranthos scoparius* extracts on acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase andurease enzymes.

The cholinesterase inhibitory activities of the studied extracts of *P.scoparius* were evaluated for the first time in this study. Table 3 shows the percentage (%) at 200 µg/mL concentration of the extracts and standard compound (galantamine). The extracts showed more inhibitory effects against BChE compared to AChE. Furthermore, the extract at the flowering stage was able to inhibit both cholinesterases enzymes (AChE and BChE) with relatively moderate activity of 12.89±2.78% and 38.18±1.26% against AChE and BChE, respectively. At the same conditions, galantamine highlighted 81.41±1.03 % and 75.54 ±1.05 % values against AChE and BChE, respectively. Cholinesterase inhibitors are known to help in boosting cholinergic deficit and maintaining good levels of acetylcholine for the prevention from Alzheimer's disease. The phenolic compounds present in this extracts as well as other possible classes of compounds can highly contribute to cholinesterase inhibition since phenolic units are well known to exhibit a suitable natural anti-cholinesterase activity [29].

Tyrosinase is the key enzyme in melanin synthesis along with in dermatological problems such as spots, age, freckles and melanoma, caused by an excess on melanin accumulation. Therefore, tyrosinase inhibitors have become progressively crucial in the treatment of skin problems [30]. The look for new natural tyrosinase inhibitors is necessary because of the side effects of synthetic inhibitors currently used in functional foods and harmless cosmetics [31, 32].The result showed that all studied extracts could inhibit tyrosinase activity. The most pronounced inhibition was observed in the ethyl acetate fraction at fruiting stage (48.40±1.52). This activity was considered as moderate in comparison to the standard kojic acid (83.6±0.2 µg/mL) at 200 µg/mL(Table 3). Our findings agrees with the research carried out by Jdey et al. [33] stating that the crude extract of *P.scoparius* slightly inhibits monophenolase.

Since urease inhibitors can be used as potential drugs in the treatment of ulcer diseases, the discovery of new inhibitors is gaining importance [34]. A large number of urease inhibitors have been identified from plants including terpenoids, polyphenols, and alkaloids and other substances that act either by competing with the substrate (urea) or by chelating the nickel co-factor [35]. The results of the anti-urease activity are depicted in Table 3. The trend in percentage inhibition at 200µg/ml of the anti-urease activity was as follow: the extract at the flowering stage (35.09 2.94)> vegetative stage (16.12 3.16%) > fruiting stage (not active). Overall, the results were all significantly different ($p<0.05$). These values were compared with the percentage inhibition of Thiourea (96.93%), which was used as the reference standard. To the best of my knowledge, this is the first study investigating the effect of *Pituranthos scoparius* extracts on the virulent enzyme urease.

Table 3. Cholinesterase, tyrosinase and urease inhibitory activities of *P. scoparius*extracts

Plant	Extracts	AChE* Assay inhibition	BChE* Assay inhibition	Tyrosinase inhibition activity	Urease Inhibition activity
P. scoparius	Vegetative stage	NA	36.27±2.09^{b}	18.27 ±0.29^{d}	16.12 ±3.16^{c}
	Flowering stage	12,89±2.68^{b}	38.18 ±1.26^{b}	33.81±1.21^{c}	35.09± 2.94^{b}
	Fruiting stage	NA	34.60±2.13^{b}	48.40±1.52^{b}	NA
Standards	Galantamin	81.41±1.03^{a}	75.54±1.05^{a}	NT	NT
	Kojik acid	NT	NT	83.6±0.2^{a}	NT
	Thiourea	NT	NT	NT	83.80 ±0.60^{a}

*Note: *The values (Inhibition%) represent means ± SDat 200 µg/mL concentration of extracts (p<0.05); NT: not tested, NA: no activity.*

Conclusion

In the present study, phenolic profile, antioxidant, anticholinesterase, urease and tyrosinase inhibitory properties of ethyl acetate extracts of *Pituranthos scoparius* at different stage were determined. This study confirms the differences exist in thecomposition of phytochemical compounds, antioxidant and enzyme capacities at different seasons of growth. Therefore, it can be indicated that the optimum period to harvest *Pituranthos scoparius* for biological activities is at flowering stage.

Acknowledgments

The authors are grateful to the Algerian Ministry of Higher Education and Scientific Research (MESRS, DGRSDT) and to Mugla Sitki Koçman University.

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