Egypt. J. Bot. **Vol. 62**, No. 1, pp. 255-273 (2022)

Egyptian Journal of Botany
http://ejbo.journals.ekb.eg/

Seasonal Variation in the Secondary Metabolites and Antimicrobial Activity of *Plantago major* L. from Egyptian Heterogenic Habitats

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PLANTAGO major has been used to treat various diseases since ancient times. The present study aimed to assess the seasonal and habitat-related variations in the secondary metabolite content and antimicrobial activity of *P. major* leaf extracts. Eight habitats were selected for sampling plants in four seasons. The phytochemical screening of the ethanolic and chloroform extracts of *P. major* leaves from different habitats in different seasons showed the presence of cardiac glycosides, flavonoids, and phenolics. The highest cardiac glycoside and total flavonoid contents were observed in urban habitats and cultivated crops during winter, whereas the highest total phenolic content was observed in fallow lands during summer. Compounds separated and identified using high-performance liquid chromatography included phenolics such as ellagic acid, catechol, resorcinol, gallic acid, and phloroglucinol and flavonoids such as apigenin, luteolin, chrysoeriol, rutin, quercetin, kaempferol, and avicularin. The methanolic extracts of *P. major* leaves showed higher antimicrobial activity than aqueous and ethanolic extracts; the methanolic extract of *P. major* leaves from canal banks showed the highest activity against *P. aeruginosa*, followed by that from orchards against *S. aureus*. The results suggest that *P. major* contains phenolics and flavonoids that have potential medicinal applications. Moreover, the antimicrobial activity of the methanolic extract differed with habitat. Therefore, the present study confirms previously reported findings and indicates that the phytochemical contents and antimicrobial activity of the plant extracts differed with environmental factors (habitat and season). In future studies, the phytochemicals with antimicrobial activity need to be extracted, separated, purified, identified, and tested as pure or mixed compounds.

Keywords: Antimicrobial activity, Flavonoids, HPLC, Phenolic compounds, *Plantago major.*

Introduction

Plantago major L. (common plantain) is a perennial herbaceous plant belonging to the family Plantaginaceae (Samuelsen, 2000). It is a weed that grows better in compacted soils than most other plants, and it is commonly found along walkways and roadsides and in grasslands, farms, and other sites where soil compaction is common (Nazarizadeh et al., 2013; Burger et al., 2019). It has a wide geographical distribution and is clearly identifiable.

Plants have been used in traditional medicine for thousands of years because of their medicinal efficacy, low toxicity, and abundance of natural anticancer agents (Ozaslan et al., 2007; Prakash et al., 2011). *Plantago* species have been traditionally used as medicinal plants for various purposes including wound treatment because of their anti-inflammatory, analgesic, antitumoral, antispasmodic, hepatoprotective, antiviral, antibacterial, antifungal, and antiulcerogenic properties (Reina et al., 2013; Mashaly et al., 2019).

Many ecological studies have focused on the response of a species to changes in environmental conditions (Hegazy et al., 2010; Salama et al., 2018). The production of secondary metabolites

#Corresponding author email: profkhalafallah@gmail.com
Received 13 /09/ 2021; Accepted 19/12/2021
DOI: 10.21608/ejbo.2021.94145.1778
Edited by: Prof. Dr. Fawzy M. Salama, Faculty of Science, Assuit University, Assuit, Egypt.

is regarded as an acclimation strategy by which a plant species adapts to environmental changes within microhabitats at the same site or between specialized habitats (Telascrea et al., 2007; Rahimmalek et al., 2009). Among the many secondary metabolites present in the plant kingdom, phenolics and flavonoids are the most common (Slima et al., 2021). They are referred to as "eco-molecules" because they exhibit significant alterations under varying environmental conditions and are formed and accumulated as a plastic response to environmental limitations (del Valle et al., 2015; Sampaio et al., 2016). Furthermore, the antioxidant activity of certain phenolic and flavonoid compounds is related to their protective functions in plants against stress conditions (Nakabayashi et al., 2014; Roux et al., 2017). Few field investigations have been conducted to establish a link between plant productivity and metabolite accumulation (Hofmann & Jahufer, 2011). Research to estimate the secondary metabolites in a species, particularly free and total phenolics and flavonoids, to establish a link between them and the plant environment is currently lacking. Therefore, the present study intends to investigate the impacts of habitat change and seasonal fluctuations on the variations in the content of phytochemical compounds and antimicrobial activity of *P. major* in the Nile Delta region.

Materials and Methods

Study area

The Nile Delta, an ancient delta in northern Egypt, is a triangular region where the Nile River extends out and flows into the Mediterranean Sea (Shalaby, 2012). It covers over 22,000km^2 and accounts for approximately 63% of Egypt's agricultural land (Shehata, 2014). It is home to Egypt's most populous governorates, accounting for over half of the country's population (Haars et al., 2016). Plants were sampled in the governorates of Cairo, Giza, Kalyoubiya, Minofiya, Sharkiya, and Gharbiya, which are in the middle and south of the Nile Delta (Fig. 1).

The research sites are designated as semiarid; the rainy season is from December to May. The monthly average temperature ranges from 13.7°C to 17.4°C during the winter and 24.7°C–28.0°C during the summer. The average relative humidity is 46.35% in June and 70.18% in January. Climatic data over several years indicate that the highest average rainfall (1.04 mm) was observed in December (Fig. 2).

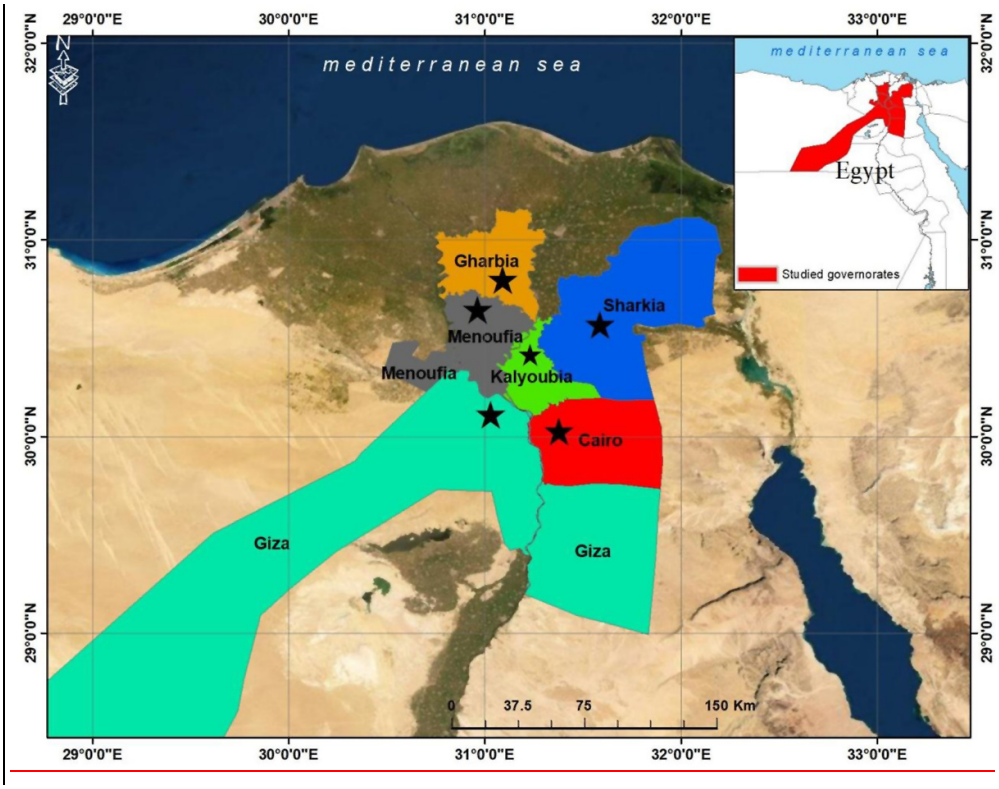

Fig. 1. Location map of the study area (modified from Google Earth on January 17, 2021)

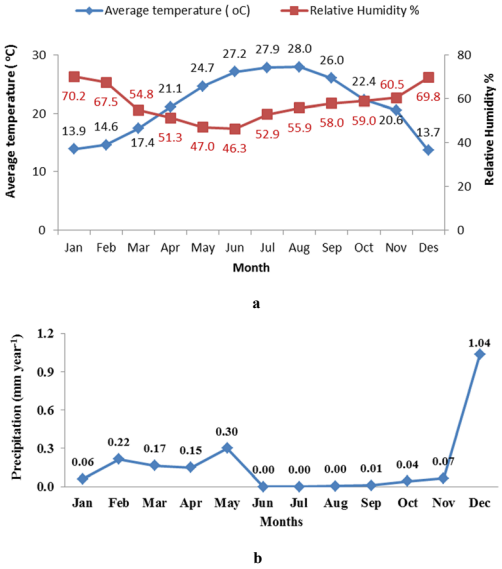

Fig. 2. Climatic data of the study area over 36 years (from 1979 to 2014) as obtained from https://climatedataguide.ucar.edu/climate-data/climate-forecast-system-reanalysis-cfsr [a) Average temperature and relative humidity and b) Average precipitation]

Phytochemical study

Plant sampling and analysis

Plantago major samples were collected seasonally from winter 2018 to autumn 2019 from eight different habitats (ditches, orchards, cultivated crops, field edges, fallow lands, canal banks, urban areas, and roadsides) in six Egyptian governorates: Cairo, Giza, Kalyoubiya, Minofiya, Sharkiya, and Gharbiya.

Three composite leaf samples were collected from each habitat during the four seasons for plant analysis. Plant materials were rinsed with tap water and then distilled water and air-dried in the shade at room temperature before being homogenized with a metal-free plastic mill.

Determination of cardiac glycosides

Cardiac glycosides were quantified using the methods described by Solich et al. (1992) and Tofighi et al. (2016).

Determination of total flavonoid content

Total flavonoid content (TFC) was calculated using the techniques describes by Solich et al. (1992) and Tofighi et al. (2016).

Equation (1) was used to determine the total content of flavonoids such as quercetin in the herbal material (mg g^{-1}; adjusted for moisture content), and TFC represents the average of three determinations.

$$TFC_{herbal\ material} = (TFC_{test\ solution} \times 1.25 \times 50)/(w\text{-}ld) \quad (1)$$

where, $TFC_{test\ solution}$ is the total concentration of flavonoids in the test solution (mg mL^{-1}), 1.25 corresponds to the dilution factor, 50 is the volume of the stock solution (mL), *w* is the weight of the herbal material (g), and *ld* is the weight loss on drying of the herbal material (g).

Determination of total phenolic content

Spectrophotometry was used to evaluate the total phenolic content in the ethanolic extract of the plant using the method described by Singleton et al. (1999) and Tofighi et al. (2016). For each analysis, the samples were used in triplicate, and the mean absorbance value was calculated. A calibration curve was constructed using the standard gallic acid solution. The concentration of phenolics (mg mL^{-1}) was estimated from the calibration curve on the basis of the measured absorbance.

Identification of phenolic and flavonoid compounds using high-performance liquid chromatography

Using the high-performance liquid chromatography (HPLC) technology, the flavonoid and phenolic components of *P. major* were estimated. The HPLC–mass spectrometry (MS) system comprised a quaternary pump, a photodiode array detector, a UV/Vis detector, and a single quadrupole MS detector with an ion source (Agilent 1100).

Flavonoids were separated in 70min using a gradient solvent system of 0.1% formic acid solution with a flow rate of 1.0mL/min, detection at 280 nm, and identification by ESI-MS (Schütz et al., 2005). Phenolic acid was separated in 60min using a gradient mobile phase of water/acetonitrile/glacial acetic acid (980:20:5, v/v/v; pH 2.68) and acetonitrile/glacial acetic acid (1000:5, v/v), with a flow rate of 3mL min^{-1} and detection at 325nm (Zheng & Clifford, 2008).

Biological activity

Preparation of extracts

Approximately 250g of plant powder was steeped in 1.25–1.50L of 95% ethanol, methanol, or hot water at room temperature for 5 days. The

mixtures were blended on a daily basis to obtain a consistent infusion. The extracts were filtered using Whatman filter paper no. 1 after 5 days. A rotary evaporator at 60°C was used to dry the filtrates. The dried extracts were kept at −20°C in sterile glass vials until further use (Kandil et al., 1994).

Microorganisms used

The following microorganisms were obtained from Ain Shams University, Faculty of Women's for Arts, Science and Education, Botany Department: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and fungal strains *Candida glabrata* and *C. albicans*. The bacterial and fungal strains were cultured in nutrient agar and malt extract, respectively.

In-vitro evaluation of the antimicrobial activity

Antimicrobial susceptibility testing was conducted using the agar plate well diffusion method. Plant extracts were tested against bacterial isolates as antimicrobial agents. The diameter of the inhibition zone was used to estimate the antimicrobial potential.

Inoculum suspensions of all bacterial and fungal isolates were spread on a surface medium. The agar plate well diffusion method was then used to investigate the antimicrobial activity. Using a 6 mm cork borer, wells were made into the medium. The dried plant extracts were dissolved in dimethylsulfoxide (DMSO) to make a 100mg mL^{-1} final extract. The well in each plate (well diameter 6 mm) was filled with 50L of plant extract. The inoculated agar plates were incubated for 24h at 37°C for bacterial strains and for 48h at 28°C for fungal strains. After 24–48h of incubation, the diameter of the inhibition zones caused by active extract components was measured. The experiment was conducted in triplicate, and the inhibition zone diameter was measured using a standard scale (Abd-El-Kader et al., 1995). DMSO, which has no antibacterial effect against the microbiological isolates, was used as a negative control.

Statistical analysis

After the data were validated for normality, the differences in plant properties across different habitats over four seasons were evaluated using a two-way analysis of variance (ANOVA-2) using SPSS software (SPSS, 2016). A post-hoc test (Duncan's test) was used when significant differences were detected among habitats or seasons.

Results

Chemical constituents

The phytochemical screening of the ethanolic and chloroform extracts of *P. major* collected from different habitats during different seasons demonstrated the presence of cardiac glycosides, flavonoids, and phenolic compounds (Table 1). ANOVA-2 indicated a significant effect of different habitats, seasons, and their interaction on the TFC in *P. major* leaves; the total phenolic content did not differ significantly among the four seasons. Additionally, the cardiac glycoside content did not significantly differ among the study habitats. The highest average TFC over four seasons (22.7mg g^{-1}) was observed in field edges, whereas the lowest average TFC over four seasons (6.5mg g^{-1}) was observed in canal banks. The highest average cardiac glycoside and phenolic contents over four seasons (18.1 and 31.8mg g^{-1}, respectively) were observed in urban habitats and canal banks, respectively, whereas the lowest average values over four seasons (12.0 and 17.1mg g^{-1}, respectively) were observed in ditches. The highest cardiac glycoside content and TFC (31.2 and 51.2mg g^{-1}, respectively) were observed in *P. major* samples from urban habitats and cultivated crops, respectively, during winter, whereas the lowest values (0.2 and 2.6mg g^{-1}, respectively) were observed in plant samples from ditches and urban habitats, respectively, during spring. The highest total phenolic content (43.2mg g^{-1}) was observed in plants from fallow lands during summer, whereas the lowest (2.9mg g^{-1}) was observed in plants from ditches during spring.

Phenolic compounds

The phenolic compounds ellagic acid, catechol, resorcinol, gallic acid, and phloroglucinol in *P. major* extract were separated and identified using HPLC (Table 2). ANOVA-2 detected a significant effect of habitat on the content of phenolic compounds in *P. major* leaves, except catechol and phloroglucinol, and a significant effect of season on the content of resorcinol and gallic acid, in addition to a significant effect of the interaction between habitats and seasons on the content all phenolic compounds. It was found that plants collected from urban habitats had the highest contents of ellagic acid and phloroglucinol (18.4 and 14.7mg g^{-1}), whereas fallow land plants

had the highest gallic acid content (20.7mg g^{-1}). Additionally, plants collected from cultivated crops had the highest resorcinol content (23.0mg g^{-1}) but the lowest catechol content (10.5mg g^{-1}), and those along canal banks had the highest catechol content (20.7mg g^{-1}) but the lowest ellagic acid content (6.1mg g^{-1}). Plants collected from ditches had the lowest gallic acid content (6.9mg g^{-1}), those from field edges had the lowest resorcinol content (6.0mg g^{-1}), and those from orchards had the lowest phloroglucinol content (6.2mg g^{-1}).

The highest contents of ellagic acid and phloroglucinol (39.5 and 32.5mg g^{-1}, respectively) were found in plants from urban habitats during summer, whereas the lowest (1.6 and 1.3mg g^{-1}, respectively) were observed in plants from field edges during spring. Additionally, the highest contents of catechol and resorcinol (41.6 and 40.3mg g^{-1}, respectively) were found in plants from field edges and cultivated crops during winter, whereas the highest gallic acid content (45.9mg g^{-1}) was observed in plants of fallow lands during autumn. The lowest contents of resorcinol and gallic acid (5.1 and 4.7mg g^{-1}, respectively) were found in plants from fallow lands and canal banks during spring, whereas the lowest catechol content (2.3mg g^{-1}) was observed in those from orchards during summer.

Flavonoid compounds

The separated flavonoid compounds were identified as follows: apigenin, luteolin, chrysoeriol, rutin, quercetin, kaempferol, and avicularin (Table 3). Habitats had a significant effect on the content of flavonoids in *P. major* leaves, except luteolin and rutin; seasons had a significant effect on apigenin and quercetin content; and the interaction between habitats and seasons had a significant effect on the content of flavonoids, except luteolin and quercetin. Plants along the canal banks showed the highest contents of chrysoeriol, rutin, and kaempferol (31.5, 19.4, and 27.8mg g^{-1}, respectively), whereas those from urban habitats had the highest luteolin and quercetin contents (21.3 and 22.3mg g^{-1}, respectively). Plants from ditches had the highest apigenin content (24.4mg g^{-1}) but the lowest quercetin content (7.6mg g^{-1}), and fallow land plants had the highest avicularin content (3.6mg g^{-1}) but the lowest apigenin, luteolin, and chrysoeriol contents (1.8, 10.5, and 14.7mg g^{-1}, respectively).

It was found that the highest contents of chrysoeriol, quercetin, and kaempferol (54.2, 50.2, and 37.1mg g^{-1}, respectively) were observed in plants from urban habitats, orchards, and fallow lands during autumn, whereas the highest apigenin and avicularin contents (45.2 and 14.2mg g^{-1}, respectively) were found in plants from urban habitats and fallow lands during winter. Additionally, the highest contents of luteolin and rutin (41.4 and 35.4mg g^{-1}, respectively) were found in plants from orchards and field edges during summer. The lowest apigenin and rutin contents (7.3 and 4.0mg g^{-1}, respectively) were observed in plants from fallow lands and canal banks during autumn, whereas the lowest chrysoeriol and quercetin contents (2.7 and 2.1mg g^{-1}, respectively) were found in plants from fallow lands and orchards during spring. The lowest luteolin and avicularin contents (6.9 and 2.4mg g^{-1}, respectively) were observed in plants from ditches and orchards during winter, but the lowest kaempferol content (8.6mg g^{-1}) was found in plants from orchards during summer.

In vitro antimicrobial activity of *Plantago major* extracts

As shown in Table 4 and Plate 1, *S. aureus* was moderately sensitive to the ethanolic extract (95%) of *P. major* leaves from all habitats except roadsides, which showed no antimicrobial effect, whereas *P. aeruginosa* was moderately sensitive to the ethanolic extract (95%) of *P. major* leaves from only urban habitats. The largest inhibition zone diameter (15mm) was observed for the ethanolic extract of plant leaves from field edges, whereas the lowest (10mm) was observed for the ethanolic extracts of plants from ditches and fallow lands against *S. aureus*. By contrast, the fungal strains were resistant to all ethanolic extracts.

The bacterial strain *S. aureus* was sensitive to the methanolic extracts of *P. major* leaves from the different habitats, with the inhibition zone diameter ranging between 11.0 (ditches) and 19mm (orchards). *P. aeruginosa* was sensitive to the methanolic extracts of plants from canal banks, cultivated crops, and field edges, with inhibition zone diameters of 20.0, 10.5, and 15.0mm, respectively (Table 5 and Plate 2). The fungal strain *C. glabrata* was moderately sensitive to the methanolic extract of plants only from ditches, with an inhibition zone diameter of 12.0mm. By contrast, *C. albicans* was resistant to all methanolic extracts.

TABLE 1. Seasonal variation in the contents of chemical constituents (cardiac glycosides, total flavonoids, and total phenolic compounds; mean ± SD) of *Plantago major* leaves from different habitats

Chemical constituent (mg g^{-1})	Season	Habitat							F-value		
		Ditches	Urban	Fallow lands	Canal banks	Cultivated crops	Field edges	Orchards	Habitat	Season	H × S
Cardiac glycosides	Aut	22.2 ± 4.2	31.2 ± 7.5	8.1 ± 2.1	18.2 ± 4.1	21.2 ± 4.6	11.1 ± 3.2	19.1 ± 5.4	**1.0**	**5.1***	**53.3*****
	Win	18.2 ± 3.4	12.1 ± 2.4	23.5 ± 6.2	19.5 ± 3.2	16.2 ± 3.7	17.1± 6.1	26.0 ± 6.1			
	Spr	0.2 ± 0.1	ND	4.5 ± 1.4	1.0 ± 0.2	11.4 ± 2.3	26.4 ± 8.3	17.3 ± 4.6			
	Sum	7.5 ± 1.2	29.1 ± 5.2	24.6 ± 5.1	10.2 ± 2.1	15.1 ± 4.2	ND	2.2 ± 0.5			
Habitat average		12.0 ± 3.1a	18.1 ± 6.3a	15.2 ± 3.3a	12.2 ± 4.2a	16.0 ± 3.7a	13.6 ± 3.9a	16.1 ± 5.1a			
Total flavonoids	Aut	19.1 ± 5.1	41.1 ± 9.5	30.1 ± 7.3	5.2 ± 1,3	51.2 ± 9.4	25.6 ± 5.4	7.7 ± 2.6	**2.7***	**9.8****	**49.0*****
	Win	4.6 ± 1.3	3.0 ± 1.1	15.2 ± 2.5	5.2 ± 1.5	14.4 ± 3.5	12.4 ± 1.6	15.3 ± 3.5			
	Spr	24.0 ± 7.2	2.6 ± 0.8	ND	13.3 ± 5.1	14.2 ± 3.2	35.0 ± 8.2	24.5 ± 4.9			
	Sum	19.5 ± 6.1	28.6 ± 4.6	13.8 ± 4,1	2.5 ± 0.7	6.2 ± 2.1	17.7 ± 5.3	25.6 ± 6.1			
Habitat average		16.8 ± 4.6a	18.8 ± 5.3a	14.8 ± 3.1ab	6.5 ± 2.1b	21.5 ± 6.2a	22.7 ± 6.4a	18.3 ± 6.1a			
Total phenolics	Aut	35.1 ± 6.7	27.4 ± 5.3	15.0 ± 3.2	25.1 ± 4.4	28.2 ± 3.5	31.8 ± 7.2	10.4 ± 1.7	**3.7****	**0.6**	**39.5*****
	Win	17.1 ± 4.1	5.0 ± 1.3	19.5 ± 5.4	28.3 ± 6.4	9.1 ± 2.3	29.1 ± 5.4	33.0 ± 5.3			
	Spr	2.9 ± 0.9	23.3 ± 6.1	5.0 ± 1.1	34.3 ± 7.1	25.6 ± 6.1	19.4 ± 2.6	29.6 ± 4.2			
	Sum	13.1 ± 3.1	22.3 ± 4.2	43.2 ± 9.1	39.6 ± 8.2	10.4 ± 3.1	8.5 ± 1.3	15.1 ± 2.9			
Habitat average		17.1 ± 5.1b	19.5 ± 5.9b	20.7 ± 6.2b	31.8 ± 5.8a	18.3 ± 5.1b	22.2 ± 7.2b	22.0 ± 6.9b			

*: P < 0.05, **: P< 0.01, ***: P< 0.001.

Means with the same letters are not significantly different (Duncan's test).

ND: Not detected, Aut: autumn, Win: winter, Spr: spring, Sum: summer. Maximum and minimum values are underlined.

TABLE 2. Seasonal variation in the phenolic content (mean ± SD) of *Plantago major* leaves collected from different habitats

Phenolic content (mg g^{-1})	Season	Habitat							F-value		
		Ditches	Urban	Fallow lands	Canal banks	Cultivated crops	Field edges	Orchards	Habitat	Season	H × S
Ellagic acid	Aut	21.7 ± 5.1	17.6 ± 4.6	15.2 ± 3.3	14.2 ± 2.9	19.7 ± 3.7	31.0 ± 7.1	34.6 ± 9.2			
	Win	28.0 ± 6.7	ND	22.0 ± 6.3	10.3 ± 1.8	8.6 ± 2.1	0.0	30.1 ± 4.9			
	Spr	ND	16.6 ± 4.5	9.2 ± 2.9	ND	24.4 ± 5.9	1.6 ± 00.4	ND	**2.3***	**1.3**	**21.0*****
	Sum	ND	39.5 ± 8.7	15.0 ± 4.2	ND	17.1 ± 3.6	ND	7.2 ± 2.1			
Habitat average		**12.4 ± 3.5ab**	**18.4 ± 4.6a**	**15.4 ± 3.7ab**	**6.1 ± 2.1b**	**17.4 ± 3.4a**	**8.2 ± 1.7ab**	**18.0 ± 3.3a**			
Catechol	Aut	15.2 ± 2.8	23.7 ± 4.2	ND	10.3 ± 2.1	20.5 ± 4.5	8.6 ± 2.1	ND			
	Win	22.6 ± 6.1	ND	26.1 ± 8.4	28.6 ± 5.2	ND	41.6 ± 9.2	32.6 ± 6.8			
	Spr	6.5 ± 1.4	22.1 ± 5.7	ND	8.5 ± 1.9	21.5 ± 5.6	9.4 ± 2.6	20.2 ± 4.8	**1.2**	**0.3**	**15.1*****
	Sum	14.5 ± 3.1	ND	21.5 ± 4.6	35.6 ± 7.6	ND	15.1 ± 3.1	2.3 ± 0.5			
Habitat average		**14.7 ± 4.1a**	**11.5 ± 2.7a**	**11.9 ± 2.3a**	**20.7 ± 4.2a**	**10.5 ± 2.1a**	**18.7 ± 4.3a**	**13.8 ± 3.6a**			
Resorcinol	Aut	11.3 ± 2.4	19.6 ± 5.1	38.1 ± 8.9	21.1 ± 5.1	33.5 ± 9.2	24.1 ± 5.1	38.6 ± 8.5			
	Win	35.5 ± 6.8	ND	6.9 ± 1.7	ND	40.3 ± 10.2	ND	22.1 ± 6.2			
	Spr	12.0 ± 3.1	ND	5.1 ± 1.5	ND	32.1 ± 7.3	ND	6.1 ± 1.9	**3.6****	**6.0****	**10.3****
	Sum	13.8 ± 3.9	13.5 ± 2.8	ND	7.5 ± 1.6	10.2 ± 1.6	ND	7.5 ± 2.2			
Habitat average		18.1 ± 3.9ab	9.0 ± 2.1bc	12.5 ± 3.1abc	7.2 ± 1.8c	23.0 ± 6.2a	6.0 ± 1.1c	18.6 ± 4.4ab			
Gallic acid	Aut	ND	27.6 ± 6.8	45.9 ± 11.3	14.6 ± 3.1	29.0 ± 6.7	33.5 ± 9.4	ND			
	Win	19.5 ± 5.2	7.7 ± 1.9	14.5 ± 2.8	31.5 ± 6.5	ND	12.0 ± 3.0	ND			
	Spr	ND	17.2 ± 4.3	13.1 ± 3.1	4.7 ± 1.2	15.9 ± 2.9	14.6 ± 4.1	17.3 ± 3.5	**3.5****	**4.5****	**15.2*****
	Sum	8.1 ± 1.9	24.1 ± 7.2	9.4 ± 2.6	13.2 ± 3.2	ND	10.3 ± 1.8	10.9 ± 1.7			
Habitat average		6.9 ± 12b	19.1 ± 4.8a	20.7 ± 5.3a	16.0 ± 3.8ab	11.2 ± 2.4ab	17.6 ± 3.5a	7.1 ± 1.2b			
Phloroglucinol	Aut	31.9 ± 6.8	15.7 ± 3.2	ND	7.3 ± 1.3	ND	12.5 ± 3.4	10.1 ± 2.4			
	Win	8.6 ± 1.3	ND	10.7 ± 2.2	9.7 ± 2.5	14.6 ± 4.3	17.6 ± 5.4	ND			
	Spr	7.6 ± 1.8	10.4 ± 1.9	ND	27.2 ± 6.6	ND	1.3 ± 0.3	ND	**1.7**	**1.1**	**20.2*****
	Sum	ND	32.5 ± 9.3	29.6 ± 8.4	ND	14.3 ± 2.7	ND	14.7 ± 3.1			
Habitat average		12.0 ± 2.9a	14.7 ± 3.2a	10.1 ± 2.2a	11.0 ± 2.4a	7.2 ± 1.6a	7.8 ± 2.1a	6.2 ± 0.9a			

*: P < 0.05, **: P< 0.01, ***: P< 0.001.

Means with the same letters are not significantly different (Duncan's test).

ND: Not detected, Aut: autumn, Win: winter, Spr: spring, Sum: summer. Maximum and minimum values are underlined.

TABLE 3. Seasonal variation in the flavonoid content (mean ± SD) of *Plantago major* leaves collected from different habitats

Flavonoid content (mg g^{-1})	Season	Habitat							F-value		
		Ditches	Urban	Fallow lands	Canal banks	Cultivated crops	Field edges	Orchards	Habitat	Season	Habitat × Season
Apigenin	Aut	11.5 ± 3.2	19.3 ± 3.6	7.3 ± 1.6	ND	ND	ND	ND	**9.7*** **	**4.3***	**6.1***
	Win	32.1 ± 6.4	45.2 ± 9.5	ND	9.5 ± 2.1	11.5 ± 3.1	27.1 ± 7.1	22.1 ± 4.6			
	Spr	34.6 ± 9.1	10.4 ± 2.4	ND	ND	14.9 ± 4.2	ND	ND			
	Sum	19.2 ± 4.2	14.6 ± 3.3	ND	25.5 ± 5.4	33.2 ± 6.7	29.4 ± 8.2	ND			
Habitat average		24.4 ± 5.4a	22.4 ± 5.1ab	1.8 ± 0.4d	8.8 ± 2.4cd	14.9 ± 3.4abc	14.1 ± 4.6bc	5.5 ± 1.1cd			
Luteolin	Aut	7.3 ± 1.4	29.4 ± 7.3	14.0 ± 4.1	24.7 ± 6.4	19.5 ± 3.2	21.6 ± 6.4	ND	**0.9**	**1.6**	**0.2**
	Win	6.9 ± 1.2	12.6 ± 2.8	17.2 ± 4.0	ND	18.5 ± 4.0	11.3 ± 1.8	ND			
	Spr	28.1 ± 6.1	33.3 ± 8.7	ND	13.3 ± 2.9	12.5 ± 2.5	24.6 ± 4.7	15.1 ± 2.8			
	Sum	15.7 ± 3.6	9.9 ± 2.3	10.8 ± 1.8	23.7 ± 4.6	ND	ND	41.4 ± 6.9			
Habitat average		14.6 ± 3.3a	21.3 ± 4.6a	10.5 ± 2.7a	15.4 ± 3.6a	12.6 ± 2.1a	14.4 ± 3.8a	14.1 ± 3.2a			
Chrysoeriol	Aut	ND	54.2 ± 9.7	ND	42.5 ± 10.2	20.6 ± 4.1	15.6 ± 5.2	ND	**2.3***	**1.8**	**21.6*** **
	Win	50.4 ± 11.2	ND	17.5 ± 4.8	38.2 ± 7.9	27.5 ± 4.9	39.8 ± 8.9	25.5 ± 4.8			
	Spr	ND	31.2 ± 7.2	2.7 ± 0.6	ND	14.1 ± 2.9	17.8 ± 6.1	16.1 ± 3.6			
	Sum	ND	15 ± 3.1	38.7 ± 8.3	45.3 ± 6.9	ND	28.5 ± 5.9	19.3 ± 5.3			
Habitat average		12.6 ± 2.6b	25.1 ± 5.8ab	14.7 ± 4.3b	31.5 ± 7.8a	15.5 ± 2.9b	25.4 ± 5.6ab	15.2 ± 3.1b			
Quercetin	Aut	25.0 ± 5.1	ND	26.2 ± 4.9	ND	13.6 ± 3.3	ND	50.2 ± 10.8	**3.0***	**6.4***	**1.9**
	Win	ND	12.9 ± 3.4	ND	16.1 ± 4.8	ND	37.4 ± 9.8	18.2 ± 3.6			
	Spr	ND	35.9 ± 7.1	ND	19.4 ± 5.3	18.0 ± 5.7	8.2 ± 1.8	2.1 ± 0.6			
	Sum	5.4 ± 1.2	40.2 ± 8.9	10.2 ± 2.3	18.4 ± 3.7	33.2 ± 6.4	26.4 ± 7.1	ND			
Habitat average		7.6 ± 1.4b	22.3 ± 5.8a	9.1 ± 3.0ab	13.5 ± 3.7ab	16.2 ± 4.1ab	18.0 ± 4.9ab	17.6 ± 4.5ab			

TABLE 3. Cont.

Flavonoid content (mg g^{-1})	Season	Habitat							F-value		
		Ditches	Urban	Fallow lands	Canal banks	Cultivated crops	Field edges	Orchards	Habitat	Season	Habitat × Season
Rutin	Aut	26.4 ± 6.0	ND	22.1 ± 4.8	4.0 ± 1.1	8.5 ± 2.1	ND	14.6 ± 3.2	**0.9**	**0.8**	**18.1*** **
	Win	17.6 ± 4.1	24.1 ± 5.7	18.5 ± 3.9	20.2 ± 4.3	15.7 ± 3.5	24.2 ± 6.2	19.1 ± 4.3			
	Spr	19.9 ± 5.2	ND	5.6 ± 1.5	19.0 ± 5.1	23.6 ± 4.1	ND	8.5 ± 1.9			
	Sum	ND	27.7 ± 6.8	15.1 ± 3.1	34.5 ± 8.2	ND	35.4 ± 9.1	14.2 ± 4.1			
Habitat average		16.0 ± 4.5a	12.9 ± 3.2a	15.3 ± 3.8a	19.4 ± 5.1a	11.9 ± 2.5a	14.9 ± 2.7a	14.1 ± 2.6a			
Kaempferol	Aut	ND	15.6 ± 1.9	24.3 ± 6.1	37.1 ± 7.1	ND	20.3 ± 6.1	22.5 ± 5.2	**5.8****	**1.1**	**24.4*** **
	Win	33.1 ± 4.9	18.4 ± 3.4	ND	18.1 ± 3.1	14.5 ± 1.9	ND	11.2 ± 1.8			
	Spr	13.1 ± 2.0	10.4 ± 1.8	ND	34.6 ± 6.3	10.4 ± 1.1	20.0 ± 3.9	ND			
	Sum	ND	14.0 ± 3.6	26.0 ± 4.3	21.3 ± 5.4	18.1 ± 4.3	ND	8.6 ± 1.1			
Habitat average		11.5 ± 3.2b	14.6 ± 4.3b	12.6 ± 3.5b	27.8 ± 6.2a	10.7 ± 2.1b	10.1 ± 1.8b	10.6 ± 1.8b			
Avicularin	Aut	ND	ND	ND	ND	ND	ND	ND	**2.8***	**1.4**	**4.8***
	Win	ND	10.5 ± 2.1	14.2 ± 3.5	ND	ND	ND	2.4 ± 0.7			
	Spr	ND	ND	ND	ND	ND	ND	ND			
	Sum	ND	ND	ND	ND	ND	ND	ND			
Habitat average		ND	2.6 ± 0.6ab	3.6 ± 0.9a	ND	ND	ND	0.6 ± 0.1b			

*: P < 0.05, **: P< 0.01, ***: P< 0.001.

Means with the same letters are not significantly different (Duncan's test).

ND: Not detected, Aut: autumn, Win: winter, Spr: spring, Sum: summer. Maximum and minimum values are underlined.

TABLE 4. Antimicrobial activity of ethanolic extracts of *Plantago major* leaves collected from different habitats against pathogenic bacterial and fungal strains

Habitat	Inhibition zone diameter (mm)			
	Bacteria		Fungi	
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Candida glabrata*	*Candida albicans*
Ditches	10.0	0	0	0
Urban	12.0	12.5	0	0
Fallow lands	10.0	0	0	0
Canal banks	12.0	0	0	0
Cultivated crops	13.0	0	0	0
Field edges	15.0	0	0	0
Orchards	14.0	0	0	0
Roadsides	0	0	0	0

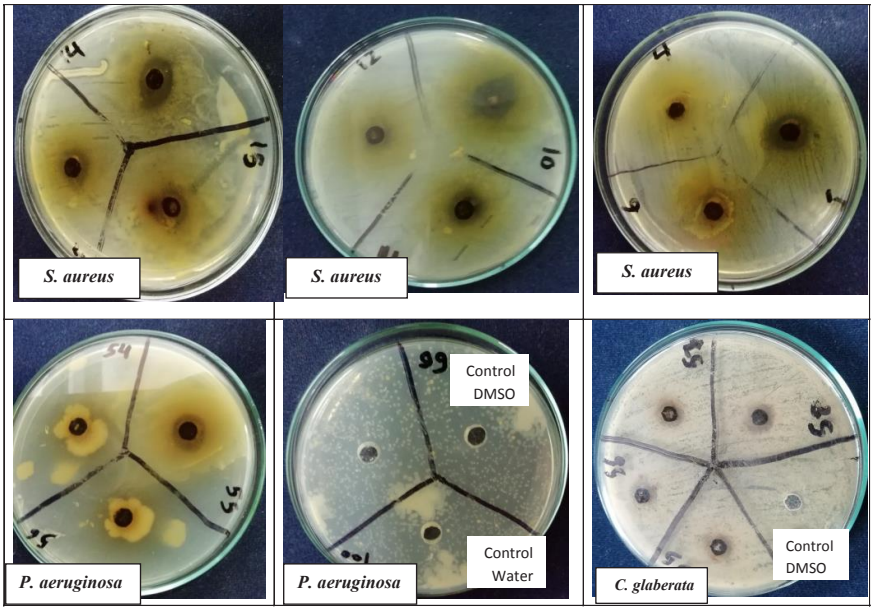

Plate 1. Antimicrobial activity of ethanolic extracts of *Plantago major* leaves collected from different habitats against pathogenic bacterial and fungal strains

TABLE 5. Antimicrobial activity of methanolic extracts of *Plantago major* leaves collected from different habitats on pathogenic bacterial and fungal strains

Habitat	Inhibition zone diameter (mm)			
	Bacteria		Fungi	
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Candida glabrata*	*Candida albicans*
Ditches	11.0	0	12.0	0
Urban	12.0	0	0	0
Fallow lands	12.0	0	0	0
Canal banks	12.5	20.0	0	0
Cultivated crops	17.0	10.5	0	0
Field edges	11.5	15.0	0	0
Orchards	19.0	0	0	0
Roadsides	15.0	0	0	0

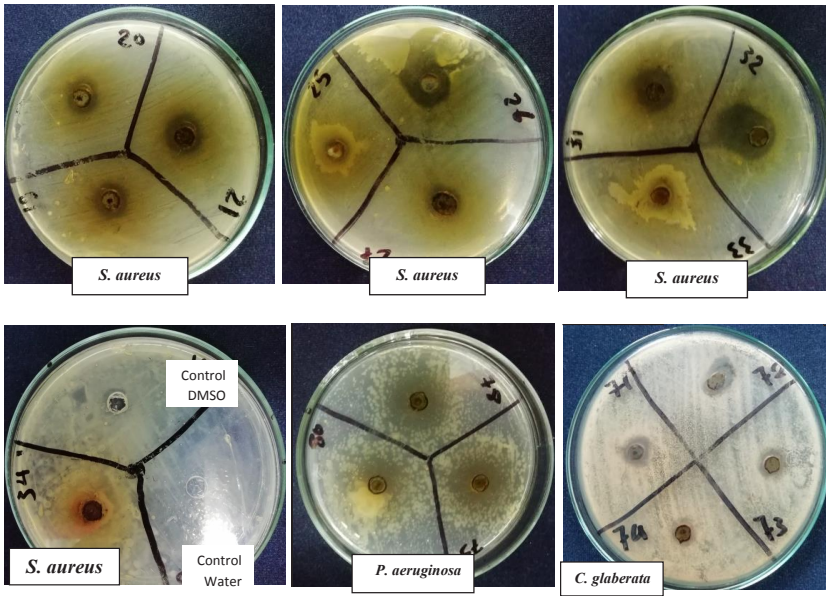

Plate 2. Antimicrobial activity of methanolic extracts of *Plantago major* leaves collected from different habitats against pathogenic bacterial and fungal strains

As shown in Table 6 and Plate 3, *S. aureus* was sensitive to the aqueous extract of *P. major* plants from ditches, urban habitats, field edges, and orchards and showed inhibition zone diameters of 11, 10, 16, and 9mm, respectively. *P. aeruginosa* and *C. glabrata* were moderately sensitive to the aqueous extract of plant leaves only from orchards and showed inhibition zones 12.0 mm in diameter. By contrast, *C. albicans* was resistant to all aqueous extracts.

TABLE 6. Antimicrobial activity of aqueous extracts of *Plantago major* leaves collected from different habitats against pathogenic bacterial and fungal strains

Habitat	Inhibition zones diameter (mm)			
	Bacteria		Fungi	
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Candida glabrata*	*Candida albicans*
Ditches	11	0	0	0
Urban	10	0	0	0
Fallow lands	0	0	0	0
Canal banks	0	0	0	0
Cultivated crops	0	0	0	0
Field edges	16	0	0	0
Orchards	9	12	12	0
Roadsides	0	0	0	0

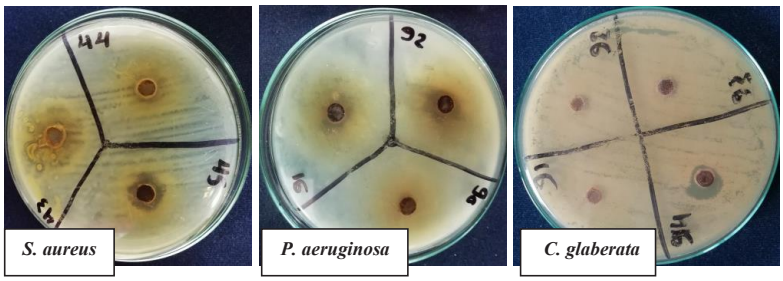

Plate 3. Antimicrobial activity of aqueous extracts of *Plantago major* leaves collected from different habitats against pathogenic bacterial and fungal strains

Discussion

To confirm the nutritional and medicinal value of plants, the determination of the phytochemical compounds (cardiac glycosides and total phenolic and flavonoid compounds) is important (Alzletni et al., 2020). Although plant metabolite production is driven by genetic factors, it is also influenced by developmental stage, functionally distinct plant organs, and environmental factors (Mala et al., 2008; El-Bakry et al., 2014). Accordingly, significant variation in the concentration of phytochemical compounds was observed in *P. major* leaves among different habitats and seasons. These results agree with those reported by Lamien-Meda et al. (2010) for *Salvia officinalis* and Farhat et al. (2013) for *Salvia* species. They attributed these differences to climatic and geographical factors, vegetative phase, and genotype. *P. major* leaves from urban habitats and cultivated crops had the highest cardiac glycoside content and TFC during winter, which may be attributed to the heat sensitivity of these compounds (Zubair et al., 2011; Mazzutti et al., 2017). By contrast, the highest total phenolic content was observed in fallow lands during summer, consistent with the findings reported by Tamura and Nishibe (2002), who observed an increase in phenolic compounds with increasing drought stress.

The average phenolic and flavonoid contents in *P. major* leaves were 2.44- and 3.55-fold higher than those reported by Kobeasy et al. (2011) for the same species. However, Al-Obaidi (2020) reported higher phenolic content but lower flavonoid content than those observed in the present study. Moreover, the cardiac glycoside and flavonoid contents in *P. major* from different habitats were greater than those observed in the medicinal plant *Calotropis procera* by El-Bakry et al. (2014). Cardiac glycosides are a group of secondary metabolites, traditionally used to increase the cardiac contractile force in patients with cardiac arrhythmia or congestive heart failure (Abarquez, 2001). The present study showed significant habitat-related and seasonal variation in cardiac glycoside, flavonoid, and phenolic contents, which indicates significant polymorphism with habitat heterogeneity. The variation in the phytochemical composition suggests that *P. major* populations exhibit phytochemical polymorphism, which agrees with the findings reported by Mala et al. (2008) in *Calotropis procera*.

Apart from being ecologically significant, flavonoids are effective antioxidants that assist in neutralizing hazardous free radicals and prevent oxidative stress, which damages cells and DNA and leads to aging and degenerative diseases such as cancer, Alzheimer's disease, and Parkinson's disease (El-Bakry et al., 2014). Furthermore, flavonoids are known to improve the effects of other antioxidant vitamins.

The flavonoid and phenolic compounds detected in *P. major* extracts using HPLC were similar to those reported by Beara et al. (2009), Jankovic et al. (2012), and Mesquita et al. (2017); they found that *P. major* contained flavonoids such as luteolin, apigenin, rutin, mangiferin, and quercetin. Moreover, Kobeasy et al. (2011) identified flavonoid compounds such as luteolin, kaempferol, and rutin and phenolic compounds such as ferulic acid, caffeic acid, gallic acid, and vanillic acid. In the present study, the contents of catechol, gallic acid, luteolin, quercetin, and rutin were higher and the ellagic acid content was lower than those reported by Kobeasy et al. (2011) in the same species. However, Alzletni et al. (2020) reported higher contents of flavonoids (except avicularin) and phenolic compounds (except phloroglucinol) in the aboveground tissues of the medicinal plant *Malva parviflora* in some of the habitats than those observed in the present study.

High contents of ellagic acid, kaempferol, and quercetin were observed in summer and autumn (during which highest temperatures and lowest relative humidity were observed); these compounds have the potential to mitigate the osmotic stress in plants (Chaves et al., 1997; Hofmann & Jahufer, 2011; Abu El-Soud et al., 2013). Additionally, ellagic acid is an important compound used as an anticarcinogenic agent (Mirsane & Mirsane, 2017), multifunctional protective agent against oxidative stress (Galano et al., 2014), and anti-inflammatory agent in the treatment of chronic ulcerative colitis (Kunzmann et al., 2015; Ceci et al., 2016) and antibacterial and antifungal activity (Hassan et al., 2021). It is reported to be present in higher quantities in raspberries, strawberries, walnuts, pecans, cranberries, and other plant-based foods (Bala et al., 2006) than the maximum values observed in the present study. Similarly, Ereifej et al. (2015) reported that gallic acid has many biological activities such as antioxidant activity (scavenging free radicals and protecting against oxidative

stress) and antimicrobial, anti-inflammatory, antimutagenic, and anticancer activities (You et al., 2011; Daglia, 2012; Yao et al., 2017; Goszcz et al., 2017; Perazzoli et al., 2017). Gallic acid also has anti-obesity properties, making it useful in lipid-related diseases including fatty liver disease (Roche et al., 2017), and antibiofilm activity against *S. aureus* (Liu et al., 2016).

According to Tchinda et al. (2014), flavonoids such as luteolin, apigenin, catechin, kaempferol, and quercetin are found in spices and are a key source of antioxidants with potential health value. Quercetin has several health-promoting properties, including anti-cancer properties, strong vasodilatory properties, anti-asthmatic properties, and anti-inflammatory properties (Kumar et al., 2017). It provides antioxidant protection by scavenging free radicals and limiting the oxidation of different molecules and by combating aging and inflammation (Lin et al., 2012). Moreover, quercetin has been reported to be effective against viruses such as herpes simplex type I, para-influenza type III, respiratory syncytial virus, and cardiovirus (Kumar et al., 2017). Additionally, quercetin, luteolin, and apigenin inhibit the viability of leukemic cells, ovarian carcinoma cells, colon cancer cells, and human breast cancer cells (Oto et al., 2011; Adom et al., 2017) and reduce the occurrence of mouth sores and induce mild symptomatic relief (Sharma & Gupta, 2010; Oto et al., 2011; Adom et al., 2017).

The antibacterial activity of a plant depends on the phytogeographical region, plant organ, and method of extraction (Fons et al., 2008; zkan et al., 2009; Metiner et al., 2012). Using the disc diffusion method, Kahyaolu and Türkolu (2008) investigated the antimicrobial effectiveness of several plants, including *P. major* in Elaz district, against *P. aeruginosa*, *S. aureus*, and *Candida spp.* They found that an ethanolic extract of *P. major* was effective against *S. aureus*. In agreement with the present findings, Samuelsen (2000) reported that the antifungal activity (against *C. albicans*) of *P. major* was weaker than its antibacterial activity (against *S. aureus*). However, Sharma et al. (2016) reported that *P. major* ethanolic extract has acceptable potency against the fungus *C. albicans* as compared with synthetic antifungal agents. The present study demonstrated stronger antibacterial activity of the ethanolic extract of *P. major* leaves from the habitat field edges against *S. aureus* and *P. aeruginosa* than against other microbes; these results are in agreement with those reported by Kahyaoğlu et al. (2008) and Stanisavljevic et al. (2008). However, weak or no antibacterial activity of the ethanolic extract of *P. major* leaves against *S. aureus* and *P. aeruginosa* was reported by Samuelsen (2000) and Nazarizadeh et al. (2013), respectively.

Notably, the methanolic extracts showed stronger antimicrobial activity than the ethanolic extracts, which is consistent with the findings reported by Samuelsen et al. (2000), Hoj et al. (2001), Sharifa et al. (2008), and Adom et al. (2017). Abd Razik et al. (2012) reported noticeable antibacterial activity of the methanolic extract of *P. major* leaves, whereas Samuelsen (2000) observed low antimicrobial activity against *S. aureus*, which is methicillin resistant. The methanolic and ethanolic extracts of *P. major* (100 mg mL^{-1}) showed antimicrobial activity against gram-positive and gram-negative bacteria. Likewise, Sharifa et al. (2008) and Adom et al. (2017) found that 100–200 mg mL^{-1} extracts showed antimicrobial activity. Moreover, the weak antimicrobial activity of the aqueous extract of *P. major* leaves against bacteria and fungi is in agreement with the findings by Velasco-Lezama et al. (2006). Similarly, Hetland et al. (2000), Sharifa et al. (2008), and Adom et al. (2017) observed no antimicrobial activity of the aqueous extract. The main finding of the present study was that *P. major* leaf extracts showed antimicrobial activity against gram-positive and gram-negative bacteria but weak or no antimicrobial activity against fungi; these results are similar to the findings reported by Samuelsen (2000) and Abd Razik et al. (2012), who attributed the antimicrobial activity to the presence of some intermediately polar or nonpolar substances with relatively low molecular weight in the plant extract.

The ethanolic and aqueous extracts of *P. major* leaves from the habitat field edges demonstrated strong antibacterial activity against *S. aureus*, whereas the methanolic extract of plant leaves from orchards and canal banks showed the highest activity against *S. aureus* and *P. aeruginosa*, respectively. Moreover, the aqueous extract of *P. major* leaves from orchards showed the highest activity against *P. aeruginosa* and *C. glabrata*.

The results of antimicrobial investigation are significant in pharmaceutical and medical

applications to inhibit the growth of *S. aureus*, which is well known for its resistance to diverse phytochemical substances and for its ability to produce various enterotoxins that cause gastroenteritis (Halpin-Dohnalek and Marth, 1989). Therefore, pharmaceutical studies are required to separate, purify, and identify the phytochemical compounds in the ethanolic, methanolic, and aqueous extracts of *P. major* leaves to investigate the antibacterial activity of each compound to identify the compounds that exert antibacterial activity against *S. aureus* and *P. aeruginosa*.

Conclusion

The present study showed that the contents of cardiac glycosides, phenolics, and flavonoids in *P. major* leaves collected from different habitats were greater than those reported in other medicinal plants. The HPLC analysis of *P. major* leaf extract indicated that it contained flavonoids such as luteolin, apigenin, rutin, kaempferol, avicularin, chrysoeriol, and quercetin in most of the studied habitats and seasons. However, phenolics (ellagic acid, catechol, resorcinol, gallic acid, and phloroglucinol) were detected in all habitats. These flavonoid and phenolic compounds have the potential to treat many human diseases. Additionally, *P. major* extracts showed biological activity against the tested gram-positive and gram-negative bacteria but weak or no activity against fungi. The methanolic extracts showed higher antimicrobial activity than the ethanolic extracts. The ethanolic extract from the habitat field edges showed strong activity against *P. aeruginosa* and *S. aureus*, whereas the methanolic extract of *P. major* leaves showed antibacterial and antifungal activity against *S. aureus*, *P. aeruginosa*, and *C. glabrata*. Moreover, the aqueous extract of *P. major* had weak activity against the tested bacteria and fungi. The effective antimicrobial activity of the ethanolic and methanolic extracts may be attributed to the various compounds in *P. major* leaves, which need to be extracted, separated, purified, and identified in future research for use in pharmaceutical applications.

Conflict of interests: The authors declare no conflict of interest.

Authors contribution: A.A. Khalafallah: suggest the idea, plan of the work and shared in the collection of plants, practical part, statistical analysis, writing the manuscript. M.A. Soliman: implements the plan, collection of the plants and Scientific references. T.M. Galal and M.A. Naim shared in the idea of the paper and reviewed the manuscript.

Ethical approval: Not applicable

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التباين الموسمي في مركبات الأيض الثانوي والنشاط المضاد للميكروبات للسان الحمل من موائل مصرية غير متجانسة

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يستخدم نبات لسان الحمل لعلاج العديد من الأمراض منذ العصور القديمة. هدفت الدراسة الحالية إلى تقييم التغيرات الموسمية والمتعلقة بالموائل في محتوى مركبات الأيض الثانوي والنشاط المضاد للميكروبات لمستخلصات أوراق النبات. تم اختيار ثمانية موائل لأخذ العينات من النباتات في أربعة مواسم. أظهر الفحص الكيميائي الموسمى للمستخلصات الإيثانولية ومستخلص الكلوروفورم لأوراق النبات من البيئات المختلفة وجود الجليكوسيدات القلبية والفلافونويدات والفينولات. لوحظ أعلى محتوى من الجليكوسيد القلبي ومحتوى الفلافونويد الكلي في الموائل الحضرية والمحاصيل المزروعة خلال فصل الشتاء، بينما لوحظ أعلى محتوى من الفينولات الكلية في الأراضي البور خلال فصل الصيف. تشتمل المركبات التي تم فصلها وتحديدها باستخدام الكروماتوجرافيا السائلة عالية الأداء (HPLC) على الفينولات مثل حمض الإيلاجيك والكاتيكول والريسورسينول وحمض الجاليك والفلوروجلوسينول وكذلك الفلافونويد مثل الأبجينين واللوتولين والكريسوريول والروتين والكيرسيتين والكايمبفيرول والأفيولارين. أظهرت المستخلصات الميثانولية لأوراق لسان الحمل نشاطًا مضادًا للميكروبات أعلى من المستخلصات المائية والإيثانولية. أظهر المستخلص الميثانولي لأوراق النبات من ضفاف الترع أعلى فعالية ضد *Pseudomonas aeruginosa*، يليه المستخلص الميثانولي للأوراق ضد *Staphylococcus aureus*. تشير النتائج إلى أن لسان الحمل يحتوي على مركبات الفينول والفلافونويدات التي لها تطبيقات طبية عديدة. في الدراسات المستقبلية، يجب استخلاص المواد الكيميائية النباتية ذات النشاط المضاد للميكروبات وفصلها وتنقيتها وتحديدها واختبارها كمركبات نقية أو مختلطة ارتباطا بأفضل الموائل والمواسم لتركيزها ونشاطها الحيوى.