

Phytochemical Analysis and Antimicrobial Activity of *Pergularia tomentosa* in the North East of The Kingdom of Saudi Arabia

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The increment in numbers of antimicrobial resistant strains along with the scarcity of new targets for drug industry has forced scientists to investigate deeply in the natural resources for new compounds with antimicrobial activity. *Pergularia tomentosa* is a member of the Apocynaceae family found in a wide geographical region including the Gulf region, Africa, and the Middle East. It is used as a remedy for the treatment of skin sores, asthma, and bronchitis. Dried plants of *Pergularia tomentosa* were subjected to extraction by using a Soxhlet extractor process to obtain essential oil and characterized by HPLC- Mass Spectroscopy (GC-MS). The essential oil was evaluated for antibacterial activity against pathogenic microorganisms by well diffusion method and confirmed by microdilution method. Additionally, we measured the antioxidant activity of the extracts using DPPH reagent. Phytochemical analysis has revealed variation in compositions and concentrations of *P. tomentosa* constituents grown in Hail from other agricultural regions. The lowest MIC was recorded with ethylacetate extract MIC of 6.25 mg/ml against *S. typhi*, whereas, the ethanolic extract had the broadest effective against the five strains with a MIC of 25 mg/ml. In conclusion, we summarize a variation between the phytochemical constituents of *P. tomentosa* plants grown in the district of Hail and other geographical regions. In addition, there are several natural phytochemicals with an antimicrobial activity could be a good target for the antimicrobial and antioxidants industry.

Keywords: *Pergularia tomentosa*, Antimicrobial, Phytochemical, Antioxidants, HPLC-MS.

The emergence of superbug strains between health care workers has obliged the scientific society to investigate deeper in the natural products for alternative compounds with

antimicrobial activity^{1, 2}. According to a report by the Infectious Diseases Society of America, around 70% of new cases administered to the hospitals in the US are involved with strains that show a potential no susceptibility to at least one drug^{3, 4}. In the UK, nowadays, methicillin-resistant *Staphylococcus aureus* (MRSA) is considered a real concern in infection control accounting for around more than 50% of all *S. aureus* isolates where as it had a low value a decade ago².

Since the beginning of mankind, there is a wide use of herbal plants in the folk medicine due to their therapeutic and pharmaceutical properties⁵⁻⁸. *Pergularia tomentosa* is a member of the Apocynaceae family (subfamily: Asclepiadeae) a yearly green plant with a distinctive odor, known in Hail city as "Aloonah"^{9, 10}. The plant is a climbing to semi erect perennial herb of around 30 cm, the stem is pale green-white in color, highly branched and usually grow vertically and milky latex is extracted from the plant¹¹.

This plant is found in a wide geographical region including Gulf region (Saudi Arabia and Oman), Africa (north Sudan, Egypt, Ethiopia, Algeria, Niger and Kenya) and Middle East (Jordan, Iraq, Iran, Pakistan, and Afghanistan,^{5, 12-14}. *Pergularia tomentosa* is used as a remedy for the treatment of rheumatic fever, asthma, bronchitis, helminthiasis and skin sores as cutaneous leishmaniasis^{15, 16}. Furthermore, several publications have reported the cytotoxic, antioxidant and antibacterial activity of *P. tomentosa*¹⁷⁻¹⁹. Up to date, this is the first study conducted on *P. tomentosa* in the region of Hail north east KSA that is focusing on the analysis of phytochemical

components and elucidating the antioxidant and antimicrobial activity of this perennial.

MATERIALS AND METHODS

Sampling

This study was performed in the northeast region of KSA at Hail district. *Pergularia tomentosa* (Fig. 1) was collected from the surrounding regions of Naqbeen village (a mountainous area) about 25 km from Hail.

Leaves stem, and roots of *P. tomentosa* were collected locally in March 2017 in Hail, Southeast of KSA. Samples were collected from the water ways and slope in hilly areas and mixed together. The authenticity of the plant was confirmed by Dr. Mohammed Ahmed, and voucher specimens are maintained at the University of Hail (UOH) herbarium.

Preparation of plant extracts

The dust-free leaves stem and roots of *Pergularia tomentosa* were shade dried for five days and finely grounded. 30 grams of dried powder were mixed in equal amounts from each part and extracted with 200 ml of ethanol, chloroform, and ethylacetate, consecutively, using Soxhlet extractor in order to separate phytochemical compounds based on their polarities. Filter paper Whatman No.1 was used to filter the crude extracts to remove impurities and debris and then the yield was concentrated by applying a vacuum at 35°C using a rotary evaporator. The concentrated extracts were subsequently dried aseptically using Lyophilization. Millipore distilled water was used to constitute inappropriate volume to obtain a final concentration of 200 mg/ml solution.

Phytochemical studies

For choosing the best solvent system (gradient of solvents (A and B)), we setup the method according to the TLC and analytical HPLC/UV. The concentration of the extract for the LC-MS analysis was 1 mg/2 ML²⁰.

Ethanol layer of the extract of *P. tomentosa* was subjected to qualitative analysis to determine the phytochemical composition for the plant. Liquid Chromatography-Mass Spectrometry (UHPLC system) with an autosampler and Waters nano Acquity HSS T3, 1.8 μ m, 100 μ m \times 100 mm column was used in the analysis. H₂O 0.1 % formic acid (A) (v/v, pH=2.17) and 90 % acetonitrile in



Fig. 1. *Pergularia tomentosa*

H₂O. 0.1 % formic acid (B) was used as mobile phases at a flow rate of 0.4 ml/min. 1 µm injection volume was used, the gradient elution for the injection was 5 % B during 0–2.5 min, a linear increase from 5 to 25 % B during 2.5–20 min, from 25 to 40 % B during 20–40 min and from 40 to 50 % B during 40–50 min, finally from 50 to 95 % B during 50–65 min followed by 15 min of maintenance. For identification of the eluent, we used Thermo Electron LTQ-Orbitrap XL mass spectrometer equipped with a nanoelectrospray ion source (Thermo Fisher Scientific, Bremen, Germany) and operated under Xcalibur 2.1 version software, in positive ionization mode for the MS analysis using data-dependent automatic switching between MS and MS/MS acquisition modes (Table 1)¹².

Determination of antioxidant activity

Antioxidant properties of *P. tomentosa* extracts was determined in terms of scavenging free radical using DPPH method²¹⁻²³.

The reduction in the concentration of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals by the activity of the antioxidants in *P. tomentosa* was measured and quantified by a colorimetric method. 0.02 g of DPPH reagent was dissolved in 1 L methanol; 3.9 mL of the solution is added to 0.1 mL of each sample that was diluted in a pure solvent of extraction at different concentrations. The mixtures were incubated for 90 minutes in a dark place at room temperature and the absorbance for each sample was measured at 517 nm using a UV/VIS spectrophotometer in triplicates.

The activity of free radical scavenging was disclosed as IC₅₀ (µg/mL). The following equation was used to calculate the reduction in the amount of free DPPH radical by the extract:

$$\text{DPPH scavenging effect (\%)} = ((A_0 - A_1) / A_0) * 100$$

A₀ is the control absorbance at 90 min, and A₁ is the sample absorbance at 90 min. All samples were analyzed in triplicate.

Antimicrobial activity

We have conducted a preliminary study using the anti-bacterial susceptibility test to determine the minimal inhibitory concentrations (MICs) for the three herbal extracts (ethylacetate, ethanol, and chloroform) against gram-positive, gram-negative and fungal strains. The protocol was conducted as defined in guidelines of National Committee for Clinical Laboratory Standard (NCCLS)²⁴. Further, we compared their activities to reference compounds, fluconazole, and ciprofloxacin. We have determined MICs for a strain collection consisting of *Staphylococcus aureus* ATCC®35556, *Staphylococcus epidermidis* ATCC®12228, *Escherichia coli* ATCC® 25404, *Salmonella typhi* ATCC® 700931 and *Candida albicans* SC5314. We covered a range of 6.25-100 mg/mL for the herbal extract against the strains. Bacterial strains were propagated on nutrient plates at 37°C for 24 hours. Where as, we propagated *C. albicans* strains on sabouraud dextrose (Sab) plates at 25 °C for 24 hours. Row number 1 on each microdilution plate was used as viability control of microbial cells. Rows 2 to 12 contained a decreasing amount of the herbal

Table 1. Chromatographic conditions of the HPLC MS

HPLC Conditions	Pump Flow Rate	Auto-sampler Injection Volume		Auto-sampler Temperature			Column Oven		
	1.00 mL/minute	25.00 µL		10.0 °C			30.0 °C		
Chromatography	Mobile Phase	Mixture of (60% ACN, +40% (675 µL Triethylamine / 1L of mixture), pH adjusted to 7.1 with phosphoric acid							
	Column Type	Sepax GP-C18, (150 × 4.6 mm, 5 µm)							
	Expected Retention Time	As seen in Table 230 nm			As seen in Table 230 nm				
MRM-Detection Conditions	Anlytes Category1	Q1 86	Q3 68.5	Dwell 150	FP 75	DP 80	EP 10	CE 20	CXP 25
	Category2	440	265	150	75	80	10	20	25
Mass-Spectra Conditions	CUR 10	CAD 6		IS 5500	TEM 400		NEB 5		

extract in a 1:1 serial dilution scheme resulting in a range of final concentrations from 6.5mg/mL to 100 mg/mL. Ciprofloxacin was used as a reference drug for the antibacterial activity of the extracts whereas Fluconazole was used for the comparison of antifungal activity. TECAN Microtiter plate reader was used for obtaining the optical density at 450nm for the microtiter plates after incubation at 37°C for 22 ± 2 hours, results were analyzed with Magellan software.

RESULTS

Phytochemical compounds

Characterization of the ethanol fraction of *P. tomentosa* was carried out using mass spectrometry. We identified 18 compounds in the aqueous extract; 14 compounds belonged to phenols and flavonoids whereas the last four had cardenolides structures. The results of the identified compounds were shown in Table 3. We also identified 3 flavonoids (Quercetin 3-O-galactoside (8), Kaempferol 3-O-glucoside (11) and Kaempferol 3-O-malonylhexoside (14)) and one cardenolide (17) with LCMS techniques. This had also been isolated before in *P. tomentosa* [ref] and three other cardenolides (5, 17 and 18)

which were not reported in this plant. Most of the identified flavonoids were belong to the flavone and flavonol^{25, 26} (Table 2, Fig. 2).

The DPPH free radical scavenging activity

The property of *P. tomentosa* extracts to scavenge free radicals was measured using a UV-visible spectrophotometer. Samples with plant extracts were analyzed covering a concentration range of 0.10-2.0 mg/mL. In the same conditions, Butylhydroxytoluene (BHT) was also measured as a reference compound for the radical scavenging activity. The proportion of DPPH free radicals scavenging for both tested extracts and the positive control BHT are depicted in Table 3. The antioxidants properties of the extracts were expressed by the IC₅₀ values, showing the sample concentration required to reduce fifty percent of DPPH free radicals (Table 3).

Linear regression analysis of the dose-response curve was used to determine the sample concentration required to reduce DPPH radical by 50% (IC₅₀ value). The values are the mean of three determinations ± standard error.

Antimicrobial activity

The three fractions of *P. tomentosa* showed different antimicrobial activity against the study strains; ethanol layer showed a broad

Table 2. Phytochemical components identified in the ethanolic extract of *P. tomentosa*

Identified Compounds in negative ion mode	Molecular formula	Antimicrobial activity	RT (min)	References
1 Phenolic glycosid: Scrophenoside D	C ₂₈ H ₃₄ O ₁₇	NO	4.12	Li et al, 2014
2 Phenolic: Synapoyl hexoside	C ₁₇ H ₂₂ O ₁₀	NO	4.7	Orqueda et al, 2017
3 Flavone: Luteolin-di C-lucoside	C ₂₇ H ₃₀ O ₁₆	YES	5.12	Singh et al, 2015
4 Phenolic: Feruloyl Glucoside	C ₁₆ H ₂₀ O ₉	NO	9.1	Frig et al, 2016
5 Cardenolide: antiaroside G	C ₂₉ H ₄₂ O ₁₂	NO	10.6	Shi et al, 2013
6 Flavonole: Kaempferol-malonyl-dihexosid	C ₃₀ H ₃₂ O ₁₉	YES	12	Salced et al, 2016
7 Flavonole: Kampferol + glucose	C ₂₇ H ₂₈ O ₁₇	YES	12.5	Heneidak et al, 2006
8 Flavonole: Quercetin 3-0-galactoside	C ₂₁ H ₂₀ O ₁₂	YES	13.5	Valente et al, 2016
9 Flavonole: Isorhamnetin-3-0-glucoside	C ₂₂ H ₂₂ O ₁₂	NO	13.6	Haijuan et al, 2013
10 Flavonole: Kaempferol 3-0-glucoside	C ₂₁ H ₂₀ O ₁₁	YES	13.9	Hettwer, 2016
11 Phenolic: Ferulylmalic acid	C ₁₄ H ₁₄ O ₈	NO	14.4	Song et al, 2016
12 Phenolic: glucuronic acid	C ₁₇ H ₁₄ O ₁₀	NO	14.5	Heneidak et al, 2006
13 Flavonole: Kaempferol 3-0-malonylhexoside	C ₂₄ H ₂₂ O ₁₄	NO	15.3	Dugo et al, 2009
14 Flavonole: Kaempferol-3-0-6 -acetyl-b-Dglucopyranoside	C ₂₃ H ₂₂ O ₁₂	YES	15.7	Ojwang, 2012
15 Flavonole: quercetin-3-(6"-succinoyl)-glucoside	C ₂₅ H ₂₄ O ₁₅	YES	16.3	Hamed et al, 2006;
16 Cardenolide: Hydroxycalactin	C ₂₉ H ₄₀ O ₁₀	NO	16.9	Piacente et al, 2009
17 Cardenolide: Antiaroside E	C ₂₉ H ₄₂ O ₁₀	NO	18	Shi et al, 2010
18 Cardenolide: Antiaroside F	C ₃₅ H ₅₂ O ₁₅	NO	18.5	Shi et al, 2010

inhibition activity against the five strains (*S. aureus*, *S. epidermidis*, *E. coli*, *S. typhi* and *C. albicans*) whereas ethyl acetate fraction had the most effective MIC values against only three strains (*S.*

epidermidis, *S. typhi*, and *C. albicans*) as depicted in Fig (4). The lowest MIC value was 6.25 mg/mL for the collection strains. A summary of the MIC distribution for the three fractions against the test strains is given in Table 4. At the level of antibacterial activity, ethyl acetate fraction has the most effective value (MIC 6.25 mg/mL). It was evident from the MIC results that the lipophilicity and the low solubility profile have affected the activity of the prepared compounds and consequently their antimicrobial effect.

Table 3. DPPH radical scavenging activity expressed as IC₅₀ values (ig/mL) of various extracts from *P. tomentosa*

Extract	IC ₅₀
Ethanol	0.63 (0.993)*
Methanol	0.58 (0.98)
Ethylacetate	0.54 (0.981)
Butylhydroxytoluene (BHT)	0.61 (0.99)

* R²: Correlation coefficient

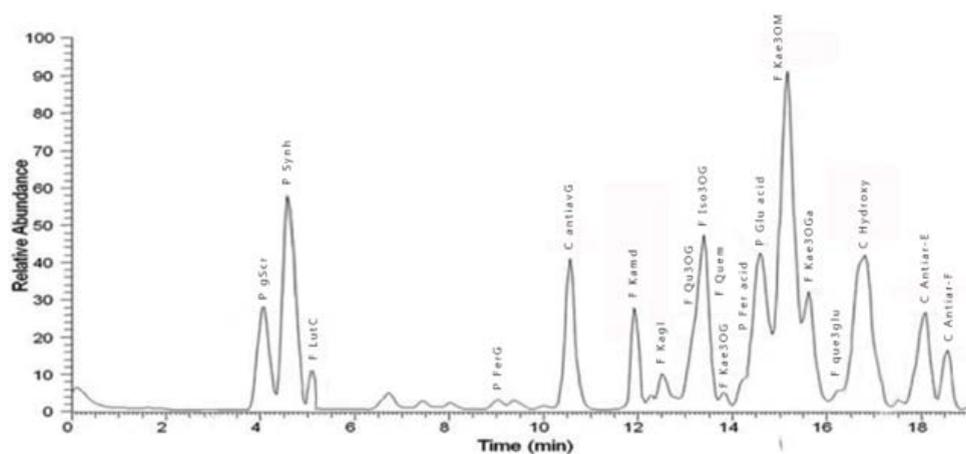


Fig. 2. Profile of LC-MS of *P. tomentosa* ethanolic extract. The names are according to the identified compounds in Table 2

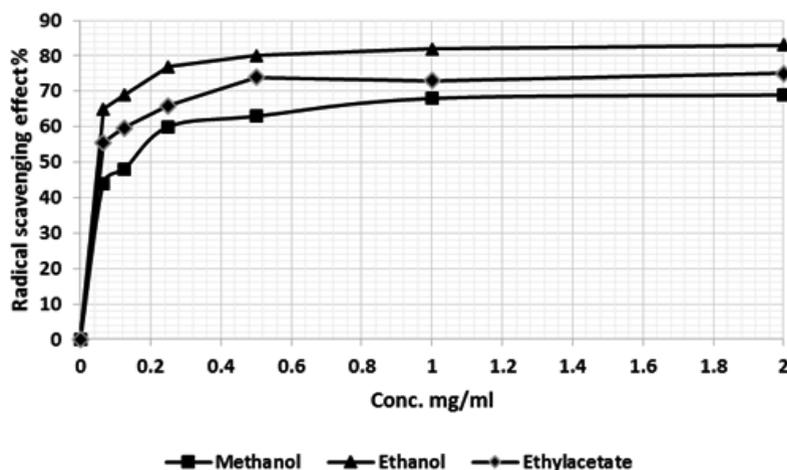


Fig. 3. Radical scavenging effect (%) on DPPH radicals of *P. tomentosa* organs

DISCUSSION

This study has investigated the phytochemical composition and the biological properties of *Pergularia tomentosa* grown in the district of Hail. The results of metabolite profiling

showed that there are 18 compounds (phenolics and cardenolides) in the extract. Fourteen compounds belonging to the phenolics and flavonoids, and 4 cardenolides were identified from leaves' aqueous extract of *P. tomentosa*. Given that *P. tomentosa* is in Asclepiadaceae, we expected that it would

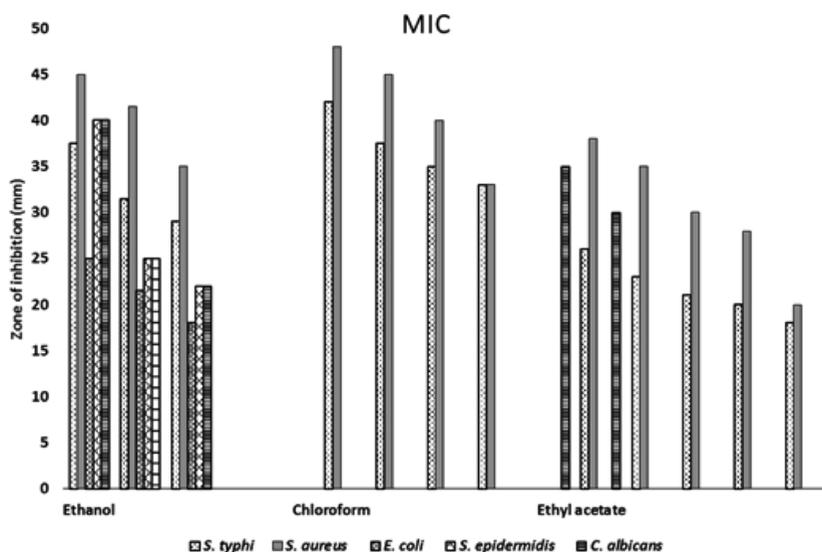


Fig. 4. MIC of *P. tomentosa* extracts against the pathogenic microorganisms

Table 4. MIC of *Pergularia tomentosa* extracts against pathogenic microorganisms

	Conc. mg/ml	<i>S. typhi</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. epidermidis</i>	<i>C. albicans</i>
Ethanol	100	37.5	45	25	40	28
	50	31.5	41.5	21.5	25	26
	25	29	35	18	22	19
	12.5	-	-	-	-	-
	6.25	-	-	-	-	-
Chloroform	100	42	48	-	-	-
	50	37.5	45	-	-	-
	25	35	40	-	-	-
	12.5	33	33	-	-	-
	6.25	-	-	-	-	-
Ethyl acetate	100	26	38	-	-	35
	50	23	35	-	-	30
	25	21	30	-	-	-
	12.5	20	28	-	-	-
	6.25	18	20	-	-	-
Ciprofloxacin	8 µg/ml	25	28	24	30	-
	4 µg/ml	20	23	19	20	-
	2 µg/ml	15	14	13	18	-
Fluconazole	10 µg/ml	-	-	-	-	17
	5 µg/ml	-	-	-	-	13

have contained many cardenolides, but 79% of the identified compounds are phenolic, and 21% belong to the cardenolides. Other reports which investigated the aerial parts of this plant⁹ demonstrated that its aerial parts are a rich source of flavonoids. Almost all studies showed that the roots of *P. tomentosa* are cardenolide-bearing part⁶. We identified 4 cardenolides in leaves of this plant. These results suggested that the leaves of *P. tomentosa* are a rich source of flavonoids and could be a suitable source for valuable cardenolides. For example, one of the identified cardenolides is Ghalakino side, which has a potent effect on cancer cells²⁷. The other three identified cardenolides (Antiaroside E-G), from the results of Shiet al (2010), exhibited strong cardiotoxic activity, with a potent inhibitor of Na⁺/K⁺-ATPase. In addition, its phenolic-compounds can play a strong antioxidant role^{8, 28}. In this field, Yakubu et al (2015) and Al Jabri (2013) confirmed the antioxidant effect of the extract of this plant^{2, 10, 11, 18}.

The antimicrobial activity for ethanol extract was the broadest whereas the lowest MIC was 6.25 mg/mL for ethyl acetate but surprisingly the inhibition was noticed in all strains the gram-positive, gram-negative and fungus. This points to inhibitory effect applied on a metabolic pathway or a process in gene transcription especially that the collection of pathogenic microorganism has different constituents in the cell membrane and ribosomal subunits^{8, 23, 29-31}.

Additionally, phytochemical analysis has revealed variation in components and concentrations of *P. tomentosa* constituents grown at different agricultural regions³². Interestingly, we can enumerate two important sources of antioxidants in *P. tomentosa*, firstly, the high concentrations of phenolic compounds in *P. tomentosa* which is considered to be a good source of powerful antioxidants, secondly, the hydroxyl groups in flavonoids which have the capability to react with DPPH radical by hydrogen atom donation to free radicals^{7, 33}, while a highly positive correlation between total phenolic content and antioxidant activity was established in case of many plant species^{7, 23}.

CONCLUSIONS

In conclusion, we summarize a variation between the phytochemical constituents of *P. tomentosa* plants grown in the district of Hail and other geographical regions. In addition, there are several natural phyto compounds with an antimicrobial activity could be a good target for the antimicrobial and antioxidants industry.

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