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Phytochemical analysis, chemical composition and antioxidant activity of different *Pergularia tomentosa* extracts

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Abstract: *Pergularia tomentosa (P. tomentosa)* is widely used as anti-rheumatic, laxative, abortive, and as a treatment for some skin disease in the traditional medicine. Our study aims to investigate the antioxidant properties of different extracts of *P. tomentosa*, study the major phenolic and flavonoids components of these extracts. Three *P. tomentosa* samples were collected from Sohag, Egypt. These samples were extracted in methanol (PTi, PTii and PTiii) and ethanol (PTiv). Total phenolic content (TPC), total flavonoid content (TFC) and the DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant activity of *P. tomentosa* extracts were investigated using colorimetric assays. PTi has the highest TPC (32.46±1.29 mg GAE/g DW), followed by PTiii sample (24.56 ± 1.97 mg GAE/g DW). On the other hand, PTiii revealed the highest TFC (11.48 ± 0.16 QE/g DW) and showed the potent antioxidant activity against DPPH with the IC_{50}= 0.340 mg/mL, while the IC_{50} of PTiv was 0.640 mg/mL. High performance liquid chromatography (HPLC) analysis of PTiii extract exhibited the presence of Gallic acid (16.4%), Ferulic acid (11.5%), Chlorogenic acid (9%), Vanillin (7%), Naringenin (3.4), Syringic acid (2.9%), Caffeic acid (2.1%), Hesperetin (1.6%), and Coumaric acid (1.55%), however, PTiv showed different concentrations of these components, such as Gallic acid (28.7%), Ferulic acid (14.14%), Caffeic acid (11.66%), Syringic acid (10.5%), Naringenin (9.9%), Catechin (6.5%), Chlorogenic acid (5.0%), Hesperetin (3.6%), Coumaric acid (2.6%), Kaempferol (2.6%), Cinnamic acid (1.5%), and Vanillin (1.3%). These results indicated that the *P. tomentosa* may be used in therapeutic applications with a potential to reduce the oxidative stress.
Keywords: *Pergularia tomentosa*; HPLC; TPC; TFC; DPPH.

1. Introduction

Medicinal plants are primary health care in many cultures, especially in Africa [1]. The *P. tomentosa* belongs to the genus *Pergularia* and family Apocyanaceae. Several members of this genus have had economic uses in the past and nowadays. Many of these species are sources of important natural products, that are used as hepatoprotective agents, analgesics, anti-inflammatory, antipyretics, antioxidants, antidiabetics and anti-cancer agents [2]. *P. tomentosa* can be found around the desert and eastward across Africa's horn through the Sinai (Egypt), southern Palestine, Jordan, Saudi Arabia, southern and eastern Iran, and Pakistan. In traditional medicine, this plant is used as an anti-rheumatic, laxative, abortive, for asthma and bronchitis and for the treatment of some skin diseases [3]. Leaves of this plant showed the presence of three cardenolides, in addition to β-sitosterol glucosides and cardenolide glycosides, which are used in cardiovascular disease, arrhythmia, cancer treatment, and decreasing muscle atrophy [4].

Moreover, several compounds that were isolated from the whole plant, in addition to lupane and ursane [5], enzymes such as (glutathione peroxidase, glutathione-s-transferase, superoxide dismutase, polyphenoloxidase, catalase, and ascorbate oxidase), α-amylase [6-8] protease, β-amylase, lipoxygenase, L. asparaginase, tyrosinase, and lipase [6]. The importance of this plant against diseases is due to the presence of bioactive compounds which include, antioxidant [9], antiproliferative [10], antitumor [11], anti-Kaposi sarcoma cells [12], anti-angiogenic agent [13], anti-hepatic damage [8], have healing effect on burns [14], and induce cellular immunity [15]. Oxidative stress is related to many diseases and health problems, including diabetes, cardiovascular diseases, cancer, and inflammations.

Therefore, the development of antioxidant treatments is very important to control diseases [16]. Medicinal plants contain large amounts of antioxidants, such as phenolic and flavonoid compounds, for which they have an important role in defense system against free radicals and oxidative stress. Several previous studies reported the value of TPC, TFC and antioxidant activity of *P. tomentosa* extracts based on the part of plant and the method of extraction [17-20]. In this work, the main goals were to evaluate the effect of different extracts of the aerial parts of *P. tomentosa* as antioxidant agents and to compare and identify phenolic compounds in the methanolic and ethanolic extracts using HPLC fingerprint analysis.

2. Materials and methods

2.1. Plant material

The aerial parts of *P. tomentosa* **(Fig. 1)** plants were collected from three places in the way of Sohag- Gohayna Desert (Hosni Mubarak Road). The first sample *P. tomentosa*

(PTi) was collected from the location 26°23'56.0"N 31°33'34.9"E, the second (PTii) was collected from the location 26°24'15.2"N 31°33'02.0"E, while the third (PTiii) was collected from the location 26°24'59.7"N 31°32'10.0"E. All samples were collected in March (2020) at the flowering stage. The identification was performed via using Cross-longitudinal section of *P. tomentosa* at Assiut University Herbarium **(Fig. 2)** to obtain an accurate identification of the plant because the plant was unusually present in these locations. However, slides were identified at Cairo University Herbarium with a voucher number (366.1067) by Prof. Dr. Hasnaa Hosny (Professor of Taxonomy, Department of Botany, Faculty of Science and Microbiology, Cairo University). The collected parts of the *P. tomentosa* plant were washed well by water to remove environmental factors, such as dust, sand or animal and insect wastes. The plant materials were dried in the open air and in the absence of the sunlight**.**

Fig. 1: Photograph of *P. tomentosa* collected from Sohag-Gohayna Desert (Egypt).

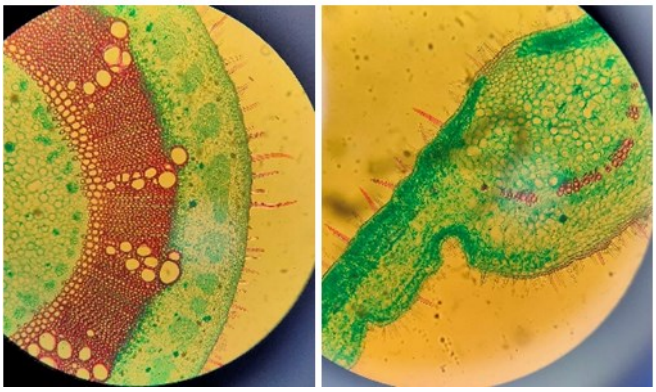

Fig. 2: Cross-longitudinal section of *P. tomentosa*.

2.2. Chemicals and apparatus

The chemicals and reagents used in this work are of analytical grade. Gallic acid (GA), Folin-Ciocalteu reagent (FCR), DPPH, Anhydrous sodium carbonate and Sodium hydroxide were purchased from Sigma Aldrich (St. Louis, MO, USA). Ascorbic acid (AA) was obtained from Alpha (Cairo, Egypt), Aluminum chloride ($AlCl_3$) was procured from HPLC (TM) (Mumbai, India), while Quercetin was acquired from NAWAH (Cairo, Egypt). Methanol and ethanol were procured from Biochem (Cairo, Egypt). HPLC analysis was performed using an Agilent 1260 series (USA).

2.3. Samples preparation and extraction

Dried plants were grounded in the mechanical grinder. PTi and PTii were extracted dividedly by the maceration approach with 100% methanol, while the third sample was extracted with 100% methanol and 95% ethanol to obtain PTiii and PTiv, respectively. All samples were filtered by Whatman filter paper NO. 1 and later were concentrated using a rotatory evaporator at 40ºC to obtain the crude extracts.

2.4. Qualitative analysis of *P. tomentosa* extracts

Qualitative phytochemical screening analysis of *P. tomentosa* extracts for the presence of phenolics, flavonoids, alkaloids, saponins, tannins, glycosides and anthraquinones was carried out according to a guide of phytochemical preliminary screening analysis of plant extracts [21], with some recent modifications.

2.4.1. Phenols

Different extracts of *P. tomentosa* were mixed with few drops of ferric chloride solution. A green color was appeared, that indicate the presence of phenols [22].

2.4.2. Flavonoids

One mL from different *P. tomentosa* extracts were added to 2% sodium hydroxide. A yellow color precipitation was formed and then disappeared later by adding diluted hydrochloric acid, which indicated the presence of flavonoids [23].

2.4.3. Alkaloids

A 100 mg of each extract of *P. tomentosa* was reacted with 0.5 mL HCl (1%), heated, and filtered. Subsequently, 0.5 ml from each filtrates was treated with 0.5 ml of Wagner's reagent [24]. Brown reddish precipitate was suggested the presence of alkaloids.

2.4.4. Tannins

Two drops of 5% $FeCl_3$ were added to *P. tomentosa* extracts. The appearance of a green precipitate confirmed the presence of tannins [25].

2.4.5. Saponins

Five ml from each *P. tomentosa* extract was shaken for a few minutes with 5 ml of distilled water in a clean test tube and warmed. The appearance of stable foam indicated the presence of saponins [22].

2.4.6. Anthraquinone

Few drops of each *P. tomentosa* extract was boiled with 10% HCl for few minutes. The solution was kept to cool, then a chloroform with few drops of ammonia were added to the filtrate, then the solution was heated. No rose pink color indicated the absence of anthraquinone [26].

2.5. Quantitative analysis of *P. tomentosa* extracts

TPC of four extracts of *P. tomentosa* (PTi, PTii, PTiii, and PTiv) was estimated spectrophotometrically through the FCR assay, while gallic acid was used as a standard [27]. A 100 µL of diluted extracts of *P. tomentosa* or standard solution of GA (0.0125, 0.025, 0.05, 0.10, 0.20 mg/mL) was added to glass

test tubes and mixed with 500 µL of 10 % FCR solution (diluted ten-fold in ultra-pure H_2O). Afterwards, 2 mL of 4% aqueous Na_2CO_3 was added to the mixture and shaken for 30 sec. Then the mixture was incubated in the dark for 30 min at room temperature. The absorbance of standards/samples was measured at 760 nm using methanol as a blank using a Taisite Spectrophotometer (New York, USA). All samples and standards were measured in three replications and the mean value calculated.

2. 5.2. Total flavonoid content (TFC)

TFC of *P. tomentosa* extracts was determined spectrophotometrically using the aluminum chloride approach, while quercetin was used as a standard. One mL of diluted extracts or standard solutions of quercetin (5, 10, 15, 20, 25 µg/mL) was mixed with 1 mL of $AlCl_3$ (2% in methanol) and shaken for 30 s. The absorbance was measured at 430 nm vs methanol as a blank [28]. The experiments were performed in triplicates.

2.6. Antioxidant activity by DPPH assay

Antioxidant activity for four extracts and standard was performed spectrophotometrically method as following; 1 mL of DPPH solution (0.1 mmol/L in methanol) was mixed with 1 mL of diluted PT extracts (0.0625, 0.125, 0.25, 0.5, 1.0 mg/mL) and then incubated in the dark at room temperature for 30 min. The absorbance was recorded at 517 nm [29]. A stock solution of the Ascorbic acid was prepared in methanol to prepare different concentrations (10, 8, 6, 5, 4, 3, 2, and 1 µg/mL) [30]. Finally, the experiment was repeated in triplicates for extracts and standards.

2.7. HPLC conditions

HPLC fingerprint analysis of two *P. tomentosa* extracts (PTiii and PTiv) was performed according to the described method in [31] through using an Agilent 1260 series. The separation was done using Zorbax Eclipse Plus C8 column (250 mm x 4.6 mm i.d., 5 µm). The mobile phase formed as follows: water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 0.9 mL/min. The program of mobile phase was consecutively in a linear gradient; 0 min (82% A); 0–1 min (82% A); 1-11 min (75% A); 11-18 min (60% A); 18-22 min (82% A); 22-24 min (82% A). The detector (multi-wavelength detection) was recorded at 280 nm. The injection volume was 5 µL for *P. tomentosa* extracts solutions. The column temperature was adjusted at 40°C.

2.8. Statistical analysis

TPC, TFC and DPPH assays were measured in triplicates, while the results were expressed as mean value and standard deviation (M ± SD). Further statistical analysis for DPPH findings was evaluated with One-way ANOVA (Analysis of Variance) and Tukey's to compare the IC_{50} values of all PT extracts with standard (Ascorbic acid), while the results were considered statistically significant at *p* value less than 0.05. All statistical analysis was performed using GraphPad Prism (version 10.3.0).

3. Results and discussion

3.1. Qualitative analysis of *P. tomentosa* extracts

Phytochemical screening analysis of four crude extracts of *P. tomentosa* exhibited their rich in bioactive components, such as phenols, flavonoids, alkaloids, saponins, and tannins **(Table 1).** These results appropriated with another study as mentioned in [32], excepting anthraquinone was not found in these extracts.

Table 1: Preliminary screening analysis of chemical constituents of *P. tomentosa* extracts.

no.	Phytochemical component	PTi	PTii	PTiii	PTiv
1	Alkaloids	+	+	+	+++
2	Anthraquinone	-	-	-	-
3	Flavonoids	++	++	+++	++
4	Phenols	++	++	++	+++
5	Saponins	+	+	+	+
6	Tannins	++	++	++	++

3.2. Quantitative analysis of *P. tomentosa* extracts

3.2.1.Total phenolic contents

The calibration curve equation of Gallic acid was y= 5.1038x + 0.0373, R^2= 0.9993. The TPC value of various extracts of *P. tomentosa* was stated as mg GAE/g DW. The TPC of four extracts of *P. tomentosa* were exhibited PTi extract to have more content of phenols with the TPC value 32.46 ±1.29 mg GAE/g DW, the second was PTiii (24.56 ± 1.97 mg GAE/g DW) as presented in **Table 2**.

On the other hand, the comparison of methanolic and ethanolic extracts of *P. tomentosa* revealed PTiii to contain higher content of phenolic components more than the ethanolic extract PTiv. These results are similar to the methanolic extracts that were collected from other countries [17, 19], however, the PTiv extract is considered to have a high amount of polyphenol more than any samples that were collected, which may due to the difficult environment or stress around this location. Conversely, the variation in the TPC values between our samples may be due to their variety in the collection place of each plant or used solvent and/or other environmental factors.

Table 2: TPC of four extracts of *P. tomentosa* (Results expressed as M ± SD, n = 3).

Samples	TPC (mg GAE/g DW)
PTi	32.46 ± 1.29
PTii	19.17 ± 0.35
PTiii	24.56 ± 1.97
PTiv	22.31 0.35

3.2.2. Total flavonoid contents

TFC test indicated the activity of medicinal plants, especially against oxidative stress [33]. *P. tomentosa* extracts have high amounts of flavonoids which gave us an impression about the plant that has antioxidant activity. The best one in TFC, was PTiii 11.48 ± 0.33 mg QE/g DW, which collected

from the third location and extracted in 100% methanol. By comparing methanolic and ethanolic extracts of the sample collected from the same location, PTiii revealed a great content of flavonoids comparing to PTiv with TFC values; 11.48 ± 0.16 and 5.40 ± 0.29 mg QE/g DW, respectively.

On the other hand, our samples showed high TFC value in comparison with samples that were collected from Algeria [34]. Another study was performed on the aqueous-methanolic extract of P. tomentosa, while the results revealed high TFC value [35]. The differences between our findings and other studies may be due to the difference in extraction approach, plant parts or locations or variation in the environment conditions.

Table 3: TFC of *P. tomentosa* extracts (Results expressed as M ± SD, n = 3).

Samples	TFC (mg QE/g DW)
PTi	**5.51 ± 0.28**
PTii	**9.63 ± 0.33**
PTiii	**11.48 ± 0.16**
PTiv	**5.40 ± 0.29**

3.3. Antioxidant activity

The DPPH method was used to determine the antioxidant activity of *P. tomentosa* extracts [36]. The scavenging activity of the extracts was detected by the equation I (%) = $[(A_{Control} - A_{Sample})/A_{Control}] \times 100$, where A_{Sample} is the absorbance of a sample solution, $A_{Control}$ is the absorbance of the control solution (methanol + DPPH), and I (%) is the percentage of inhibition. The IC_{50} of any extract/compound has been related to its antioxidant capacity, as it means the amount of antioxidant required to inhibit the DPPH concentration by 50%, which determined from a linear regression analysis [37]. The lower IC_{50} value reflects the best and higher antioxidant activity. **Table 4** presented the IC_{50} values of different *P. tomentosa* extracts. Both methanolic and ethanolic extracts of *P. tomentosa* were found to contain varied flavonoids and phenolic compounds, which may be responsible for the antioxidant activity of this plant against DPPH radicals [18].

The best radical scavenging activity appeared with PTiii (IC_{50}= 0.34 mg/mL) as compared to other methanolic extracts and its ethanolic extract. Antioxidant activity of plant extracts is directly proportional to its phenolic and flavonoid contents [38]. In our study, there was a correlation between the total phenolic and flavonoid content with the antioxidant activity of *P. tomentosa* extracts. TFC results were highly correlated with the antioxidant activity, where the activity of extract increased by rising flavonoid contents. A little correlation was found between TPC results and antioxidant activity compared with the TFC. This may due to the non-flavonoid and phenolic compounds in the extract, which may be caused competitive reactions and reduce the effectiveness of the extract.

Overall, our findings indicated that all the test PT samples are likely to possess significant levels of antioxidant capacity although comparatively less than Ascorbic acid ($p < 0.001$). In addition, there are highly significant differences between the different PT extracts (PTi-PTiv), suggesting varying antioxidant activity levels among them. Thus, our research study suggested that all PT samples, especially PTiii extract may be efficaciously applied as an substantial and natural antioxidant source in the inhibition and treatment of numerous ailments, such as inflammatory, diabetes, cancer and other degenerative conditions.

Table 4: DPPH radical scavenging activities of various *P. tomentosa* extracts. (Results expressed as M ± SD, n = 3)

Samples	IC_{50} (mg/mL)
PTi	**1.86 ± 0.129*****
PTii	**0.43 ± 0.024*****
PTiii	**0.34 ± 0.011*****
PTiv	**0.62 ± 0.090*****
AA (standard)	**0.01 ± 0.002**

The significance difference between the IC_{50} of different PT extracts and Ascorbic as standard was evaluated via ANOVA analysis, followed by Tukey's test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

HPLC approach, was used to quantify and identify the flavonoid and phenolic constituents of plant extracts via comparing the retention time of the detected peaks with those of the pure standard compounds [39]. Eighteen phenolic and flavonoid standards, such as Caffeic acid, Catechin, Chlorogenic acid, Cinnamic acid, Coumaric acid, Daidzein, Ellagic acid, Ferulic acid, Gallic acid, Hesperetin, Kaempferol, Methyl gallate, Narigenin, Quercetin, Rosmarinic acid, Rutin, Syringic acid, and Vanillin were used to identify the major compounds in *P. tomentosa* extracts. The results of the HPLC analysis of PTiii and PTiv were presented in **Table 5**.

According to our results, phenolic and flavonoid contents have a direct effect on antioxidant activity of our extracts. Scientists and researchers describe the use of HPLC for characterization and detection of secondary metabolites in plant extracts, mainly phenol compounds and flavonoids [40-44]. To identify the major bioactive components in *P. tomentosa* extracts, phenolic and flavonoid standards were injected to HPLC system **(Fig. 3)**.

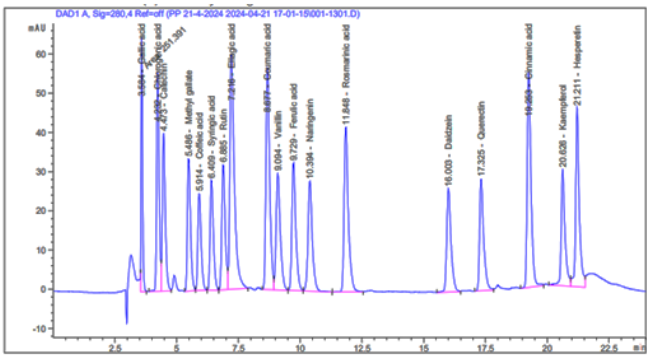

Fig. 3: HPLC fingerprinting analysis of phenols and flavonoids standards.

The major bioactive compounds detected in PTiii were GA (16.4%), Ferulic acid (11.5%), Chlorogenic acid (9%), and Vanillin (7%), while the rest of 18 compounds appeared as minor amounts. The highest intensity peak (37%) was detected in PTiii extract **(Fig. 4)** at retention time 10.31 min, was an unknown compound.

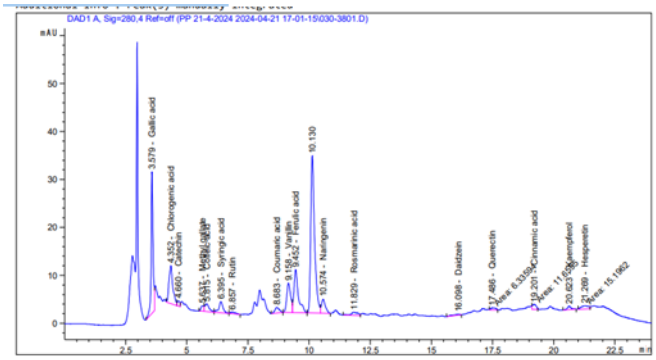

Fig. 4: HPLC fingerprinting analysis of PTiii extract.

The bioactive components that detected in the HPLC chromatogram of PTiv were different as shown in **Fig. 5**, while GA was the major component with value (28.7%), followed by Ferulic acid (14%), caffeic acid (11.7%), syringic acid (10.5%), Narengenin (9.9%), catechin (6.5%), and chlorogenic acid (5%). The rest of other compounds appeared as minor but more that presented in PTiii.

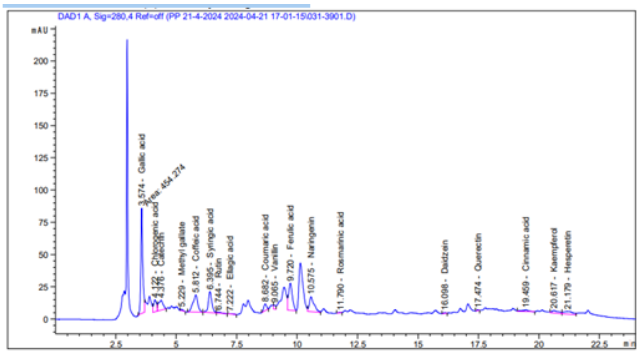

Fig. 5: HPLC fingerprinting analysis of PTiv extract.

It's obvious that the concentration of bioactive substances presented on the samples depend on the solvent used and vary in bioactive contents and antioxidant activity. PTiv has Gallic, Catechin, Caffeic acid, Ferulic acid, Syringic acid, and Naringenin more than PTiii, while Vanillin and Chlorogenic acid presented in PTiii were considered to be more than PTiv. Both *P. tomentosa* extracts (methanolic & ethanolic) are found to contain rich contents of phenolic and flavonoid compounds which are known for their potent antioxidant capacity against free radicals [45, 46]. As far as we know, this work is the first study to investigate the phenolic and flavonoid compounds of aerial parts of *P. tomentosa* by HPLC technique. We used HPLC analysis to determine the concentration of phenols and flavonoids quantitatively as presented in **Table 5**. One literature only [18] indicated using the HPLC analysis to identify phenolics and flavonoids in the crude latex of the plant, while results demonstrated the presence of Chlorogenic acid, Quercetin, and p-Coumaric acid. The other researches in this plant care about crude latex and cardenolides in this latex [13, 47, 48], although the whole plant is rich in bioactive contents.

Table 5: Chemical constituents of PTiii and PTiv extracts.

no	RT	Components	PTiii peak area	Conc. (µg/mL)	PTiv peak area	Conc. (µg/mL)
1	3.57	Gallic acid	16.44	12.47	28.73	36.14
2	4.12	Chlorogenic acid	9.05	11.06	4.97	10.07
3	4.38	Catechin	0.80	1.73	6.51	23.25
4	5.23	Methyl gallate	0.92	0.45	0.399	0.32
5	5.81	Caffeic acid	2.13	1.62	11.66	14.68
6	6.39	Syringic acid	2.90	1.84	10.50	11.06
7	6.77	Rutin	0.53	0.83	0.80	2.08
8	7.22	Ellagic acid	0.00	0.00	0.25	0.36
9	8.68	Coumaric acid	1.54	0.50	2.61	1.39
10	9.07	Vanillin	7.09	2.49	1.27	0.74
11	9.72	Ferulic acid	11.54	6.13	14.14	12.46
12	10.13	Unknown	37.54	----	----	----
13	10.58	Naringenin	3.40	2.90	9.90	13.99
14	11.79	Rosmarinic acid	1.32	1.26	0.13	0.21
15	16.10	Daidzein	0.47	0.28	0.30	0.29
16	17.47	Quercetin	0.66	0.39	0.20	0.19
17	19.46	Cinnamic acid	1.22	0.20	1.54	0.42
18	20.62	Kaempferol	0.83	0.50	2.56	2.56
19	21.18	Hesperetin	1.59	0.66	3.55	2.42

4. Conclusion

The antioxidant activities of different *P. tomentosa* extracts were evaluated using DPPH assay and the chemical constituents were characterized by HPLC analysis. Extraction using 100% methanol provided significantly better results of TFC, TPC, and antioxidant activity than those of the other solvent systems or locations. However, extract of *P. tomentosa* in 100% methanol collected from the location 26°23'56.0"N 31°33'34.9"E showed the highest amounts of TPC (32.46 ±1.29 mg GAE/g D.W) while TFC is the lowest one (5.51 ± 0.28 mg QE/g D.W) and the lowest value of IC_{50} (1.856 mg/mL). It is clear that 100% methanol extract from location 3 (26°24'59.7"N 31°32'10.0"E) exhibited the highest antioxidant properties, which it's related to flavonoid contents in the plant. The results of this work indicated that *P. tomentosa*, which collected from the least desertified is the most suitable extract as a treatment against free-radicals that cause oxidative stress. For future work, some further analysis, such as LC-MS/MS and NMR will be required to identify all unknown compounds, which are found in PT extracts.

On the other hand, an in vivo investigation on the most active *P. tomentosa* extract will be considered essential to obtain more details about the action mechanism or the biochemical pathway of its bioactive compounds and their role as antioxidant agents.

CRediT authorship contribution statement:

Nagwa Mohamed El-Sawi: Supervision, Conceptualization, Writing–review and editing. *Mohamed Olish*: Investigation, Formal analysis, Data curation, Writing–original draft preparation. *Mahmoud Hefny Gad*: Supervision, Conceptualization, Methodology, Visualization, Data curation, Writing–original draft preparation, Writing–review and editing. All authors have read and agreed to the published version of the manuscript.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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