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RESEARCH ARTICLE

Phenolic Contents and Antioxidant Activity of *Helicteres Hirsuta* Extracts

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Abstract: *Helicteres hirsuta,* which belongs to the *Malvaceae* family, is used in traditional medicine to treat malaria, diabetes and cervical cancer. While these uses have not been validated in clinical studies thus far, extracts from *H. hirsuta* exhibit confirmed antioxidant and potential anti-cancer activity that warrant critical assessment. The aim of this study was to determine the antioxidant properties of different parts of the plant *H. hirsuta.* The antioxidant capacities of methanol extracts of different plant parts, and specific solvent fractions thereof were determined on the basis of 2,2-diphenyl–1–picrylhydrazyl (DPPH) radical scavenging and total antioxidant activity assays. The ethyl acetate fraction from the leaf material of *H. hirsuta* has the most potent antioxidant activity with the lowest half maximal inhibitory concentration (IC_{50}) value of 9.50 μg/mL, that is 4 times lower than the IC_{50} of curcumin. The amounts of methyl gallate and rutin dominated the extracts at 8.62 ± 0.01 and 6.76 ± 0.02 mg/g, respectively, out of a total of 21.00 ± 0.01 mg/g active phenolic fraction. The amount of methyl gallate strongly correlated with total phenolic content and total antioxidant capacity, thus this compound may be useful as a marker of antioxidant activity in common medicinal plants

Keywords: *Helicteres hirsuta,* antioxidant activity, methyl gallate, rutin, HPLC, DPPH.

1. INTRODUCTION

Indigenous knowledge is a valuable resource for identifying new bioactive natural products. The experience of natural healers with using medicinal plants constitutes *de facto* biological screening, with long-term accumulation and the impartation of data from one generation to another in the ethnic community. *Helicteres hirsuta* belongs to the *Helicteres* genus of the Malvaceae family growing as part of native vegetation in Southeast Asian countries such as Vietnam, Laos, Cambodia, Indonesia and Thailand [1, 2]. This plant was used in traditional medicine to treat malaria, diabetes and cervical cancer [3]. Studies confirmed that the extracts from *H. hirsuta* exert strong antioxidant and potentially cancer-preventive effects [2, 4]. Recently, the chemical composition of *H. hirsuta* was documented and many of the biological activities of isolated compounds and secondary metabolites were described [5-9].

Antioxidant activity is one of the key properties of a compound considered for disease prevention [10-14]. There are several experimental methods to determine the antioxidant capacity [15, 16]. These methods differ in terms of their assay principle, experimental conditions and mechanism. The approaches based on the stable free radical DPPH and total antioxidant capacity offer the most effective way for the measurement of the antioxidant activity because of their fast and simple features [10]. The antioxidant activity of medicinal plants is attributed to phenolic compounds [17-21], supposedly due to the presence of hydroxyl groups, benzylic hydrogens or other substituents [22-25]. Among phenolic compounds, rutin, quercetin, quercitrin, methyl gallate, α–tocopherol, luteolin are antioxidants with potent radical scavenging activity [26-28]. These compounds are widely used as antioxidants in the food industry, cosmetics, and for therapeutics; hence they may serve as reference compounds when assessing radical scavenging activity.

In this work, the antioxidant potential of specific parts of *H. hirsuta* was determined. Extracts of the natural products were assessed for activity by the total antioxidant capacity, DPPH radical scavenging, total phenolic and total flavonoid content methods. To link antioxidant activity to chemical

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composition, six typical phenolic compounds (rutin, quercetin, quercitrin, methyl gallate, α-tocopherol and luteolin) were quantified in the extracts by HPLC.

2. RESULTS AND DISCUSSION

2.1. *In Vitro* Evaluation of Antioxidant Potential of Methanol Extractions

2.1.1. The DPPH Radical Scavenging Activity

The antioxidant capacity of the methanol extracts was determined by using the DPPH method in the comparison with typical antioxidants *i.e*. gallic and ascorbic acid [29], the results are presented in Table **1**.

As shown in Table **1**, the DPPH radical scavenging activity of methanol extracts of *H. hirsuta* also correlated to the concentration. At the concentration of 100 µg/mL all of the extracts exhibited higher activity (82.78%) than curcumin (81.26%). The IC_{50} values of the methanol extracts of *H. hirsuta* leaf and branch samples (IC_{50} = 14.69 and 14.00 µg/mL, respectively) showed the highest activities at 2.5 times than that of curcumin (IC_{50} = 38.50 µg/mL) but much lower activity than gallic acid and ascorbic acid (IC_{50} = 3.58 and 1.60 µg/mL, respectively). The fruit had the lowest DPPH radical scavenging activity with the IC_{50} > 100 µg/mL. Thus the methanol extracts of leaf and branch of *H. hirsuta* have the best antioxidant properties.

2.1.2. Total Phenolic and Flavonoid Contents

Phenolic compounds are considered to be the dominant contributors to the antioxidant activity of medicinal plants. Therefore, total phenolic content (TPC) was defined by using Folin-Ciocalteu's reagent, expressed in terms of gallic acid equivalent [17, 30, 31]. Flavonoids are a prominent class of phenolic compounds with well-known high antioxidant activity, hence the total flavonoid content (TFC) was also measured as described above and expressed in terms of quercetin equivalent mass [32]. The total antioxidant capacity (TAC) was expressed as number equivalents of gallic acid (GA) [33] and ascorbic acid (AS) [34] and evaluated by using the phosphor-molybdenum method [29] in order to evaluate co-relations between TPC, TFC and TAC. The obtained results were shown in Table **2** and Figure **S1**, **SI**.

As shown in Table **2**, the TPC and TFC in the plant parts of *H. hirsuta* were in the range of 38.83 ± 0.04 to 72.77 ± 0.12 mg GAE/g and 5.82 ± 0.13 to 41.91 ± 0.99 mg QE/g, respectively. The leaf extracts contained the highest TPC and TFC value at 72.77 ± 0.12 mg GAE/g and 41.91 ± 0.99 mg QE/g, respectively. Thus this extraction exhibited the largest TAC value at 174.94 ± 1.56 mg GA/g and 58.35 ± 0.23 mg AS/g. However, the lowest TPC and TFC values were observed in the fruit extracts that could be the reason for the lowest TAC value (81.62 ± 1.14 mg GA/g and 27.23 ± 0.26 mg AS/g) for this extraction. This result is in good agreement with the obtained results in the DPPH testing and affirms that the leaf extract has the highest TPC, TFC as well as TAC values. Thus this extract was used in further investigation.

2.2. Evaluation of Antioxidant Potential of Fraction Extracts from Leaf of *H. hirsuta*

The patent methanol extraction of *H. hirsuta* leaf samples was further extracted with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The resulted fractions were then evaluated for antioxidant activity by using the DPPH radical scavenging and the phosphor-molybdenum methods. The results are shown in Tables **3** and **4** and Figure **S2**, **SI**.

Table 1. The DPPH radical scavenging activity rates of the plant parts of *H. hirsuta*.

Concentration* (µg/mL)	Leaf	Branch	Aerial parts	Gallic acid	Ascorbic acid	Curcumin
100.0	83.25	93.89	82.78	87.34	96.65	81.26
20.0	68.27	66.64	58.27	76.23	93.80	40.64
4.0	12.74	22.25	20.74	54.26	88.81	29.07
0.8	1.32	12.13	10.32	21.93	37.08	20.19
IC_{50} (µg/mL)	14.69	14.00	17.07	3.58	1.60	38.50

*: Fruit extracts have not exhibited any activity in < 100 µg/mL concentrations.
Aerial parts including leaf, branch and fruit.

Table 2. The TPC, TFC and TAC of the plant parts of *H. hirsuta*.

Plant Part	TPC	TFC	TAC	
	(mg GA/g)	(mg QE/g)	(mg GA/g)	(mg AS/g)
Branch	51.68 ± 0.11	38.68 ± 0.84	122.28 ± 1.23	40.79 ± 0.20
Leaf	72.77 ± 0.12	41.91 ± 0.99	174.94 ± 1.56	58.35 ± 0.23
Fruit	38.83 ± 0.04	5.82 ± 0.13	81.62 ± 1.14	27.23 ± 0.26
Aerial parts	67.59 ± 0.16	41.18 ± 0.80	112.89 ± 1.35	37.66 ± 0.22

Table 3. TAC of five fractions from the leaf of *H. hirsute.*

Fractions	TAC	
	(mg GA/g)	(mg AS/g)
n-Hexane	138.96 ± 1.24	46.35 ± 0.20
Chloroform	159.59 ± 1.35	53.24 ± 0.41
Ethyl acetate	247.07 ± 1.18	82.41 ± 0.58
n-Butanol	196.97 ± 1.44	65.70 ± 0.63
Water	176.70 ± 1.37	58.94 ± 0.24

As shown in Table **3**, the TAC values of five fractions were in the range of 138.96 ± 1.24 to 247.07 ± 1.18 mg GA/g and 46.35 ± 0.20 to 82.41 ± 0.58 mg AS/g. The antioxidant activity of ethyl acetate and *n*-butanol fractions was significantly higher than that of *n*–hexane, chloroform, and water fractions. The highest inhibition was observed in the ethyl acetate extract at 247.07 ± 1.18 mg GA/g and 82.41 ± 0.58 mg AS/g, whereas that for the *n*–hexane fraction was lowest at 138.96 ± 1.24 mg GA/g and 46.35 ± 0.20 mg AS/g.

The DPPH results (Table **4**) show that the antioxidant activities of the five fractions of the leaf extract were much higher than that of curcumin. The ethyl acetate fraction showed the highest activity with the lowest IC_{50} value of 9.50 μg/mL, which is 4 times lower than the IC_{50} of curcumin. The high activity could be ascribed to the presence and potential synergistic effect of multiple phenolic compounds and/or the presence of other, hitherto unidentified antioxidants in this fraction. Thus, based on the two *in vitro* models, all five fractions extracted from the leaf of *H. hirsuta* are antioxidants with high potential, particularly the ethyl acetate fraction.

2.3. Quantification of Some Phenolic Compounds from Methanol Extraction of Leaf of *H. hirsuta* by HPLC

It is generally observed that the antioxidant activity of plants is attributable to phenolic compounds [15]. Initial analysis by HPLC indicated that the plant contains several typical phenolic compounds (methyl gallate, rutin, quercetin, quercitrin *α*-tocopherol and luteolin) that have well characterized antioxidant activities. Former studies suggested that the amounts of these compounds play an important role in explaining the differences in the antioxidant activities of plant extracts [26-28]. Thus in this study, the amounts of these compounds in the most potent antioxidant extract (the methanol extract of leaf of *H. hirsuta*) were defined by HPLC. The results are shown in Table **5**. The separation of the different phenolic compounds was successful with good linearity, with recoveries in the range of 92.84 to 98.54% (Table S1, SI). Approximately 75% of the dry mass of the total phenolic compounds is made up of methyl gallate and rutin (8.62 ± 0.01 and 6.76 ± 0.02 mg/g, respectively), whereas that for the other phenolic compounds were in the range of 0.08 ± 0.01 to 2.73 ± 0.02 mg/g. Thus the amounts of methyl gallate and rutin in *H. hirsuta* plants are significantly higher than those reported for other plants [35-38]. Thus, the *H. hirsuta* plant is a potential source of methyl gallate and rutin compounds.

2.4. Correlation Between Methyl Gallate, Rutin or Quercitrin and Antioxidant Capacity of Leaf of *H. hirsuta*

Several studies reported on the relationships between phenolic compound content and antioxidant capacity. Some authors found a correlation [39] while others disputed that relationship [40]. Considering the varied activities of the different phenolic compounds, here the statistical correlation between the amounts of methyl gallate, rutin and quercitrin and the total antioxidant capacity was assessed in *H. hirsuta* as well as five medical plants: *H. parasitica, A. clypearia, A. bauchei, S.oleracea* and *P. venusta* for which literature data were available (Table **6**). Of the studied compounds, only the amount of methyl gallate correlated to total antioxidant capacity with high statistical significance (coefficients from 0.8389 to 0.9806, Table **S2**, **SI**). Thus, methyl gallate content was also representative of the total phenolic content.

Table 4. The DPPH radical scavenging activity rates of five fractions from the leaf of *H. hirsute.*

Con. (μg/mL)	*n*-Hexane	Chloroform	Ethyl Acetate	*n*-Butanol	Water	GA	AS	Curcumin
100.0	72.31	83.92	82.78	94.76	86.24	87.34	96.65	81.26
20.0	43.24	47.28	86.34	62.74	48.47	76.23	93.80	40.64
4.0	28.51	29.56	30.98	20.12	18.24	54.26	88.81	29.07
0.8	14.08	12.66	10.43	8.62	8.25	21.93	37.08	20.19
IC_{50} (μg/mL)	52.60	23.81	9.50	15.20	21.94	3.58	1.60	38.50

Table 5. The amounts of the dominant phenolic compounds in the leaf extract of *H. hirsute.*

Sample	Methyl Gallate (mg/g)	Rutin (mg/g)	Quercetin (mg/g)	Quercitrin (mg/g)	*α*-Tocopherol (mg/g)	Luteolin (mg/g)	TA6C-(HPLC)* (mg/g)
Leaf	8.62 ± 0.01	6.76 ± 0.02	2.51 ± 0.08	2.73 ± 0.02	0.29 ± 0.02	0.08 ± 0.01	21.00 ± 0.01

*: the amounts of six compounds were defined by HPLC.

Table 6. Methyl gallate, rutin, quercitrin, TAC and TPC from some medicinal plants.

Sample	Methyl Gallate (mg/g)	Rutin (μg/g)	Quercitrin (μg/g)	TAC	TPC	Ref.
A. clypearia	14.47 ± 0.13	86.90 ± 0.10	9.89 ± 0.14	280.27 ± 1.32	74.49 ± 1.08	[41]
A. bauchei	1.59 ± 0.01	45.98 ± 0.05	0.02 ± 0.00	233.30 ± 1.16	24.35 ± 0.41	
P. venusta	0.16 ± 0.00	3.25 ± 0.00	0.35 ± 0.01	139.63 ± 1.11	21.35 ± 0.43	
S. oleracea	0.57 ± 0.01	10.90 ± 0.01	0.18 ± 0.00	143.72 ± 1.52	18.17 ± 0.79	
H. parasitica	18.34 ± 0.00	41.88 ± 0.05	7.24 ± 0.10	301.47 ± 1.68	93.22 ± 0.34	
H. hirsuta	8.60 ± 0.02	6687.45 ± 44.12	2732.13 ± 0.02	174.94 ± 1.56	67.59 ± 0.16	this work

3. MATERIALS AND METHODS

3.1. Plant Materials

The plant parts of *H. hirsuta* were collected on 15 January 2018 in Thua Thien Hue province of Vietnam. They were taxonomically identified by the department of Biology, College of Sciences; Hue University. A voucher specimen was deposited at the department of Biology, College of Sciences; Hue University.

All of the reagents, HPLC solvents and chemicals used in this work were purchased at analytical grade from Sigma - Aldrich Co. (USA), (methyl gallate (CAS number: 99-24-1), rutin (CAS number:207671-50-9), quercetin (CAS number:117-39-5), quercitrin (CAS number:522-12-3), *α*-Tocopherol (CAS number:10191-41-0) luteolin (CAS number:491-70-3), except the ethanol that was food grade and was purchased from local suppliers.

3.2. Preparation of Methanol Extracts and Fractions

A dried sample (100 g) was soaked in 0.5 L methanol (MeOH) three times at room temperature for 60 min. The solutions were combined, filtered through Whatman No.4 paper and evaporated under reduced pressure at 50^{o}C, yielding a crude methanol extract. The MeOH extract was dissolved in water and then extracted with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The solvents were then distilled to yield five fractions: *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water.

3.3. Evaluation of the Total Antioxidant Activity Using the Phosphor-molybdenum Method

The total antioxidant activity of the samples was determined following the method described in the literature with minor modifications [29]. In brief, a 0.3 mL aliquot of the sample was mixed with 3 mL of a reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate), and then the mixture was incubated at 95^{o}C for 90 min. The mixture was then cooled down to 25^{o}C and the absorbance was measured at 695 nm wavelength against a blank that contained 3 mL of the reagent solution without the sample. The total antioxidant activity was expressed as number equivalents of gallic acid (GA) [33] and ascorbic acid (AS) [34] and as the absorbance of the sample where the higher absorbance value indicates the higher antioxidant activity.

3.4. Evaluation of DPPH Radical Scavenging Activity

The DPPH free radical scavenging activity of each sample was determined using a Jasco V-630 Spectrophotometer based on a published method [17, 29]. The samples were dissolved in 1.5 mL methanol at various concentrations (25, 50, 75 and 100 μg/ mL) and mixed with 1.5 mL of 100 μM DPPH (100 μM DPPH dissolved in methanol before using). The reaction mixture was shaken for 1 minute and incubated at room temperature for 30 minutes. The absorbance was then measured at 517 nm wavelength. Methanol was used as a blank sample. Radical scavenging activity was defined as the IC_{50} value [42].

3.5. Total Phenolic Content

Total phenolic content was determined by the Folin – Ciocalteu method. 0.5 mL of the methanolic extract solution was mixed with 2.5 mL of Folin – Ciocalteu (1:10) reagent and 2 mL saturated Na_2CO_3 solution. The tubes were incubated for 2 hours at room temperature. Absorbance was then measured at 760 nm wavelength. Gallic acid was used to construct the standard curve (with concentrations between 0.05 ÷ 3 mg/mL) and the results were expressed as mg of gallic acid equivalents (GAE) per g of sample [17, 30].

3.6. Total Flavonoid Content

The total flavonoid content was determined following a published method[43]. Briefly, 1 mL of the methanol extract solution was diluted by the mixture of 4 ml of deionized water and 0.3 mL of 5% $NaNO_2$. After 5 minutes, 0.3 mL of 10% $AlCl_3$ solution was added to the solution. Then, 2 mL of 1M NaOH solution was also added, and the mixture was filled to 10 mL by deionized water. Absorbance was then measured at 510 nm wavelength. The total flavonoid content was determined using a standard curve of quercetin at 0.02–50 mg/mL. The results were expressed as quercetin equivalents (QE) on a dry weight (DW) basis [43].

3.7. HPLC Conditions

One hundred milligrams of dry plant matter were accurately weighed and dissolved in methanol to obtain a 10 mg/mL sample solution. Chromatographic analysis was carried out in a C_{18} reversed phase Inertsil ODS-3 column (150 x 4.6 mm) packed by 5 μm diameter particles, UV-Vis detector. The HPLC parameters and chromatographic conditions are given in Table **7**. All of the solutions and the mobile

Table 7. HPLC parameters.

Compounds	Methyl Gallate	Rutin	Quercetin	Quercitrin	α-Tocopherol	Luteolin
Concentrations (mg/mL)	0.5 to 5	0.05 to 2	0.5 to 2	0.05 to 1	0.05 to 1	0.02 to 1
Mobile phase (v/v)	0.5% orthophosphoric acid (A): Methanol (B) (0 ~ 10 min, 10 → 25% A; 10 ~ 60 min, 25 → 47% A)			water (A) : acetonitrile (B) (0 ~ 20 min, 15% B → 25% B, 20 ~ 30 min, 25% B → 70%)	methanol: water (97:3)	acetonitrile: 0.1% acid phosphoric
Flow rate (mL/min)	1.0			1.0	1.2	0.5
Injection volume (μL)	20			10	20	10
Standard Rt (Min)	15.48 ± 0.12	38.33 ± 0.23	55.62 ± 0.42	15.03 ± 0.18	11.21 ± 0.11	2.80 ± 0.11
Detection wavelength (nm)	370	370	370	370	295	347

phases were filtered through a 0.45 μm cellulose filter before use and all chromatographic operations were carried out at ambient temperature.

All the experiments were performed three times (n = 3) and the results were expressed as the mean value ± standard deviation (SD). The accuracy of the method was determined by the standard addition method. The leaf extract of H. hirsuta was spiked with 0.05 mg/mL concentration of calibration solutions. Six phenolic compounds from *H. hirsuta* leaf present in the investigated leaves were previously determined. For each standard compound, the percentage of recovery was calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{amount found} - \text{amount contained}}{\text{amount added}} \times 100$$

The identification of phenolic compounds was accomplished by comparison of their retention times to those of pure standards.

CONCLUSION

Antioxidant properties of extracts of *H. hirsuta* plants have been investigated. It was shown that all of the methanol extracts of different *H. hirsuta* parts exert good antioxidant activity that is roughly 2 times higher than that of curcumin in the DPPH model. The ethyl acetate fraction from the leaf extract of *H. hirsuta* demonstrates the highest antioxidant activity with the lowest IC_{50} value at 9.50 μg/mL that is 4 times lower than that of curcumin. Quantification of six typical phenolic compounds from *H. hirsuta* leaf: methyl gallate, rutin, quercetin, quercitrin, *α*-tocopherol and luteolin revealed that the amounts of methyl gallate and rutin dominated the extracts at 8.62 ± 0.01 and 6.76 ± 0.02 mg/g, respectively, out of a total of 21.00 ± 0.01 mg/g active phenolic fraction. The amount of methyl gallate strongly correlated with total phenolic content and total antioxidant capacity, thus this compound may be useful as a marker of antioxidant activity in common medicinal plants. The results also indicate that *H. hirsuta* is a promising resource of natural antioxidants.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article and its supplementary materials.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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