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HPLC Profiling and Antioxidant Properties of the Ethanol Extract of *Hibiscus tiliaceus* Leaf Available in Bangladesh

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Authors' contributions

This work was carried out in collaboration between all authors. Authors HH, PNA, SER, TAK and IAJ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MMR and SY managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this research was to investigate the antioxidant activity and HPLC fingerprinting profiles of the ethanolic leaf extract of *Hibiscus tiliaceus* growing in Bangladesh. Catechin, rutin hydrate, ellagic acid and quercetin contents were quantified in the sample by HPLC-DAD (99.00±1.88, 79.20±1.59, 59.40±1.36 and 69.30±1.47 mg/100 g of dry extract), respectively. The antioxidant

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potential by 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activity, reducing power, total antioxidant capacity and total phenolic and flavonoid content were determined. In all the methods, the extract exhibited potent antioxidant activity. The IC_{50} value of the extract on ABTS radical was found to be 6.25±0.15 µg/ml, and at 250 µg/ml, maximum absorbance of reducing power was obtained 0.6475±0.021. The total antioxidant capacity, total phenolic and flavonoid contents were found in considerable amount (595.2±3.61 mg of ascorbic acid/g, 298.07±2.01 mg/g of gallic acid, and 13.69±0.06 mg/g of quercetin equivalent), respectively. Substantial amounts of phenols and flavonoids were noticed and thus, justify the free radical scavenging and antioxidant characteristics of the extract.

Keywords: Hibiscus tiliaceus, antioxidant, HPLC, rutin hydrate, ellagic acid, quercetin, ABTS, reducing power.

1. INTRODUCTION

Hibiscus tiliaceus L. (*H. tiliaceus*) is a coastal plant belonging to the Malvaceae family. They also go by other names like beach hibiscus, sea hibiscus, Indian hibiscus and wild cotton tree. In Bangladesh, however, *H. tiliaceus* is locally known as Bhola. These plants are aboriginal to the shorelines of the Indian and Pacific oceans and grow mainly as an ornamental tree to a height of 3-6 m.

Plants of Malyaceae family are used in a wide range of areas. They are sources of natural fibres. They are used as treatment for the irritation of mucous membranes, gargle for mouth, throat ulcers and gastric ulcers [1]. Woods, leaves, barks and flowers of *H. tiliaceus* have many medicinal purposes. Traditionally, the flower is broadly used for birth control in Asian and African countries. In Malaysia and Indonesia, the flowers are used to treat fever [2]. The flower bud and bark of *H. tiliaceus* contain a slimy, juicy sap, which when soaked can be used as medicine for congested chests, fevers, coughs, dysentery, ear infections, abscesses and as laxative [3,4]. The buds can also be chewed and eaten to treat dry-throat. An infusion of the dried wood of *H. tiliaceus* is usually used to expel the placenta and to combat post-parturition disorders [5]. An aqueous extract of wood and fresh flowers have also been reported to be useful in treating skin diseases [6,7].

Previous phytochemical investigations revealed the potential role of the plants of *Hibiscus* genus. They are a valuable source of triterpene derivatives, phytosteroids and antioxidants [8]. Flowers and leaves of *H. tiliaceus* contain stigmasterol, stigmastadienol, stigmastadienone, vanillic acid, syringic acid, n-trans-feruloyltiramine, n-cis-feruloyltyramine and beta-sitostenone. A new coumarin, hibiscusin, and a new amide, hibiscusamide along with p-hydroxybenzoic acid, scopoletin and p-hydroxybenzaldehyde were also isolated from the stem wood of *H. tiliaceus* [9].

The methanol wood extract of *H. tiliaceus* showed significant anti-inflammatory and anti-depressant activity [10] in addition to strongly inhibiting the mutagenic action of tert-butyl-hydroperoxide [11]. The extract showed antioxidant activity against oxidative DNA damage [12]. *In vivo* antioxidant properties and antimutagenic effects of the flower extract were also evaluated. The antinociceptive and anti-inflammatory activity of leaf when tested exhibited significant activity results. Other than that, *H. tiliaceus* showed selective cytotoxicity against breast cancer cells [13] and against three strains of bacteria: *S. aureus*, *E. coli* and *S. paratyphi* [14]. The plant also revealed antipyretic and antiulcer properties [15]. No antifungal activity was demonstrated [16].

In the present investigation, we attempted to determine the antioxidant properties of the ethanol leaf extract of *H. tiliaceus* growing in Bangladesh, and quantify the major bioactive polyphenolic compounds through HPLC.

2. MATERIALS AND METHODS

2.1 Plant Material

Leaves of *H. tiliaceus* were collected from Khulna, Bangladesh during January 2013 (Accession no: DACB 36722). All plant materials were properly washed, shade dried, ground, and submitted to extraction with ethanol.

2.2 Extraction

Powdered leaf sample was extracted in an orbital shaker with 95% ethanol. Ethanol extract was

obtained by continuous stirring at room temperature for 1 week. The extract was then filtered first with a cotton plug to get rid of plant debris, and afterwards through Whatman filter paper no. 1 and concentrated using a vacuum rotary evaporator (R-215, Buchi, Switzerland) at 60ºC.

2.3 Chemicals

Vanillic acid (VA), (+)-catechin hydrate (CH), caffeic acid (CA), gallic acid (GA), (-)-epicatechin (EC), rutin hydrate (RH), *p*-coumaric acid (PCA), ellagic acid (EA), quercetin (QU), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), ascorbic acid, folin-ciocalteu's phenol reagent were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol (HPLC), acetonitrile (HPLC), ethanol, acetic acid (HPLC), phosphate buffer (pH 6.6), potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), EDTA, sodium phosphate, ammonium molybdate and sodium carbonate were purchased analytical grade from Merck (Darmstadt, Germany).

2.4 HPLC Analysis

2.4.1 Instrumentation

Chromatographic analysis was performed on an HPLC system model Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC systems (RSLC) from Thermo Fisher Scientific Inc., MA, USA. They were equipped with a diode array detector (DAD: 3000RS), quaternary pump system (LPG: 3400RS) and Ultimate 3000RS autosamplier (WPS: 3000). The system was controlled by Version 6.80 RS 10 Dionix Chromeleon software. Acclaim® C18 (4.6 x 250 mm; 5 µm) column from Dionix, USA was used for the chromatographic separation of polyphenols that was maintained at 30ºC using a column compartment (TCC:3000).

2.4.2 Chromatographic conditions

A solution of *H. tiliaceus* was prepared at a concentration of 5 mg/ml in ethanol by mixing for 30 min. The solutions were allowed to stand at 5°C in the dark. These were then spiked to recognize the individual polyphenols. The sample solutions were next filtered through 0.2 µm nylon syringe filter (Sartorius, Germany) before being degassed for 15 min in an ultrasonic bath (Hwashin, Korea). Finally, the compounds were isolated with reverse-phased HPLC and their respective chromatograms were obtained [17,18].

Acetonitrile (solvent A), pH 3.0 acetic acid solution (solvent B) and methanol (solvent C) were used for the mobile phase. The gradient program consisted of a 0 min run at 5%A/95%B, a 10 min run at 10%A/80%B/10%C, a 20 min run at 20%A/60%B/20%C and a 35min run at 100%A before the column was washed and reconditioned. A flow rate of 1 ml/min was maintained throughout the analytical run, and the sample injection volume was 20 µl. The detection wavelength was: λ 280 nm held for 18.0 min, changed to λ 320 nm and held for 6 min, and finally changed to λ 380 nm and held for the rest of the analysis and the diode array detector was controlled at a wavelength of 200 nm to 700 nm.

Individual phenolic compounds were identified by comparing their retention time and UV spectrum with those obtained by injecting standards in the same HPLC conditions. The detection and quantification of CH, GA, VA, EC and CA were carried out at 280 nm, while PCA, EA, and RH were read at 320 nm, and QU at 380 nm, respectively. Analyses were performed in triplicate.

2.4.3 Standard and sample preparation

Ethanol stock solutions (100 µg/ml) containing phenolic compounds were prepared and diluted to appropriate concentrations to make standard solutions of 20 µg/ml for all the polyphenolic compounds except for caffeic acid, which was prepared to 8 µg/ml, and quercetin to 6 µg/ml. The reaction mixtures were afterwards stored in a dark place at 5°C. The calibration curves were constructed by plotting the peak under the curve area versus the concentration of the analytes.

2.5 Antioxidant Activities

2.5.1 ABTS radical scavenging test

The ABTS radical cation scavenging activity was performed with slight modifications described by Fan et al. [19]. $ABTS^+$ radical cations were formed during the reaction of 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in a dark place at rtp for 12-16 h. Right before use, the ABTS solution was diluted with pH 7.4 phosphate buffered saline (PBS) solution to an absorbance of 0.70±0.02 and read at 734 nm. Free radical scavenging activity was assessed by mixing 1 ml of the test sample with 1 ml of ABTS working standard. The reaction was allowed to

reach completion by letting it stand at room temperature for 6 min before reading the absorbance at 734 nm. The percentage scavenging activity was calculated with the formula:

Percentage inhibition (%) = $(A_o – A_s / A_o) \times 100$,
Where, A_o=absorbance of blank; A_s=absorbance of sample

2.5.2 Reducing power assay

Reducing power assay of the leaf extract of *H. tiliaceus* was evaluated using the process of Hemayet et al. and Dehpour et al. [20,21]. The extract at different concentration (2.5 ml) was mixed with ethanol (1 ml), 0.2 M phosphate buffer pH 6.6 (2.5 ml) and 1% potassium ferricyanide (2.5 ml). The reaction mixtures were incubated for 20 min at 50°C and centrifuged for 10 min at 5000 rmp after the addition of 10% solution of trichloroacetic acid (2.5 ml). 2.5 ml of the supernatant was mixed with de-ionized water (2.5 ml) and 0.1% ferric chloride (0.5 ml). Absorbance of the samples was read in triplicates at 700 nm.

2.5.3 Total antioxidant capacity

Total antioxidant capacity of the leaf extract of *H. tiliaceus* was evaluated by the process of Prieto et al. [22]. The extract was prepared in ethanol and mixed with the reagent solution (1 ml). The reaction solution included 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate mixture. These were then incubated at 95ºC for an hour and half. The mixture was cooled to rtp and their absorbance was measured at 695 nm. Ascorbic acid was used as the standard and its equivalents were calculated using the calibration curve. All values were read in triplicates.

2.5.4 Total phenolic content

Total phenolic content of the *H. tiliaceus* ethanol extract was calculated using the process of Folin-Ciocaltu [23,24] with slight alterations to it. 0.5 ml of extract (1 mg/ml) was reacted to 5 ml Folin-Ciocaltu reagent, which was prepared in the ratio 1:10 v/v with distilled water, and 4 ml (75 g/l) sodium carbonate. The solutions were left standing for the next 30 min at 40ºC for the color to develop. The absorbance was measured at a wavelength of 765 nm against a blank sample. The total phenolic content is expressed as mg per gram of gallic acid equivalents (GAE) with the equation, $y = 6.993x+0.0379$, $R^2 = 0.9995$.

2.5.5 Total flavonoid content

The total flavonoid content was calculated by the aluminium chloride colorimetric method with slight alterations to it [25,26]. Absorbance of the mixture was read with an UV spectrophotometer (Model 205, Jena, Germany) at 430 nm. Standard curve was calibrated using quercetin with the equation, $y = 6.2548x+0.0925$; $R^2 = 0.998$ and expressed as mg per gram of quercetin equivalent.

2.6 Statistical Analysis

All calculations and results were carried out in triplicates and demonstrated as mean ± standard deviation (S.D).

3. RESULTS AND DISCUSSION

3.1 HPLC Assay of *H. tiliaceus*

Phenolic compounds in the leaf extract of *H. tiliaceus* were analyzed by HPLC-DAD. As demonstrated in Fig. 1, the retention times and the standard peaks were compared and four phenolic compounds were detected: (+) catechin, rutin hydrate, ellagic acid and quercetin, respectively. The most abundant polyphenol noted in the extract of *H. tiliaceus* was (+) catechin (99.00±1.88 mg/100 g dry extract), which was followed by rutin hydrate (79.20±1.59 mg/100 g dry extract). Ellagic acid and quercetin were also detected in moderate quantities (59.40±1.36 and 69.30±1.47 mg/100 g dry extract, respectively). The HPLC chromatogram and the bioactive polyphenolic compounds in the ethanol extract of *H. tiliaceus* are presented in Fig. 1 & Table 1, respectively.

Phenolic compounds have strong antioxidant activities associated with their abilities to scavenge free radicals, donate hydrogen ions, chelate metals and break radical chain reactions *in vitro* and *in vivo* [27]. As is observed in Table 1, *H. tiliaceus* showed a rich content of these active components, and thus could be used as powerful antioxidants for industries and health care centres.

3.2 Antioxidant Activities

The antioxidant characteristics of the extract of *H. tiliaceus* were evaluated for by different *in vitro* antioxidant assays such as ABTS radical scavenging activity, reducing power assay, total phenolic and flavonoid content and total antioxidant capacity.

3.2.1 ABTS radical scavenging activity

The ABTS scavenging ability was determined based on the percentage inhibition of the ABTS cation by hydrogen donating and chain-breaking antioxidants in the sample (Fig. 2, Table 2). The sample effectively neutralised the radical cation $ABTS^{+}$. The activity was found to increase in a dose-dependent manner at a concentration of 10 to 250 µg/ml. At minimum concentration (10 µg/ml), the highest activity obtained by the leaf extract of *H. tiliaceus* was 80.86±0.15% inhibition value exceeding that of ascorbic acid (48.60±0.17%). The leaf extract exhibited an IC_{50} value of 6.25±0.15 µg/ml, which was similar to that of the ascorbic acid (12.34±0.12 µg/ml). Therefore, *H. tiliaceus* leaf extract can be said to possess strong free radical scavenging ability [28].

3.2.2 Reducing power assay

The reducing power assay is related to its electron donating ability was evaluated based on its relative maximum absorbance (Fig. 3). At 250 µg/ml, the maximum absorbance of the extract was found to be 0.6475±0.021, while ascorbic acid read an absorbance of 1.1115±0.009. Absorbance of the sample increased linearly with concentration. As Fe^{3+} oxidized to Fe^{2+} and donated a hydrogen atom, the free radical chain is interrupted. When this happens, the yellow color of the test solution changes to green and blue depending on the reducing power ability of the extract. This initiates the compounds to exert an antioxidant response [29]. Phenolic compounds are capable of donating hydrogen ions and hence, these phenolic compounds in the extract of *H. tiliaceus* might be a reason behind the high reducing ability of the extract.

3.2.3 Total antioxidant capacity

Total antioxidant capacity of *H. tiliaceus* leaf extract is based on the reduction of phosphomolybdenum, Mo (VI) to green molybdenum (V) by the extract. The total antioxidant capacity observed in the ethanol extract of *H. tiliaceus* was 595.20±3.61 mg of AAE/g of extract (Fig. 4) [30,31,32].

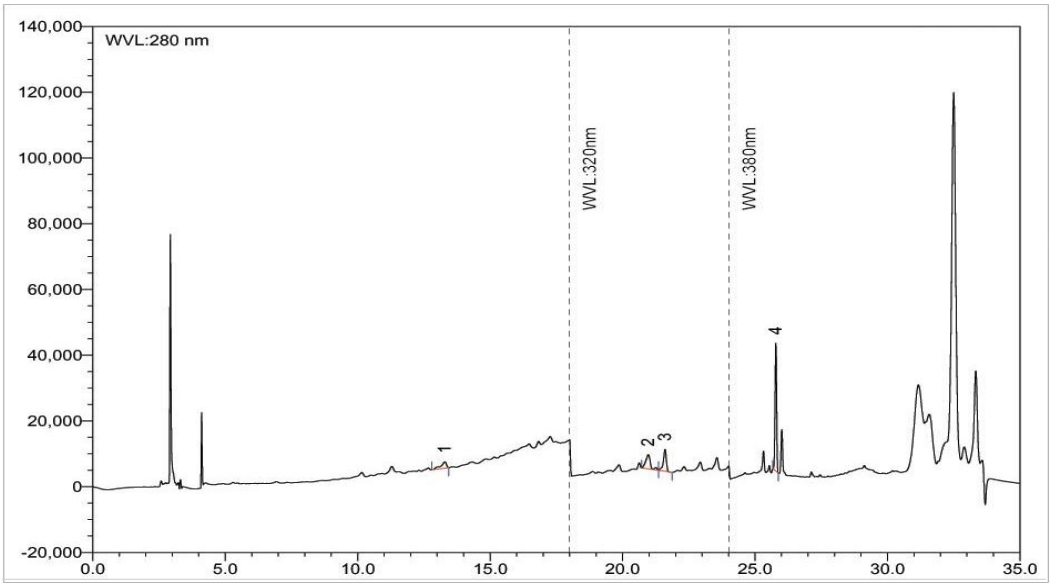

Fig. 1. Chromatogram obtained from HPLC of *H. tiliaceus* ethanol leaf extract. Peaks: 1, (+)-catechin; 2, rutin hydrate; 3, ellagic acid; 4, quercetin

Table 1. Bioactive polyphenols obtained from the ethanol extract of *H. tiliaceus* (n = 3)

Polyphenols	Ethanol extract of *H. tiliaceus* leaf	
	Content (mg/100 g dry extract)	% relative standard deviation (RSD)
Catechin	99.00	1.88
Rutin hydrate	79.20	1.59
Ellagic acid	59.40	1.36
Quercetin	69.30	1.47

3.2.4 Total phenolic and flavonoid content

Fig. 4 gives information on the total phenolic and total flavonoid content of *H. tiliaceus* leaf extract. The total phenolic and flavonoid content of the leaf extract of *H. tiliaceus* were found to be 298.07±2.01 mg per gram of gallic acid equivalent and 13.69±0.06 mg per gram of quercetin equivalent, respectively. Phenolic and flavonoid compounds are effective free radical scavengers and have convincingly demonstrated powerful antioxidant characteristics, hence, making itself useful in the field of medicine [33,34,35,36]. The presence of polyphenols in the *H. tiliaceus* leaf extract is a possible explanation for the high percentage inhibition value obtained. As a result, it can be concluded that the extract of *H. tiliaceus* shows significant antioxidant activity due to these phenolics, flavonoids, etc.

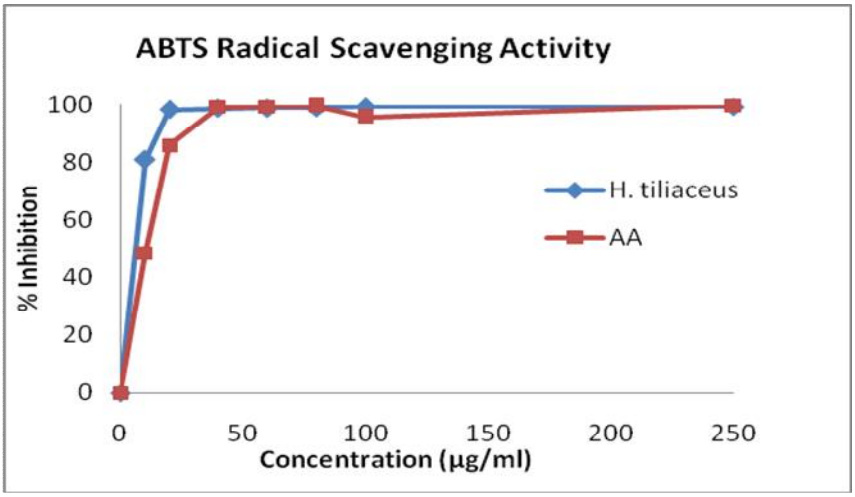

Fig. 2. ABTS radical scavenging activity of *H. tiliaceus* leaf extract with standard ascorbic acid

Table 2. IC_{50} value of *H. tiliaceus* leaf extract and standard ascorbic acid in ABTS radical scavenging activity

	***H. tiliaceus* leaf extract**	**Ascorbic acid**
IC_{50}	6.25±0.15	12.34±0.12

The values are expressed as mean ± standard deviation (n=3).

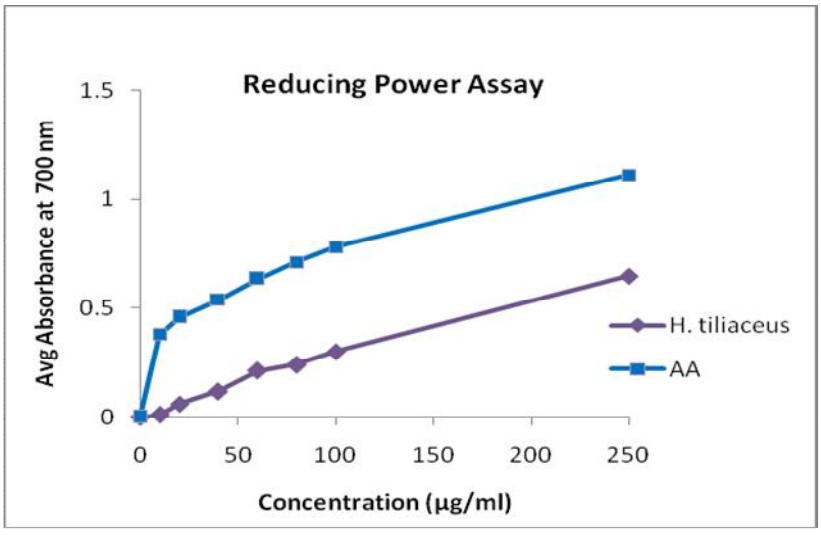

Fig. 3. Reducing power assay of *H. tiliaceus* leaf extract with standard ascorbic acid

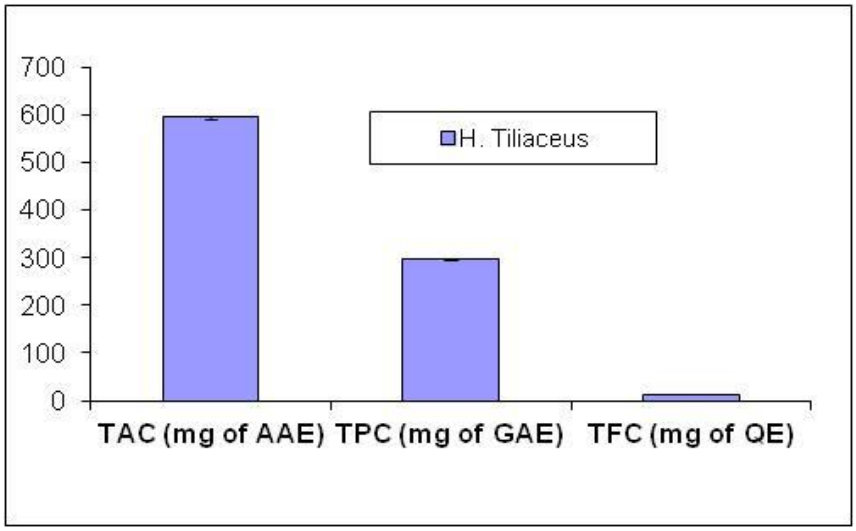

Fig. 4. Total antioxidant capacity (TAC), total phenolic content (TPC) and total flavonoid content (TFC) of *H. tiliaceus* leaf extract with standard ascorbic acid. AAE: Ascorbic Acid Equivalent; GAE: Gallic Acid Equivalent; QE: Quercetin Equivalent

4. CONCLUSION

In the present study, antioxidant activities of the ethanol leaf extract obtained from *H. tiliaceus* were investigated. The extract demonstrated effective antioxidant properties, as determined by the scavenging effect on the $ABTS^{+}$, reducing power assay, total antioxidant activity and total phenolic and flavonoid content. According to the results, the extract was effective in antioxidant properties. It was also found that the leaf extract contains a substantial amount of phenolic compounds. Therefore, it can be inferred that the phenolics present in the *H. tiliaceus* sample might be responsible for its antioxidant properties presenting meaningful information for the collection and application of *H. tiliaceus* in both healthcare and food industry. Nonetheless, a large, systematic study of *H. tiliaceus* from different sources would be helpful.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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