Original Article:
Evaluation of Antioxidant and Anticoagulation Activity of *Piper chaba* Hunter Stem

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ABSTRACT

Background: *Piper chaba* Hunter, a flowering vine of the Piperaceae family, has long been used in South Asian countries for culinary purposes and traditionally in fat-rich meat preparation. The curative potential of this herb is of great interest to be studied.

Objectives: The antioxidant and anticoagulation potential, as well as total phenolic and flavonoid content, were evaluated using cold and boiled water extract separately from the dried and ground stem.

Methods: Antioxidant potential was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and ferric reducing antioxidant power (FRAP) assay. The anticoagulation activity was evaluated by serine protease inhibition assay and prothrombin time (PT) assay. Folin–Ciocalteu (FC) reagent and aluminum complex ($AlCl_3$) were used to assess total phenolic content and total flavonoid content, respectively.

Results: DPPH scavenging assay revealed the IC_{50} value of 125.52 µg.mL^{-1} and 157.94 µg.mL^{-1} for boiled and cold water extract, respectively. Potent ferric reducing potential (FRAP) was observed as 142.87 µM and 135.37 µM of ferrous equivalent per 100 µg for boiled and cold water extract, respectively. The IC_{50} value of serine protease inhibitory activity was found as 182 µg.mL^{-1} and 161.12 µg.mL^{-1} for cold and boiled water extract, respectively. The PT time was 27.00 min for boiled water extract and 24.68 min for cold water extract. Significant phenolic and flavonoid content was also found in the test sample.

Conclusion: *P. chaba* stem extract possesses potent antioxidant and anticoagulation activity, which can neutralize oxidative free radicals and have a vasodilation effect in oxidative and inflammatory diseases.

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Introduction

Oxidative processes are involved in metabolism, which is essential for cell multiplication, development, and survival. Reactive oxygen species (ROS) are oxidizing agents generated from the metabolism of oxygen as by-products resulting from various cellular signaling pathways involved in the defense mechanism [1]. An important feature among the various ROS types is their ability to engender oxidative damage to DNA, proteins, and lipids. Excessive generation of ROS may lead to oxidative stress and eventually loss of cell function and ultimately, apoptosis or necrosis [2, 3]. Antioxidants are capable of slowing down or preventing damages caused by free radicals by intercepting, delaying, inhibiting free radical functions, or breaking the oxidation chain reaction [4]. Synthetic antioxidants, namely butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are well known to exhibit free radical inhibition properties in the human body, but these compounds can also be hazardous and may be responsible for liver damage, Alzheimer's disease (AD), cancer, cardiovascular diseases, and even facilitating the aging process and other diseases [5].

Oxidative stress may also have a direct effect on the blood coagulation system. Stress can provoke the activation of the sympathoadrenal medullary system, which in turn stimulates both the coagulation cascade and the fibrinolytic system and ultimately ends up in hypercoagulability [6]. In chronic degenerative diseases, namely atherosclerosis, fibrin clots form dense matted deposits (DMD) that are not effectively removed, generating a situation known as thrombosis, which increases the risk of cardiovascular difficulties [7]. Blood vessel blockage and fibrin clots in the body can result in ischemic stroke and may cause inevitable brain damage. Typical remedial thrombolytic agents have been examined and FDA-approved tissue plasminogen activator has low efficacy as well as has several side effects; thus, it is necessary to develop safer and more efficacious treatment candidates from natural sources [8].

For this reason, there is a particular interest in the potential health benefits of natural antioxidant-anticoagulant sources and plants that has the greatest capacity for scavenging ROS as well as in the identification of natural antioxidants from native plant sources. Scientists are trying to develop plant-based antioxidant molecules instead of synthetic ones and scientific communities are now interested in phytochemistry and the activities of plant extracts with the ultimate aim to emphasize the perspectives of the esteem of natural products [9, 10].

Piper chaba Hunter is a plant of the Piperaceae family, locally (Bangladesh) known as 'Choi jhal'. The hairless, smooth, scrambling shrub is available in India, Bangladesh, Malay Islands, and other warmer regions of Asia, including Singapore, Indonesia, and Sri Lanka. The aerial part of this plant is claimed to exhibit anti-diarrheal, antibacterial, anti-hypertensive, carminative, diuretic, analgesic, and smooth muscle relaxant properties [11]. A previous phytochemical study on *P. chaba* revealed the existence of alkaloids and lignan, namely kusunokinin, piperamine-2, 4-decadienoic acid, piperidide, and pellitorine [12, 13]. Several reports on antioxidant [14], antimicrobial [15], and anti-inflammatory [13] properties of *P. chaba* in the nonpolar extract have been documented.

Traditionally, *P. chaba* has been used in fat-rich meat preparation. Fat-rich meat ultimately results in excess oxidative stress and atherosclerosis from excess fat oxidation. Hence, the antioxidant and anticoagulation potential of this herb may have to neutralize oxidative free radicals and vasodilation effect. Considering less information about the antioxidant and anticoagulation activity of water extract, this study focused on the evaluation of the antioxidant and anticoagulation potential of its cold and boiled water extracts and a comparison between two extracts to achieve the most effective one for use in traditional meat preparation.

Materials and Method

Sample collection, identification, and extraction

Fresh *P. chaba* stems were collected from the nearest local market, the sample was identified and also a representative specimen (AA-KU-2019015) was dropped at the herbarium of the Forestry and Wood Technology Discipline, Khulna University, Khulna-9208, Bangladesh. The chopped stems were dried adequately under shade followed by grinding of the dried stem into a fine powder using a grinder. Ground powder (400 g) was saturated in a glass jar containing 1L of cold (25°C) distilled water and was kept for seven days with regular stirring and shaking. Boiled water extract of *P. chaba* was prepared by boiling the mixture of 400 g of the ground powder and 1L of distilled water for 30 minutes on a regular gas stove. The mixture solution was filtered off with clear cotton to eliminate plant debris followed by evaporation of solvent using a fridge dryer. Both of the samples were stored in a refrigerator at 4 °C for further study.

Chemicals and reagents

Ascorbic acid, sodium nitrite, aluminum chloride, sodium hydroxide, sodium carbonate, calcium chloride, ferric chloride, quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6- tripyridyltriazine (TPTZ), Folin-Ciocalteu reagent, and gallic acid were bought from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents of analytical grade were bought from local commercial suppliers.

Standard drugs

The standard blood anticoagulant drug warfarin (warin 5 mg tablet) was collected from Incepta Pharmaceuticals Limited, Bangladesh, and was used as a standard for the anticoagulation activity study.

In vitro antioxidant activity test

Because of the complex nature of various phytochemical classes, it is inappropriate to assess the antioxidant potential of plant extract precisely by one single method. Therefore, in this study, we conducted DPPH free radical scavenging assay as well as a ferric reducing antioxidant power (FRAP) assay to assess antioxidant capacity.

DPPH free radical scavenging assay

The free radical scavenging capacity was assessed by DPPH free radical scavenging assay as directed by Afrin et al. [16] with some modifications. To begin, nine concentrations (1.57, 3.13, 6.25, 12.50, 25, 50, 100, 200, and 400 µg.mL^{-1}) of plant extract and standard quercetin were prepared by serial dilution technique using ethanol as solvent. Then, 1 mL of 0.004% DPPH was added to each triplicate test tube of different concentrations that already had 1 mL of plant extract or standard. After 30 minutes of incubation period at the dark condition and room temperature, the inhibition percentage was calculated by recording absorbance at 517 nm [17] against ethanol control in a UV-visible spectrophotometer (UV-VIS Spectrophotometer - 2375, Labmatrix Manufacturing LLP). Percentage scavenging activity was calculated using the following Equations 1 and 2:

1.

$$\text{Scavenging ability (\%)} = \frac{\left(\text{absorbance of the control} - \text{absorbance of the sample}\right)}{\text{absorbance of the control}} \times 100$$

2.

$$\text{Inhibition (\%)} = \frac{\left(\text{Absorbance of sample} - \text{Absorbance of blank}\right)}{\text{absorbance of control}} \times 100$$

The 50% inhibitory concentration (IC_{50}) was calculated by a non-linear regression curve of percent inhibition against the sample concentration.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed according to Islam et al. [18]. Firstly, TPTZ solution (0.031 g TPTZ in 10 mL 40 mM HCl), Acetate buffer (300 mM, pH 3.6), and 20 mM ferric chloride ($FeCl_3.6H_2O$) solutions were prepared followed by preparing FRAP working reagent using 25 mL 300 mM acetate buffer +2.5 mL TPTZ solution + 2.5 mL H_2O. Secondly, 2mL of FRAP reagent was added to Fe^{2+} ($FeSO_4.7H_2O$) solutions of known concentrations (0, 5, 10, 20, 40, 60, 80, and 100 µM) and a standard calibration curve was plotted using the absorbance against different concentrations. FRAP reagent preparation for the sample and ascorbic acid (positive control) followed the abovementioned procedure with a slight change and 2.5 mL ferric chloride solution was used instead of H_2O. One hundred µL of plant extract (100 µg.mL^{-1}) or ascorbic acid solution, 900 µL of distilled H_2O, and 2 mL of FRAP reagent were added to the freshly prepared reaction mixture and kept at 37 °C in the water bath and a dark environment for 30 minutes. FRAP value of the sample and ascorbic acid were calculated by measuring the absorbance at 593 nm and plotting the value at the standard straight-line equation.

Quantitative determination of antioxidant

Total phenolic content determination

The total phenol content (TPC) of the extracts was assessed according to Islam, et al. (2020) using Folin–Ciocalteu (FC) reagent [18]. To begin, five concentrations (50, 100, 150, 200, and 250 µg.mL^{-1}) of gallic acid solution and 100 µg.mL^{-1} concentration of plant extracts were prepared. Then, 1 mL of FC reagent (10-fold diluted with distilled water) and 9 mL of distilled water were added to each test tube of gallic acid and plant extract solution. Next, after 5 min, 10 mL of 7% sodium carbonate and the required amount of distilled water were added to the mixture until the total volume reached 25 mL. After 30 minutes of incubation period at room temperature, the absorbance was recorded at 750 nm. The gallic acid standard calibration curve was made by plotting absor-

bance vs. concentration. Finally, TPC was calculated by the standard gallic acid curve.

Total flavonoid content determination

This assay is based on the formation of flavonoid-Al-Cl_3 complex, which is measured spectrophotometrically according to Aunjum et al. [19]. To begin, five concentrations (50, 100, 150, 200, and 250 µg.mL^{-1}) of standard quercetin solution and 100 µg.mL^{-1} concentration of plant extracts were prepared. Then, to each tube of quercetin and plant extract containing 1 mL of the test sample, 5 mL of distilled water and 0.3 mL of $NaNO_2$ (5%, w/v) were added. Next, after mixing the solution properly, 0.6 mL of $AlCl_3$ (10%) and 2 mL of NaOH (1M) were added to the reaction mixture. Then, after 5 min, the absorbance was measured at 510 nm. The total flavonoid content (TFC) value was calculated using the standard quercetin calibration curve and expressed as mg of quercetin equivalents (QEs) per gram of dry extract (mg QE/g dw).

Determination of anticoagulation activity

Serine protease inhibition activity: Protease inhibition assay was conducted according to Bijina et al. [20] with slight modifications. To begin, aliquots of five concentrations (25, 50, 100, 200, and 400 µg.mL^{-1}) for both quercetin (standard) and plant extract in ethanol were prepared using the serial dilution technique. Then, 1 mL of trypsin (0.5 mg/mL prepared in 0.1 M phosphate buffer, pH 7.0) pre-incubated at 37° C for 15 min was added to each test tube. Next, 2 mL of 1% Hammerstein casein (prepared in 0.1 M phosphate buffer) was added to the above mixture and incubated for 30 min at 37° C for 30 min. After the incubation period, the reaction was terminated by adding 3 mL of 5 % trichloroacetic acid (TCA). Finally, after 15 minutes of centrifugation at 10,000 rpm, the absorbance of the clear supernatant was measured at 280 nm in a UV–Visible spectrophotometer against appropriate blanks. Percent inhibition was calculated by using the following Equation 3:

$$3.\ Inhibition\ (\%) = \frac{Abs_{sample} - Abs_{blank}}{Abs_{blank}} \times 100$$

The IC_{50} was calculated by a non-linear regression curve of percent inhibition against the sample concentration.

Prothrombin time determination assay: The PT was measured according to a previous report [21]. Firstly, from five healthy volunteers, 5 mL of blood from the median cubital vein was drawn into an EDTA vacuum tube. Then, blood samples were centrifuged for 15 min at 3000 rpm. Plasma from each tube was pooled together. Next, aliquots of three concentrations (250, 500, and 1000 µg.mL^{-1}) for standard (warfarin) and plant extract were prepared and 200 µL of blood plasma was mixed with 100 µL of different concentrations of test samples and standards. After shaking adequately, 300 µL of CaCl2 (25 mM) was added to each test tube followed by incubation at 37°C in a water bath. During the incubation period, each test tube was shaken gently back and forth in 5 seconds intervals and coagulation time was measured using a stopwatch.

Statistical analysis

All assays were conducted multiple times in the same environment and thus multiple data were generated for the same sample and same experiment. Each assay had three different data sets, namely boiled water extract, cold water extract, and standard.

One-way analysis of variance (ANOVA)

One-way ANOVA compared the means of two or more independent groups in order to determine if there is statistical evidence that the associated group means are significantly different from other given groups. To determine variance among each of the data sets, we performed one-way ANOVA in Prism (GraphPad) software [22]. We used three separate columns for the three samples in all assays at a 95% (p = 0.05) confidence interval in the software. We kept the parameters as experimental design without matching and pairing, multiple comparisons were used to compare the mean of the columns, Tukey's test was used for multiple comparisons, and then the ANOVA test was performed. A p-value lower than 0.05 was considered significant and greater than 0.05 was considered non-significant.

Correlation analysis

We plotted two different data sets for two samples in two columns in Prism [22]. Pearson correlation coefficient and two-tailed option were selected for correlation measurement. We analyzed the R-value and p-value of the results. The value of R is always between +1 and –1 while values close to +1 denote a strong positive linear relationship and values near -1 denote a strong negative linear relationship. The P denotes significance (Sig.) in relationships. A value less than 0.05 dictates that there is less chance to occur the correlation due to random data and thus the correlation is significant.

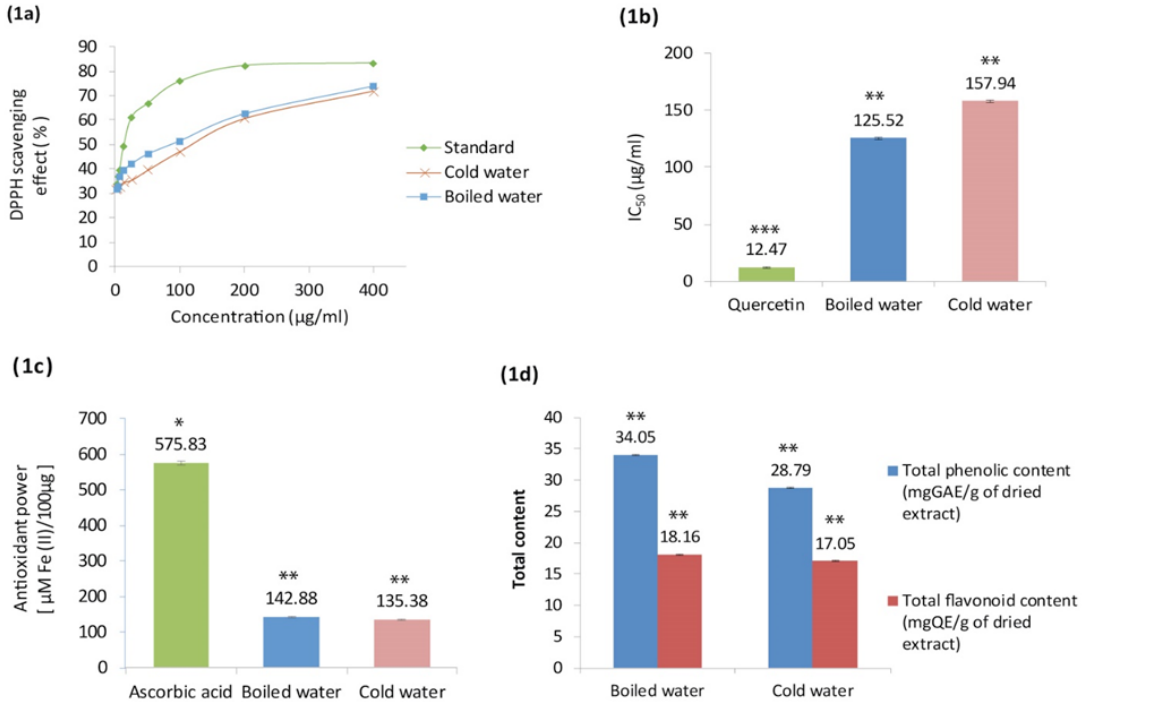

Figure 1. Antioxidant assay, total phenolic content (TPC), and total flavonoid content (TFC) of tested sample extracts and standard

(a) DPPH scavenging effect in percentage; (b) IC_{50} value of DPPH scavenging assay; (c) FRAP value; (d) Total phenolic and flavonoid content. *P<0.05, ** P<0.01, and *** P<0.001.

Standard error

Standard error measures the accuracy with which a sample distribution represents a population. The Standard Error takes the Standard Deviation (SD) and divides it by the square root of the sample size. We calculated standard error using the Microsoft excel program [23].

Results

DPPH free radical scavenging activity

The DPPH free radical scavenging activity increased in a concentration-dependent linear pattern (Figure 1a). Scavenging percentages at 400 µgmL-1 concentration of boiled water and cold water extracts were found at 74.02% and 71.74%, respectively, while reference standard quercetin showed a scavenging percentage of

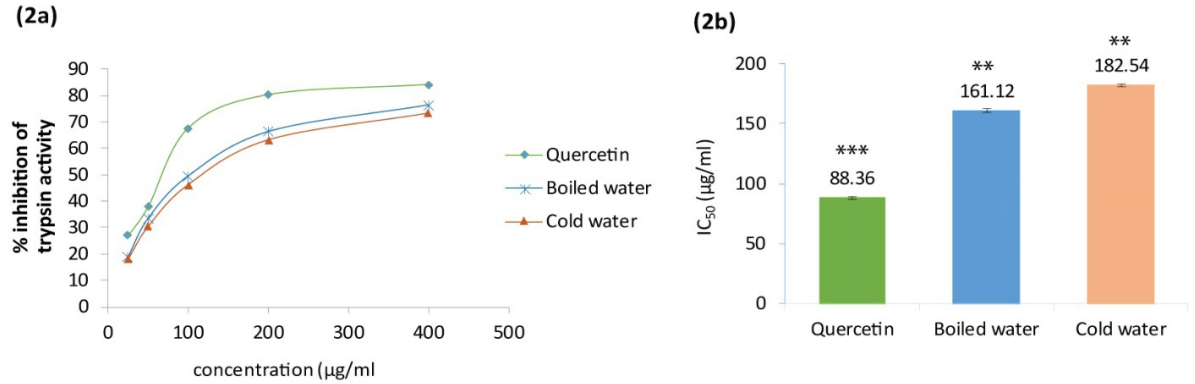

Figure 2. Anti-coagulation activity of tested sample and standard

(a) Protease inhibition effect in percentage; (b) IC_{50} value of protease inhibition assay; **P<0.01 and ***P<0.001 compared to the respective control group.

Table 1. Prothrombin time of the test samples and standard

Concentration (µg.mL^{-1})	Prothrombin Time (min)			Negative Control
	Mean±SE.			
	Warfarin (Standard)	Boiled Water Extract	Cold Water Extract	
1000	36.65±0.18	27.10±0.15	24.68±0.23	
500	30.20±0.21	18.63±0.09	17.15±0.18	1.18±0.03
250	28.15±0.18	16.10±0.15	14.50±0.09	

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83.33%. Boiled water extract showed better DPPH scavenging potential (IC_{50} 125.52 µg.mL^{-1}) compared to cold water extract (IC_{50} 157.94 µg.mL^{-1}) (Figure 1b). Both Boiled and cold water extracts demonstrated significant DPPH free radical scavenging activity (P<0.01) compared to the control group.

Ferric reducing antioxidant power (FRAP) assay

The FRAP values of boiled water and cold water extracts were found to be 142.87 and 135.37 µM Fe (II)/100µg), respectively, while the FRAP value of standard ascorbic acid was 575.83 µM Fe (II)/100µg) (Figure 1c). Significant ferric reducing potential (P<0.01) was observed in both boiled and cold water extracts compared to the control.

Total phenolic and flavonoid content

The quantitative analysis of the total amount of phenolic acid in the boiled and cold water extracts exhibited a total phenolic content of 34.05 mg and 28.78 mg gallic acid equivalent per gram of dry extract, respectively. The flavonoid content of boiled and cold water extract was 18.16 and 17.05 mg quercetin equivalent per gram of dry extract, respectively. The phenolic and flavonoid content of boiled water extract was found to be considerably higher than that of cold water extract (Figure 1d), which was statistically significant (P<0.01) based on one-way ANOVA.

Serine protease inhibitory activity

In this assay, Trypsin (used as serine protease) degraded casein by its proteolytic activity. The degradation of casein is reduced through inhibition of trypsin by the compounds present in the test sample. The protease inhibition activity of the test sample and standard increased linearly in a concentration-dependent pattern (Figure 2a). Maximum inhibition percentages at 400 µg.mL^{-1} concentration of boiled water and cold water extracts were found to be 76.48% and 73.29%, respectively, compared to standard quercetin (84.01%). Both boiled and cold water extracts exhibited significant protease inhibition activity at P<0.01 compared to quercetin.

Prothrombin time assay

In PT assay, the clotting time after using plant extracts and standard drug (warfarin) showed a concentration-dependent manner (Table 1). The highest PT was 36.65 min using warfarin at 1000 µg.mL^{-1}. The PT time at the highest concentration (1000 µg.mL^{-1}) for boiled water extract was 27.00 min and cold water extract was 24.68 min for the complete clot. One-way ANOVA revealed

Table 2. Correlations among different assays of the test extracts

Assays	FRAP		Phenol		Flavonoid		PT		Protease inhibition	
	R^2	Sig.	R^2	Sig.	R^2	Sig.	R^2	Sig.	R^2	Sig.
DPPH	0.93	< 0.001	0.99	<0.001	0.90	0.002	0.97	< 0.001	0.96	< 0.001
FRAP			0.93	0.001	0.94	< 0.001	0.98	< 0.001	0.97	< 0.001
Phenol					0.90	0.002	0.97	< 0.001	0.97	< 0.001
Flavonoid							0.96	< 0.001	0.98	< 0.001
PT									0.99	< 0.001

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that all values were statistically significant (P<0.001) compared to the control group.

Correlations among antioxidant activities, total phenolic content/total flavonoid content, and anticoagulation activities

Linear correlation was observed among antioxidant, total phenolic/flavonoid content, and anti-coagulation activity (Table 2). DPPH free radical scavenging assay and FRAP assay indicate antioxidant activity. PT and protease inhibition assays determine in vitro anti-coagulation activity. The statistical analysis was performed using GraphPad Prism 8 software. The R2 values ranged from 0.90 to 0.99. In vitro antioxidant assay showed a strong correlation with total phenolic content. Furthermore, in vitro antioxidant activity demonstrated a strong correlation with in vitro anticoagulation activity.

Discussion

In a biological situation, the excessive ROS level known as oxidative stress causes the imbalance that eventually leads to cell and tissue damage [24]. Antioxidants, as a form of plant secondary metabolites, play a vital role in neutralizing oxidative stress either by donating an electron or by inhibiting oxidation enzymes. Serine proteases (or serine endopeptidases) cleave peptide bonds in proteins, in which serine participates as the nucleophilic amino acid at the enzyme's active site, and ultimately acts in blood coagulation. Natural anticoagulants (serine protease inhibitors) can be a great alternative to commercial medicine, without any adverse effects considering the natural product as relatively safe and cost-effective.

In this study, we examined the antioxidant and anticoagulation potential of boiled water (regular cooking conditions) as well as cold water extracts of *P. chaba* stem by several antioxidant and anticoagulation assays. Strong antioxidant capacity was observed compared to the reference standard. Researchers also found potent antioxidant activity in several studies while using non-polar extract of *P. chaba* [14, 25]. The in vitro anticoagulation assays (PT and serine protease) revealed the potential of both extracts, which is comparable to the reference standard. A linear correlation was found between antioxidant and anticoagulation activity of both samples. Potent phenolic and flavonoid content also showed a linear relationship with antioxidant and anticoagulation capacity. Polyphenols show both antioxidative and anticoagulant properties simultaneously [26, 27]. Hence, the antioxidant and anticoagulation activity of the polyphenol molecules present in the plant extracts can be substantiated by the linearity in correlation. Flavonoids are a class of polyphenolic secondary metabolites found in plants. Most phenolic compounds of the extract were flavonoids. For all tests, boiled water extract showed better capacity than cold water extract. This is because the aqueous solubilities of organic solids vary exponentially with temperature [28]. The increase in kinetic energy that comes with higher temperatures allows the solvent molecules to more effectively break apart the solute molecules that are held together by intermolecular attractions.

Conclusion

This study validated the presence of potent antioxidant and anticoagulation metabolites in *P. chaba* Hunter. We demonstrated strong antioxidant and anticoagulation capacity as well as potent phenolic and flavonoid content, which were also statistically significant compared to the reference group. Further analyses would reveal the bioactive metabolite profile of *P. chaba*. The bioactivity of *P. chaba* might be used in pharmaceutical and cosmeceutical implications alone or in combination with food.

Ethical Considerations

Compliance with ethical guidelines

All the procedures and rules of Bangladesh Medical Research Council were strictly obeyed during human blood drawing (for prothrombin timing assay).

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Authors' contributions

Each author contributed equally during this lab work and corresponding study.

Conflict of interest

The authors declared no conflicts of interest.

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