Clinical Phytoscience

ORIGINAL CONTRIBUTION

Open Access

Antioxidant, cytotoxic, antibacterial and thrombolytic activities of *Centella asiatica* L.: possible role of phenolics and flavonoids

Md. Harun-Or- Rashid[1*], Mst. Mahmuda Akter[1], Jalal Uddin[1], Saziya Islam[1], Mousumy Rahman[1], Khurshid Jahan[1], Md. Moklesur Rahman Sarker[2] and Golam Sadik[3]

Abstract

Background *Centella asiatica* L. (Apiaceae), a green leafy vegetable, has been used as a popular folk medicine in Bangladesh in the treatment of various ailments. The plant is reported to contain phenolics and flavonoids, but their bioactive potentials are not yet completely known. The present study was designed to investigate the role of the phenolic and flavonoids in the antioxidant, cytotoxicity, antibacterial and thrombolytic activities.

Methods Four solvent fractions viz. methanol (MSF), ethyl acetate (ESF), chloroform (CSF) and petroleum ether (PSF) were prepared from the dried powder of the whole plant by the modified Kupchan method. Total phenolic content and flavonoid content were determined by Folin Ciocalteu method and aluminum chloride colorimetric method, respectively. The antioxidant activity was assessed by the DPPH radical scavenging and total antioxidant capacity assays. The antibacterial activity was determined by the disc diffusion method and cytotoxicity was evaluated by the brine shrimp lethality bioassay. Thrombolytic activity was assayed using streptokinase as standard.

Results Qualitative analysis of phytochemical revealed the presence of phenolics and flavonoids along with other bioactive constituents. Among the extractives, CSF contained the highest content of phenolics (155.46 ± 0.52 mg GAE/g) and flavonoids (345.17 ± 1.12 mg QE/g) and exhibited the most potent antioxidant activity in terms of total antioxidant capacity (179.01 ± 0.89 mg AAE/g) and DPPH scavenging ability (IC_{50}; 15.31 ± 0.32 µg/mL). Similarly, CSF showed the highest cytotoxicity with LC_{50} values of 13.80 ± 0.23 µg/mL, and thrombolytic activity with 43.94 ± 0.62% clot lysis. The fraction also exhibited broad spectrum antibacterial activity. A significant correlation was observed between the flavonoid content and total antioxidant activity ($r^2 = 0.894$, $p < 0.05$), while high correlation was seen between phenolic and flavonoid content and DPPH radical scavenging, total antioxidant capacity and cytotoxicity ($r^2 = 0.612–0.928$). Similarly, a positive correlation was found between phenolic and flavonoid content with thrombolytic and antibacterial activities.

Conclusion These results revealed that *C. asiatica* is a rich source of phenolics and flavonoids and correlated with antioxidant, cytotoxicity, antibacterial and thrombolytic activities. Hence isolation of phenolics and flavonoids from this plant may offer potential candidates which may be effective in the prevention of many chronic diseases.

Keywords *C. asiatica*, Apiaceae, Antioxidant, Antibacterial, Thrombolytic and Cytotoxic effects

*Correspondence:
Md. Harun-Or- Rashid
drmhrashid9092@gmail.com
[1] Department of Pharmacy, World University of Bangladesh, Avenue 6, Lake Drive Road, 17/H, Uttara, Dhaka 1230, Bangladesh
[2] Department of Pharmacy, State University of Bangladesh, 77 Satmasjid Road, Dhanmondi, Dhaka 1205, Bangladesh
[3] Department of Pharmacy, University of Rajshahi, Rajshahi 6205, Bangladesh

Introduction

Fruits and vegetables rich in nutrition and nutraceuticals have received a great deal of attention due to their potential health benefits. Epidemiologic studies suggest that consumption of fruits and vegetables reduce the risk of cancer, cardiovascular disorder and many other chronic diseases [1–3]. They contain different classes of phytochemicals such as phenolics and flavonoids, alkaloids and saponins, steroids and glycosides which are attributable to the bioactivities. Among the phytoconstituents, phenolics and flavonoids have attracted much interest as they contain hydroxyl groups and able to neutralize the free radicals, quenching singlet and triplet oxygen, or decomposing peroxides by donating an electron or a hydrogen atom. They are commonly known as natural antioxidant and found effective in the prevention and repair of oxidative damage [4–6]. It is well known that free radicals and reactive oxygen species (ROS) liberated from the cell are capable of destroying cellular constituents and lead to the development of numerous chronic diseases, such as cancer, Alzheimer's disease, Parkinson's disease, diabetes and coronary heart diseases [7]. A large number of studies have shown that malignant cells have greater levels of ROS which can promote carcinogenesis by causing oxidative damage to DNA and macromolecules within cells, altering signal transduction pathways, and promoting a malignant phenotype [8]. Dietary antioxidants such as flavonoids have been shown to prevent or slow down malignancy by reducing oxidative damage and promoting DNA repair [9].

Thrombosis is the pathophysiological process of the formation of a blood clot within the blood vessel obstructing the flow of blood through the circulatory system. The consequence of thrombosis is embolism, ischemia, heart attack, stroke and so forth [10]. It is one of major cause of mortality and morbidity. A handful of drugs including tissue plasminogen activator, urokinase, streptokinase is currently in use [11], thus demands for development of new or alternative drugs for its treatment. Plants with high content of polyphenols have been reported to exhibit good thrombolytic activity [12, 13].

The burden of bacterial infection is high in the globe and the condition has been exacerbated due to increasing resistance to antimicrobial agents [14]. Indiscriminate use of antimicrobial agents and mutational capacity of bacteria results in the development of antimicrobial resistance strain. Edible plants rich in antioxidants have shown therapeutic potential in the treatment of bacterial infection [15]. Hence search of plant for high content of phenolic and flavonoid with potential bioactivity is important to develop alternative medicine or functional food for prevention and management of the diseases.

Centella asiatica L., locally known as Thankuni is a member of the Family Apiaceae and found in marshy areas all over Bangladesh. The plant is widely considered as a health food due to high content of nutrients and health promoting properties [16]. Its leaves are eaten as raw or mixed with other green vegetable. In traditional medicine, the leaves are commonly used in the treatment of dysentery, cholera, tuberculosis, common cold, urinary tract infection, leprosy, psoriasis and eczema, bronchitis and asthma, wound healing and gastrointestinal problem [17, 18]. The plant has been described in Ayurvedic medicine to improve cognitive function and to treat mental and neurological disturbances [19]. Biological investigations of this plant have shown a multitude of bioactivities including antioxidant, antimicrobial, neuroprotective, thrombolytic, and cytotoxic activities [20–25]. Phytochemical analysis revealed the presence of triterpenoids, steroids, alkaloids, saponins, phenolics and flavonoids [26, 27]. Much work has been done on the neuroprotective properties and found the triterpenes as the major active constituents for cognitive activity [28, 29]. Although the plant has been reported to contain the phenolics and flavonoids [30, 31], their biological activities are not yet clearly known.

In the present work, we have prepared four solvent fractions of different polarity from the dried powder of *Centella asiatica*, determined their phenolics and flavonoids content, and evaluated the antioxidant, cytotoxicity, antibacterial, and thrombolytic activities. Finally, Pearson's correlation studies were performed to analyze the relationship between phenolics and flavonoids with the bioactivities.

Materials and methods

Collection of plant materials

The whole plant thankuni (*Centella asiatica*, Family: Apiaceae) was collected from rural areas of Narayanganja district, Bangladesh when it became mature during April–May, 2018. The plant was taxonomically identified by the expert of Bangladesh National Herbarium (BNH), Dhaka, Bangladesh where a voucher specimen has been deposited (DACB 41,559) for future reference. The plant materials were cleaned, cut into pieces, sun-dried and finally dried in an oven at 45 °C and crushed.

Preparation of plant extract

The 250 g powdered materials were soaked in 1.5 L of methanol in an amber-colored glass bottle at room temperature for 7 days with occasional shaking. The extracts were filtered to a clear solution and concentrated with a rotary evaporator under reduced pressure at 50 °C and evaporated to dryness (MSF: 18.0 gm, 7.2%w/w). An aliquot (10.0 g) of the methanol extract (MSF) was

partitioned with petroleum ether, chloroform, and ethyl acetate according to the modified Kupchan protocol [32]. Yield was calculated based on the starting material. The resultant fractions were evaporated to dryness to yield pet-ether (PSF, 2.13 g, 1.534%), chloroform (CSF, 2.2 g, 1.584%), and ethyl acetate (ESF, 3.12 g, 2.26%) soluble fractions. All these fractions were then stored in a refrigerator until further use.

Organisms and chemicals

Cultures of bacteria and shrimp eggs were collected from the Institute of Nutrition and Food, University of Dhaka, and International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Kanamycin disc (K-30) from BBL and Cookeville (TN, USA); nutrient agar media was purchased from Becton, Dickinson and Company (Sparks, MD, USA). Dimethyl sulphoxide (DMSO), DPPH, gallic acid, quercetin and Folin–Ciocalteau (FC) reagent were from BDH chemicals Ltd., Poole, England. The standard drugs streptokinase, ascorbic acid, and vincristine sulfate (VC) used in this study were obtained as kind gifts from Beacon Pharmaceuticals Ltd., Bangladesh. All other chemicals and organic solvents were of the highest grade.

Phytochemical analysis

Qualitative tests were performed on the crude methanol extract (MSF) to detect the presence of different phytochemicals including phenolics, flavonoids, alkaloids, saponins, and phytosterols by using the standard phytochemical methods as described earlier [33].

Determination of total phenolic content

The total phenolic contents of the fractions of *C. asiatica* were assayed by Folin-Ciocalteu method as described earlier [34]. To a mixture of 2.5 ml of 10% (v/v) Folin-Ciocalteu reagent and 2.5 mL of 7.5% (w/v) sodium carbonate solution, 0.5 ml sample was added and left in the dark for 20 min at 25 °C. The absorbance of the reaction mixture was recorded by a spectrophotometer (Shimadzu, Japan) at 760 nm. A standard curve was obtained for gallic acid and the phenolic content was determined from extrapolation of this curve.

Determination of total flavonoid content

The total flavonoid contents of the fractions of *C. asiatica* were estimated by aluminum chloride colorimetric method as described [35]. To a mixture of methanol (3.0 ml), 10% w/v $AlCl_3$ (0.2 mL), 1 M potassium acetate (0.2 ml) and 5.6 mL of distilled water, individual fraction (1.0 mL) was added and left at room temperature for 30 min. The absorbance of the reaction mixture was recorded by a spectrophotometer at 420 nm. A standard curve was obtained for quercetin and the flavonoid content was determined from extrapolation of this curve.

Antioxidant activity

Total antioxidant capacity

The antioxidant capacity of the fractions of *C. asiatica* was assessed by the method as described earlier [36]. To a mixture of sulphuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM), the individual fraction was added and heated in a water bath at 95 °C for 90 min. After cooling to room temperature, the absorbance of the mixture was recorded at 695 nm against blank. A standard calibration curve was prepared using ascorbic acid and the total antioxidant capacity was determined from extrapolation of this curve.

DPPH free radical scavenging assay

The ability of the fractions of *C. asiatica* to scavenge DPPH radical was determined by the method as described by Brand-Williams et al., [37]. Ascorbic acid was used as positive control. Methanolic solution of fraction or standard compound was mixed with 0.135 mM of methanolic DPPH and left in dark for 30 min. The absorbance of the reaction mixture was recorded at 517 nm. The percent scavenging was calculated using the equation:

$$\left[\left(A_{control} - A_{sample}\right)/A_{control}\right] \times 100$$

where, $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of extract or standard compound. IC_{50} values were determined from the straight-line equation obtained from the plot of % inhibition against log of sample concentration.

Cytotoxicity

The cytotoxic activity of the fractions of *C. asiatica* was determined by the Brine shrimp (*Artemia salina* Lech) lethality bioassay [38]. Briefly, shrimp eggs were allowed for 36 h in simulated seawater to hatch and mature as nauplii (Larvae). Five milligrams of each fraction was dissolved in 1.0 ml DMSO. Test solutions 5, 10, 20, 40, and 80 µL were taken in separate vials and 5 mL of the seawater was added to each vial containing 20 nauplii. A control group was used containing 80 μL of DMSO and 20 nauplii in 5 mL of seawater. After 24 h, the number of survivors in each vial was counted. The LC_{50} (median lethal concentration) values were determined from the straight-line equation obtained from the plot of % mortality against log of sample concentration.

Antibacterial activity

In-vitro antibacterial activity of the fractions of *C. asiatica* was performed against five gram-positive (*Bacillus subtilis, Sarcina lutea, Staphylococcus aureus, Sarcina sarcinaceae*, and *Bacillus cereus*) and six gram-negative (*E. coli, Shigella dysenteriae, Shigella shiga, Pseudomonas aeruginosa, Vibrio mimicus,* and *Salmonella typhi*) bacteria by disc diffusion method [39]. The fractions MSF, PSF, CSF, and ESF were dissolved in a sufficient amount of methanol individually, so that 10 µL solution contains 100 µg of crude extract. The inhibitory activity was performed with 400 µg of extractives per disc. A disc with 40 µL methanol was used as the negative control. The test bacteria were inoculated in sterilized nutrient agar media, mixed thoroughly and transferred to an aseptic condition immediately. The disc containing test fraction and standard drug were placed in the petri dish and were incubated at 37 °C for 24 h. Clear zones of inhibition were measured in millimeter. The antibacterial activity of the fractions was compared with the standard kanamycin disc (K-30).

Thrombolytic activity

The thrombolytic activity of the fractions of *C. asiatica* was evaluated by the method as described earlier [40]. To prepare the sample, each fraction (100 mg) was suspended in 10 ml of sterile isotonic solution and shaken vigorously. The suspension was kept overnight and the supernatant was collected by filtration through a filter paper for in vitro evaluation of clot lysis activity. Streptokinase (SK) was used as positive control and isotonic solutions as negative control. Venous blood from volunteer was distributed in pre-weighted sterile microcentrifuge tubes and then incubated at 37 °C for 45 min. Serum was removed completely after clot formation and the weight of clot was measured. To each tube containing clot, sample or standard drug or isotonic solution were added separately. After incubation at 37 °C for 90 min, the released fluid was removed and tubes were again weighed. The differences in weights were used to calculate the percent of clot lysis according to the following equation:

$$\text{Percent of clot lysis} = \left(\text{weight of released clot} \,/\, \text{clot weight}\right) \times 100\%$$

Statistical analysis

All the assays were done in three replicates of each sample and values were taken as mean ± SD by statistical analysis. Microsoft excel 2010 were used to calculate the experimental results. Significant differences (p-value < 0.05) between the means were determined using the t-test. Correlation studies were performed using Pearson's correlation test.

Results

Phytochemical analyses

Qualitative phytochemical analysis

Qualitative analyses of the crude methanol extract (MSF) of *C. asiatica* indicated the presence of phenolics, flavonoids, alkaloids, tannins, steroids, and glycosides (Supplementary Table S1).

Total phenolic content (TPC)

The total phenolic content of the four solvent fractions of *C. asiatica* was estimated by Folin-Ciocalteu method and the result is shown in the Table 1. The phenolic content of PSF, CSF, ESF and MSF were found to be 34.76 ± 0.21, 155.46 ± 0.52, 116.59 ± 0.57, and 88.62 ± 0.41 mg of GAE/g of sample, respectively. The results revealed the highest phenolic content in CSF followed by ESF, MSF and PSF.

Total flavonoid contents (TFC)

The flavonoid content of the different fractions of *C. asiatica* was determined by aluminium chloride colorimetric method and the result is presented in the Table 1. The total flavonoid content of MSF, PSF, CSF and ESF were 211.34 ± 0.98, 90.52 ± 0.52, 345.17 ± 1.12, and 193.68 ± 0.99 mg of QE/g of extract, respectively, suggesting that CSF exhibited the highest content of flavonoid followed by MSF, ESF, and PSF which is similar to the phenolic content.

Antioxidant activity

Total antioxidant capacity (TAC)

The total antioxidant capacity of the different fractions of *C. asiatica* was determined based on their ability to reduce Mo (VI) to Mo (V) and the result is given in the Table 2. The total antioxidant capacities of MSF, PSF, CSF, and ESF of *C. asiatica* were 102.32 ± 0.58, 68.97 ± 0.48, 179.01 ± 0.89, and 80.11 ± 0.53 mg of AAE/g of extract, respectively. Among the four fractions, CSF showed the highest capacity and PSF, the lowest (Fig. 1A). Using

Table 1 Total phenolic and flavonoid content of the different fractions of *C. asiatica*

Samples	TPC (mg GAE/g of extract)	TFC (mg QE/g of extract)
MSF	88.62 ± 0.41	211.34 ± 0.98
PSF	34.76 ± 0.21	90.52 ± 0.52
CSF	155.46 ± 0.52	345.17 ± 1.12
ESF	116.59 ± 0.57	193.68 ± 0.99

TPC Total phenolic content, *TFC* Total flavonoid content, *MSF* Crude methanol extract, *PSF* Petroleum ether soluble fraction, *CSF* Chloroform soluble fraction, *ESF* Ethyl acetate soluble fraction

Table 2 Total antioxidant capacity and DPPH scavenging activity of the different fractions of *C. asiatica*

Fraction/ Standard	TAC (mg AAE/g of fraction)	IC_{50} value (µg/mL)
MSF	102.32 ± 0.58	24.19 ± 0.52
PSF	68.97 ± 0.48	373.16 ± 0.81
CSF	179.01 ± 0.89	15.31 ± 0.32
ESF	80.11 ± 0.53	28.83 ± 0.61
AA	-	9.94 ± 0.42

MSF Crude methanol extract, *PSF* Petroleum ether soluble fraction, *CSF* Chloroform soluble fraction, *ESF* Ethyl acetate soluble fraction, *AA* Ascorbic acid, *TAC* Total antioxidant capacity

Pearson's correlation analysis, a significant correlation was observed between TFC and total antioxidant capacity ($r^2 = 0.894$, $p < 0.05$), whereas a high correlation was seen between TPC and total antioxidant capacity ($r^2 = 0.649$) (Table 3).

DPPH radical scavenging assay

The free radical scavenging potential of the fractions of *C. asiatica* was evaluated by spectrophotometric method using the synthetic DPPH free radicals. The percent scavenging of DPPH radical at various concentrations of the fraction was calculated and the results have been shown in the Fig. 1B. Our results demonstrated the ability of the fractions to scavenge the free radical. For comparison of potency, the IC_{50} values of each of the fractions were calculated and shown in the Table 2. Among the fractions, CSF showed highest activity with the IC_{50} value of 15.31 ± 0.32 µg/mL followed by MSF, ESF and PSF. Ascorbic acid was used as standard whose IC_{50} was found to be 9.94 ± 0.42 µg/ml. From correlation analysis, we found high correlation of TPC and TFC with DPPH scavenging activity ($r^2 = 0.728$ & 0.612) (Table 3).

Table 3 Correlation of total phenolic and flavonoid contents with antioxidant activity and cytotoxicity

Activities	Correlation value (r^2)	
	Total phenolic content	Total flavonoid content
Total antioxidant capacity	0.649	0.894
DPPH scavenging	0.728	0.612
Cytotoxicity	0.928	0.473

Cytotoxicity

The *in-vivo* cytotoxicity of the fractions of *C. asiatica* was determined against brine shrimp by brine shrimp lethality bioassay. The cytotoxicity of the fractions was expressed in terms of LC_{50} values which were estimated from the straight line obtained from the plot of % mortality versus the log of sample concentrations (Fig. 2A & 2B). The LC_{50} values of MSF, CSF, and ESF were found to be 25.47 ± 0.45 µg/mL, 13.80 ± 0.23 µg/mL and 17.82 ± 0.34 µg/mL, respectively. These results indicated the highest cytotoxicity in the CSF followed by ESF and MSF. Under the same experimental condition, the standard cytotoxic agent vincristine showed an LC_{50} of 1.85 ± 0.23 µg/mL. Correlation studies showed a significant correlation between total phenolic content and cytotoxicity ($r^2 = 0.928$).

Antibacterial screening

The antibacterial activity of the fractions of *C. asiatica* was screened against eleven Gram-positive and Gram-negative bacteria by disc diffusion method. The results of antibacterial activity have been shown in the Table 4. All the fractions exhibited inhibition against test bacteria having the zone of inhibition ranging from 5 to

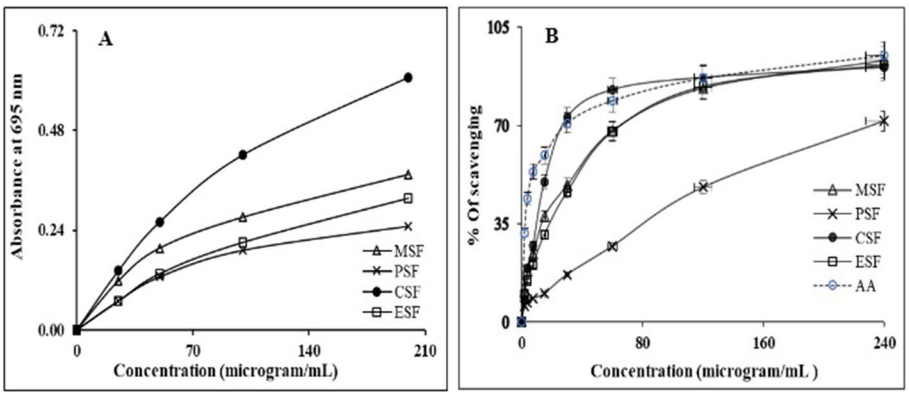

Fig. 1 Evaluation of *in-vitro* antioxidant activities of the different fractions of *C. asiatica*. **A** Total antioxidant capacity; **B** DPPH free radical scavenging activity. Ascorbic acid (AA) was used as the standard antioxidant. Data was expressed as mean ± SD ($n = 3$). PSF, Petroleum ether soluble fraction; CSF, Chloroform soluble fraction; ESF, Ethyl acetate soluble fraction; MSF, Crude methanol extract

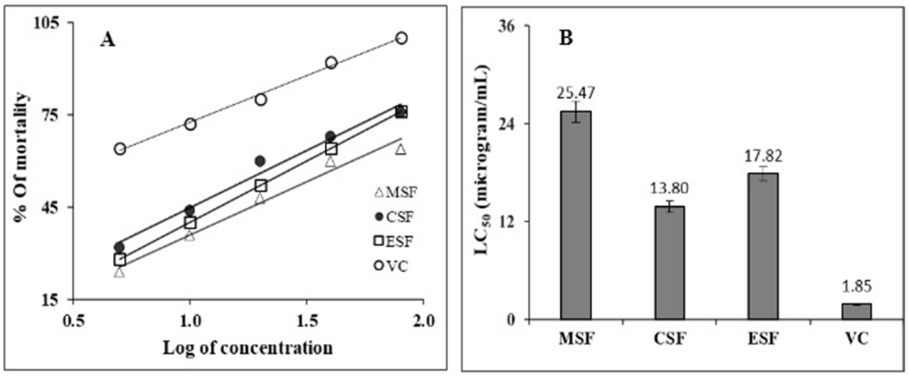

Fig. 2 Cytotoxicity of the different fractions of *C. asiatica* against brine shrimp. **A** Graph plotted for log of concentration versus % of mortality of nauplii; **B** LC_{50} values of the different fractions. Vincristine (VC) was used as the standard drug for comparison. Data expressed as mean ± SD ($n = 3$). PSF, Petroleum ether soluble fraction; CSF, Chloroform soluble fraction; ESF, Ethyl acetate soluble fraction; MSF, Crude methanol extract

Table 4 Antibacterial activity of the different fractions of *C. asiatica*

Test organism	Diameter of zone of inhibition (mm)				
	MSF	PSF	CSF	ESF	K-30
Gram positive bacteria					
Bacillus subtilis	6	7	5	-	37
Sarcina lutea	19	17	20	18	35
Staphylococcus aureus	18	19	20	16	37
Bacillus cereus	9	11	12	11	38
Sarcina sarcinaceae	14	-	23	20	32
Gram negative bacteria					
Shigella dysenteriae	16	18	30	16	37
Vibrio mimicus	12	15	13	18	35
E. coli	18	12	22	24	31
Shigella shiga	18	12	21	22	30
Pseudomonas aeruginosa	19	-	20	23	27
Salmonella typhi	21	-	22	24	28

MSF Crude methanol extract, *PSF* Petroleum ether soluble fraction, *CSF* Chloroform soluble fraction, *ESF* Ethyl acetate soluble fraction. (-), sign indicates no activity. K-30, Kanamycin 30 µg /disc. All measurements were done in triplicate and average value was taken

30 mm. Among the fractions, CSF was found to be the most active against both the Gram-positive and Gram-positive bacteria. It showed the highest zone of inhibition (30 mm) against *S. dysentriae*. While ESF was comparatively less active against the Gram-positive bacteria than CSF but showed more active against few Gram-negative bacteria including *E. coli*, *S. shiga*, *P. aeruginosa* and *S. typhii*. MSF showed activity against all the test bacteria. Whereas PSF did not show activity against the organisms. The standard antibiotic kanamycin exhibited the highest inhibition against all test bacteria.

Table 5 Thrombolytic activity of the different fractions of *C. asiatica*

Samples	% Of clot lysis
MSF	17.58 ± 0.23
PSF	35.14 ± 0.42
CSF	43.94 ± 0.62
ESF	27.60 ± 0.52
Blank	8.16 ± 0.23
SK	63.34 ± 0.58

MSF Crude methanol extract, *PSF* Petroleum ether soluble fraction, *CSF* Chloroform soluble fraction, *ESF* Ethyl acetate soluble fraction, *SK* Streptokinase

Data expressed as mean ± SD ($n = 3$)

Thrombolytic effect

The different fractions of *C. asiatica* were assayed for thrombolytic activities by determining the ability to clot lysis. Streptokinase (SK) was used as a positive control that showed 63.34 ± 0.58% lysis as compared to the isotonic solution that had 7.0 ± 0.23% lysis activities. In this study, the different fractions of *C. asiatica* exhibited varying degrees of thrombolytic activities ranging from 17.58 ± 0.23% to 43.94 ± 0.62% (Table 5). Highest clot lysis activity (43.94 ± 0.62%) was found in CSF followed by PSF, ESF and MSF.

Discussion

Natural foods or food derived nutraceuticals are considered to be the promising approach at present for the prevention and treatment of various chronic diseases. Interest in foods has increased so much in recent times because they are safe and exhibit multiple biological and therapeutic activities due to the presence of high content of diverse secondary metabolites [41, 42]. Many food

plants have been used in traditional medicine to treat different ailments. Scientific investigation of these plants may aid in the development of alternative medicine or functional foods. *C. asiatica* is a popular vegetable and folk medicinal plant used in Bangladesh for the treatment of a variety of aliments [17–19]. Phenolics and flavonoids are important classes of phytochemicals that exhibit antioxidant and other biological activities [4–6]. Although the occurrence of these phytochemicals is evident in this plant [30, 31], little is known about their roles in the bioactivities. Herein we report for the first time that *C. asiatica* is a rich source of phenolics and flavonoids and correlated with antioxidant, cytotoxicity, thrombolytic and antibacterial activities.

The methanol extract (MSF) of *C. asiatica* were fractionated into three different fractions PSF, CSF and ESF for phytochemical and biological investigation. Preliminary analysis of MSF revealed the presence of steroids, glycosides, tannins, alkaloids, phenolics and flavonoids as the potential bioactive constituents (Supplementary Table S-1). Quantitative analysis of phenolic and flavonoid content revealed that the CSF contained the highest content of phenolics (155.46 ± 0.52 mg GAE/g dried extract), flavonoids (345.17 ± 1.12 mg QE/g dried extract) followed by ESF (116.59 ± 0.57 mg GAE/g dried extract and 193.68 ± 0.99 mg QE/g dried extract), MSF (88.62 ± 0.41 mg GAE/g dried extract and 211.34 ± 0.98 mg QE/g dried extract), and PSF (34.76 ± 0.21 mg GAE/g dried extract and 90.52 ± 0.52 mg QE/g dried extract) (Table 1). The phenolic and flavonoid content in the methanol extract were reported earlier by Zainol et al. [20] and Mustafa et al. [21] which is consistent with our result. Numerous phenolic compounds such as ferulic acid, chlorogenic acid, isochlorogenic acid, 3,5-di-*O*-caffeoyl quinic acid, 1,5-di-*O*-caffeoyl quinic acid, and 3,4-di-*O*-caffeoyl quinic acid have been isolated from the leaves of *C. asiatica* [30]. Similarly, two new flavonoids castilliferol and castillicetin has been identified from the plant in addition to the common flavonoids quercetin, catechin, kaempferol, kaempferol-3-*O*-*b*-D-glucoside, and quercetin-3-*O*-*b*-D-glucoside [31]. Our results revealed that *C. asiatica* is a rich source of ploy phenolics. The presence of large amount of polyphenolics in the CSF suggests that it might play a role in biological activity.

Natural antioxidants are capable of ameliorating the oxidative damage induced by free radicals and thus considered to be the potential candidates for treating many chronic disorders [4–6]. In this study, we have assessed the antioxidant activity of the fractions using the two widely accepted antioxidant assays such as DPPH radical scavenging and total antioxidant activity assays. In both assays, CSF showed the highest activity followed by MSF and ESF (Fig. 1). The IC_{50} values of the fractions for DPPH radical scavenging was 15.31 ± 0.32, 24.19 ± 0.52 and 28.83 ± 0.61 µg/mL, respectively, while the same were 179.01 ± 0.89, 102.32 ± 0.58, and 80.11 ± 0.53 mg of AAE/g of fraction for total antioxidant activity. The antioxidant activity of the methanol extract of *C. asiatica* has been reported earlier by Zainol et al., [20] and Mustafa et al., [21] which is consistent with our result. In many plants, the antioxidant activity has been found to be correlated with the phenolics and flavonoids content [6–8]. We also found a strong correlation of TPC and TFC with DPPH scavenging and total antioxidant capacity (Table 3). Our results suggested that CSF possesses strong antioxidant activity and that might be able to prevent or reduce the oxidative damage in oxidative stress induced diseases.

Brine shrimp lethality bioassay is a simple method used widely for evaluating the possible cytotoxicity of the plant extract/compound. This bioassay has shown a good correlation with the human tumor solid cell lines [43]. Antioxidants from dietary sources was shown to slow down the pathogenesis of cancer [9]. In this study we found the highest cytotoxicity in the CSF with LC_{50} of 13.80 µg/mL followed by ESF and MSF (Fig. 2). Earlier Soyingbe et al., [44] reported that methanol and ethyl acetate extract of *C. asiatica* have significant anti-proliferative effect in several human cancer cell lines including human breast adenocarcinoma (MCF-7) cells, human colorectal carcinoma cells (Caco), human cervical cancer cells (HeLa) [36]. From Pearson,s correlation studies, we found a high correlation between phenolic content and cytotoxicity ($r^2 = 0.928$) and a moderate correlation between flavonoid content with cytotoxicity ($r^2 = 0.473$) (Table 3). A similar relationship between flavonoid content and anti-tumor activity has been shown earlier by Pittella et al., [22] in human and mouse cancer cell lines. Our results suggest that CSF has high cytotoxicity which require investigation against these cell lines.

Plant has enormous potential to cure bacterial infection due to presence of antibacterial constituents. Antioxidants from plants have been reported to exhibit potential antibacterial activity [45]. In this study we found the antibacterial activity in all the test fractions (Table 4). A comparison of activity between the fractions revealed that CSF is the most active against both the Gram-positive and Gram-negative bacteria followed by ESF, MSF and PSF. It is worth noting that CSF displayed the highest zone of inhibition (30 mm) against *S. dysentriae* which support its traditional use in dysentery. ESF and MSF also showed good activity against the test organisms. PSF was the least active. In a previous study, Panda et al., [15] reported the antibacterial activity in the methanol and aqueous extract of *C. asiatica*, which is in good

agreement with our result. Our results revealed that CSF exhibited the highest antibacterial activity among the test fractions and the activity showed a positive correlation with phenolics and flavonoids.

Thrombus formation is the key to the development of many vascular complexities [10]. Blood clotting is a complex process that involves a series of events. Majority of the thrombolytic agents activate plasminogen that breaks down the cross-linked fibrin mesh work which is further proteolysed by other enzymes [11]. In this study, we found the highest thrombolytic activity in the CSF followed by PSF and ESF. The thrombolytic activity of the methanol extract and ethyl acetate soluble phase was shown first by Satake et al., [30] which is in accordance with our result. Similar to antibacterial activity, a positive correlation was apparent between phenolics and flavonoids and thrombolytic activity. Our data showed the highest thrombolytic activity of CSF and the activity is associated with the polyphenolic content.

Conclusion

In conclusion, this study showed that *C. asiatica* is a rich source of phenolics and flavonoids and correlated with antioxidant, cytotoxicity, antibacterial and thrombolytic activities. These findings suggest a role of phenolics and flavonoids in the bioactivities. Further exploration of this plant may lead to the development of bioactive phenolic and flavonoid compounds which may be effective in the management of many chronic diseases.

Abbreviations

MSF	Methanol soluble fraction
PSF	Pet-ether soluble fraction
CSF	Chloroform soluble fraction
ESF	Ethyl acetate soluble fraction
GAE	Gallic acid equivalent
QE	Quercetin equivalent
AAE	Ascorbic acid equivalent
DPPH	2,2-Diphenyl-1-picryldydrazyl
SK	Streptokinase
IC_{50}	Median (50%) inhibitory concentration
LC_{50}	Median (50%) lethal concentration
TPC	Total phenolic content
TFC	Total flavonoid content
TAC	Total antioxidant capacity

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40816-023-00353-8.

Additional file 1.

Acknowledgements

The authors would like to thank the authority of World University of Bangladesh for their financial support to conduct the research in the Department of Pharmacy. The authors are also thankful to the National Herbarium of Bangladesh, Mirpur, Dhaka for identification of the plant. The authors greatly acknowledge the kind donation of standard drug samples used in the study from Beacon Pharmaceuticals Ltd.

Authors' contributions

MMA, SI, and MR were involved in collection of plant parts and laboratory experiments. JU and KJ were involved in work supervision, data acquisition and interpretation of results. MMRS and GS did critical statistical analysis, experiments and report revision. Concept development, experiment design, overall monitoring, report writing and final approval of the manuscript was done by MHR. The author(s) read and approved the final manuscript.

Funding

The authors did not receive any fund from any organization or any other govt., or non-government source to conduct the study.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Declarations

Ethics approval and consent to participate

This study was an ***in-vitro*** study, so ethical approval was not required.

Consent for publication

All the authors of this manuscript have consented to publish the article.

Competing interests

The authors declare that they don't have any conflict of interest to publish this article.

Received: 8 January 2021 Accepted: 9 January 2023
Published online: 17 January 2023

References

1. Boeing H, Bechthold A, Bub A, Ellinger S, Haller D, Kroke A, Leschik-Bonnet E, Muller MJ, Oberritter H, Schulze M, Stehle P, Watzl B. Critical review: vegetables and fruit in the prevention of chronic diseases. Eur J Nutr. 2012;51:637–63. https://doi.org/10.1007/s00394-012-0380-y.
2. Veiga M, Costa EM, Silva S, Pintado M. Impact of plant extracts upon human health: a review. Crit Rev Food Sci Nutr. 2020;60(5):873–86. https://doi.org/10.1080/10408398.2018.1540969.
3. Aune D, Giovannucci E, Boffetta P, Fadnes LT, Keum N, Norat T, Greenwood DC, Riboli E, Vatten LJ, Tonstad S. Fruit and vegetable intake and the risk of cardiovascular disease, total cancer and all-cause mortality-a systematic review and dose-response meta-analysis of prospective studies. Int J Epidemiol. 2017;46(3):1029–56. https://doi.org/10.1093/ije/dyw319.
4. Zhang YJ, Gan RY, Li S, Zhou Y, Li AN, Xu DP, Li HB. Antioxidant phytochemicals for the prevention and treatment of chronic disease. Molecules. 2015;20(12):21138–56. https://doi.org/10.3390/molecules201219753.
5. Costa C, Tsatsakis A, Mamoulakis C, Teodoro M, Briguglio G, Caruso E, Tsoukalas D, Margina D, Dardiotis E, Kouretas D, Fenga C. Current evidence on the effect of dietary polyphenols intake on chronic diseases. Food Chem Toxicol. 2017;110:286–99. https://doi.org/10.1016/j.fct.2017.10.023.
6. Ramana KV, Reddy ABN, Majeti NVRK, Singhal SS. Therapeutic potential of natural antioxidants. Oxid Med Cell Longev. 2018:9471051. https://doi.org/10.1155/2018/9471051
7. Pizzino G, Irrera N, Cucinotta M, Pallio G, Mannino F, Arcoraci V, Squadrito F, Altavilla D, Bitto A. Oxidative Stress: harms and benefits for human health. Oxid Med Cell Long. 2017;8416763:13. https://doi.org/10.1155/2017/8416763.
8. Lopez-Lazaro M. Excessive superoxide anion generation plays a key role in carcinogenesis. Int J Cancer. 2007;120:1378–80.

9. Vance TM, Azabdaftari G, Pop EA, Lee SG, Su LJ, Fontham ETH, Bensen JT, Steck SE, Arab L, Mohler JL, Chen MH, Koo SI, Chun OK. Intake of dietary antioxidants is inversely associated with biomarkers of oxidative stress among men with prostate cancer. Brt J Nutr. 2016;115:68–74. https://doi.org/10.1017/S0007114515004249.
10. Monie DD, DeLoughery EP. Pathogenesis of thrombosis: cellular and pharmacogenetic contributions. Cardiovasc Diagn Ther. 2017;7(3):S291–8.
11. Mackman N. Triggers, targets and treatments for thrombosis. Nature. 2008;451(7181):914–8. https://doi.org/10.1038/nature06797.
12. Ali MS, Amin MR, Kamal CM, Hossain MA. In vitro antioxidant, cytotoxic, thrombolytic activities and phytochemical evaluation of methanol extract of the A. philippense L. leaves. Asian Pac J Trop Biomed. 2013;3(6):464–9. https://doi.org/10.1016/S2221-1691(13)60097-0.
13. Uddin MMN, Kabir MSH, Hasan M, Mahmud ZA, Bhuiya NMMA, Ahmed F, Hasan MR, Hosen MT, Alam MS. Assessment of the antioxidant, thrombolytic, analgesic, anti-inflammatory, antidepressant and anxiolytic activities of leaf extracts and fractions of Tetracera sarmentosa (L.) Vahl. J Basic Clin Physiol Pharmacol. 2018;29(1):81–93. https://doi.org/10.1515/jbcpp-2016-0173.
14. Sabrina M, Elizabeth C. Trends, epidemiology, and management of multidrug resistant gram-negative bacterial infections in the hospitalized setting. Antibiotics. 2020;9(4):196. https://doi.org/10.3390/antibiotics9040196.
15. Panda SK, Mohanta YK, Padhi L, Park YH, Mohanta TK, Bae H. Large scale screening of ethnomedicinal plants for identification of potential antibacterial compounds. Molecules. 2016;21(3):293. https://doi.org/10.3390/molecules21030293.
16. Chandrika UG, Kumarab PAASP. Gotu Kola (Centella asiatica): Nutritional properties and plausible health benefits. Adv Food Nutr Res. 2015;76:125–57. https://doi.org/10.1016/bs.afnr.2015.08.001.
17. Kirtikar KR, Basu BD. Indian medicinal plants. Dehra Dun and Periodical Experts, Delhi. 1987;II:1193–5.
18. Ghani A. Medicinal plants of Bangladesh with chemical constituents and uses. 2nd ed. Ramna, Dhaka, Bangladesh: Asiatic Society of Bangladesh; 2003.
19. Kapoor LD. CRC Handbook of Ayurvedic Medicinal Plants, (CRC Press LLC, Florida). 2005. p. 208–9.
20. Zainol MK, Hamid AA, Yusof S, Muse R. Antioxidative activity and total phenolic compounds of leaf, root and petiole of four accessions of Centella asiatica (L.) Urban Food Chemistry, 2003;81(4):575–81. https://doi.org/10.1016/S0308-8146(02)00498-3
21. Mustafa RA, Hamid AA, Mohamed S, Bakar FA. Total phenolic compounds, flavonoids, and radical scavenging activity of 21 selected tropical plants. J Food Sci. 2010;75:28–35.
22. Pittella F, Dutra RC, Junior DD, Lopes MT, Barbosa NR. Antioxidant and cytotoxic activities of Centella asiatica (L) Urb. Int J Mol Sci. 2009;10(9):3713–21. https://doi.org/10.3390/ijms10093713.
23. Park JH, Choi JY, Son DJ, Park EK, Song MJ, Hellstrom M, et al. Anti-inflammatory effect of titrated extract of Centella asiatica in phthalic anhydride-induced allergic dermatitis animal model. Int J Mol Sci. 2017;18(4):1–14. https://doi.org/10.3390/ijms18040738.
24 Puttarak P, Dilokthornsakul P, Saaokaew S, Dhippayom T, Kongkaew C, Sruamsiri R, Chuthaputti A, Chaiyakunapruk N. Effect of Centella asiatica (L.) Urb. On cognitive function and mood related outcomes: a systematic review and meta-analysis. Sci Reports. 2017;7:10646. https://doi.org/10.1038/s41598-017-09823-9.
25. Torbati FA, Ramezani M, Dehghan R, Amiri MS, Moghadam AT, Shakour N, Elyasi S, Sahebkar A, Emami SA. Ethnobotany, phytochemistry and pharmacological features of Centella asiatica: a comprehensive review. Adv Exp Med Biol. 2021;1308:451–99. https://doi.org/10.1007/978-3-030-64872-5_25.
26. Gray NE, Alcazar Magana A, Lak P, Wright KM, Quinn J, Stevens JF, Maier CS, Soumyanath A. Centella asiatica - Phytochemistry and mechanism of neuroprotection and cognitive enhancement. Phytochem Rev. 2018;17(1):161–94. https://doi.org/10.1007/s11101-017-9528-y.
27. Zheng CJ, Qin LP. Chemical components of Centella asiatica and their bioactivities. Zhong Xi Yi Jie He Xue Bao. 2007;5(3):348–51. https://doi.org/10.3736/jcim20070324.
28. Sun B, Wu L, Wu Y, Zhang C, Qin L, Hayashi M, Kudo M, Gao M, Liu T. Therapeutic potential of Centella asiatica and its triterpenes: a Review. Front Pharmacology. 2020;11:568032. https://doi.org/10.3389/fphar.2020.568032.
29. Mook-Jung I, Shin JE, Yuh SH, Huh K, Koh JY, Park HK. Protective effects of asiaticoside derivatives against beta-amyloid neurotoxicity. J Neurosci Res. 1999;58:417–25.
30. Satake T, Kamiya K, An Y, Taka TN, Yamamoto J. The anti-thrombotic active constituents from Centella asiatica. Biol Pharm Bull. 2007;30(5):935–40. https://doi.org/10.1248/bpb.30.935.
31. Subban R, Veerakumar A, Manimaran R, Hashim KM, Balachandran I. Two new flavonoids from Centella asiatica (Linn.). J Nat Med. 2008;62(3):369–73. https://doi.org/10.1007/s11418-008-0229-0.
32. Kupchan SM. Recent advances in the chemistry of terpenoid tumor inhibitors. Pure Appl Chem. 1970;21(2):227–46. https://doi.org/10.1351/pac197021020227.
33. Asaduzzaman M, Uddin MJ, Kader MA, Alam AHMK, Rahman AA, Rashid M, Kato K, Tanaka T, Takeda M, Sadik G. In vitro acetylcholinesterase inhibitory activity and the antioxidant properties of Aegle marmelos leaf extract: implications for the treatment of Alzheimer's disease. Psychogeriatrics. 2014;14(1):1–10.
34. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymology. 1999;299:152–78.
35. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 1999;64(4):555–9.
36. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolybodenum complex: specific application to the determination of vitamin E. Anal Biochem. 1999;269:337–41.
37. Brand-Williams W, Cuvelier ME, Berst C. Use of free radical method to evaluate antioxidant activity. Lebensm Wiss Technol. 1995;28:25–30.
38. Laboni FR, Batul UK, Uddin J, Karim N, Labu ZK, Rashid MH. Antioxidant, cytotoxic, sedative and anti-diuretic effect of stem and root extracts of Basak, Adhatoda vasica. World J Sci Engineering. 2016a;1(1):31–38. https://www.researchgate.net/publication/316284981
39. Bauer AW, Kirby WM, Sherris JC, Truck M. Antibiotic susceptibility testing by a standardized single disc method. Am J Clinic Pathol. 1996;45(4):493–6.
40. Laboni FR, Batul UK, Islam S, Hossain P, Rahman MM, Uddin J, Karim N, Rashid MH. Phytochemical screening, antibacterial, thrombolytic and anti-inflammatory activities of stem and root extracts of Basak, Adhatoda vasica. World J Sci Engineering. 2016b;1(1):55–63. https://www.researchgate.net/publication/316285064
41. Adefegha SA. Functional foods and nutraceuticals as dietary intervention in chronic diseases; Novel perspectives for health promotion and disease prevention. J Diet Suppl. 2018;15(6):977–1009. https://doi.org/10.1080/19390211.2017.1401573.
42. Santos-Buelga C, Gonzalez-Paramas AM, Oludemi T, Ayuda-Duran B, Gonzalez-Manzano S. Plant phenolics as functional food ingredients. Adv Food Nutr Res. 2019;90:183–257. https://doi.org/10.1016/bs.afnr.2019.02.012.
43. Ullah MO, Haque M, Urmi KF, Zulfikar AHM, Anita ES, Begum M, Hamid K. Antibacterial activity and brine shrimp lethality bioassay of methanolic extracts of fourteen different edible vegetables from Bangladesh. Asian Oac J Tropical Biomed. 2013;3:1–7. https://doi.org/10.1016/S2221-1691(13)60015-5.
44. Soyingbe OS, Mongalo NI, Makhafola TJ. In vitro antibacterial and cytotoxic activity of leaf extracts of Centella asiatica (L.) Urb, Warburgia salutaris (Bertol. F.) Chiov and Curtisia dentate (Burm. F.) CA Sm -medicinal plants used in South Africa. BMC Compl Alt Med. 2018;18:315. https://doi.org/10.1186/s12906-018-2378-3.
45. Bouarab-Chibane L, Forquet V, Lanteri P, Clement Y, Leonard-Akkari L, Oulahal N, Degraeve P and Bordes C. Antibacterial properties of polyphenols: characterization and QSAR (quantitative structure-activity relationship) models. Front Microbiol. 2019. https://doi.org/10.3389/fmicb.2019.00829

Publisher's Note