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Antioxidant, thrombolytic, cytotoxic and antibacterial activities of leaves of *Vitex peduncularis*

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Abstract

Vitex peduncularis (Verbenaceae), a perennial tree, is locally utilized for possessing multifaceted pharmacological properties including analgesic, antimalarial, anti-angina, antidiabetic, antioxidant, anti-inflammatory and hepatoprotective. Hence, this study aims to evaluate their antioxidant, thrombolytic, cytotoxic and antibacterial activities from the methanolic crude extract and its various solvent fractions. Data from antioxidant and thrombolytic assay displayed that Kupchan partitioned chloroform soluble fraction revealed the maximum phenolic content (73.39 mg of GAE/g of extract), DPPH free radical scavenging activity (26.69 µg/ml) and thrombolytic activity (37.35%). In brine shrimp lethality evaluation, the LC_{50} values indicate that the most cytotoxic compound was found to be from ethyl acetate soluble fraction (LC_{50} 6.73 µg/ml). Finally, antibacterial activity of different fractions showed that both chloroform and methanolic fraction possessed mild activity against the tested bacteria. The potential biological effects of the crude fractions have been demonstrated demanding future studies of the isolated compounds to assess these activities.

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Introduction

Since the emergence of civilization man has gathered massive knowledge on various plant derived drugs and chemicals which are being used to treat different disorders (Schultes *et al*. 1994). Plant-derived components have founded the traditional system of medicine in many countries which continue to play an undeniable role in human health care for thousands of years. Excessive production of reactive oxygen species (ROS) in various metabolic processes may cause oxidative chain reactions which affects the level of antioxidants in body and may cause numerous diseases such as cancer, allergies, neurodegenerative diseases, and various cardiac problem (Dudonne *et al.* 2009; Kaushik *et al.* 2012). The anti-oxidative effect of different plant extracts is mostly due to the function of phenolic compounds such as phenolic diterpenes, phenolic acids (Shahidi *et al*. 1992) and flavonoids (Pietta *et al*. 1998) which have been linked to reduce tumor or cancer incidence and mortality rates in many ethnic communities (Velioglu *et al*. 1998).

Cerebral sinus thrombosis (CVST) is a frequent condition that has a high rate of morbidity and mortality (Zhao *et al*. 2008). Various medicines such as heparin, tissue urokinase (t-PA), streptokinase, urokinase and others are also used to

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breakdown blood clot within blood vessels and treat patients with CVST (Baruah *et al*. 2006). However, numerous fatal disorders including hemorrhagic condition, lacked specificity, severe anaphylactic shock etc. are sometimes responsible because of these drugs (Rouf *et al*. 1996). Therefore, efforts are underway to find out suitable alternatives.

The infections caused by various microorganisms is the pressing problem by a major portion of the world population due to improper sanitation, inadequate hygiene practice and overloaded living conditions (Rasoanaivo, 1993). Since the antibiotic resistance arises from the careless use of commercially available antibiotics are rising day by day, the discovery of new drugs is essential to prevent the most inevitable medical and social catastrophe (Powers, 2004).

Vitex peduncularis belongs to the Verbenaceae family, is a medium to large sized perennial tree; growing 7 to 18 m tall; young parts sparsely pubescent, glabrescent when mature; leaves usually 3-foliate, petiole flattened, pubescent, about 10 cm long (Saran *et al*. 2019). It is conserved mainly in the tropical and subtropical regions of the world. In Bangladesh, the species is known by various local name and mainly distributed in the forest area of Chattogram, Chattogram Hill Tracts, Cox's Bazar, Khagrachari, Sylhet, Bandarban, Gazipur and Tangail (Hasnat *et al*. 2019). It is the main source of several active chemical compounds including flavonoids, triterpenoid, iridoids, alkaloids, steroids, cardiac glycosides, tannins, phenols, and free amino acids, with important traditional medicinal uses among human society. Traditionally, the treatment of joint ache is used a fresh boiled bark juice extracted from this plant. Previous studies revealed that various phytochemicals isolated from this plant have significant analgesic (Singh *et al*. 2008), anti-inflammatory (Ferreres *et al*. 2017); anxiolytic, sedative, and antioxidant (Auniq *et al*. 2021); anti-nociceptive and anthelmintic (Auniq *et al*. 2019); antibacterial and antifungal activities (Panda *et al*. 2012). Bark and leaves of *V. peduncularis* are also successfully used for the treatment of malaria, diabetes, and jaundice (Nag *et al*. 2011).

Since only about 6% of the existing medicinal plants species have been investigated for their potential biological and pharmacological properties (Cragg and Newman, 2013), there has still been a gap between the natural endowment and exploration of the same for the human health challenges (Wiland-Szymańska, 2009). Therefore, considering the enormous potentiality of the medicinal plants, the present study was designed to evaluate the antioxidant, thrombolytic, cytotoxic, and antibacterial activities of the different solvent extracts of *V. peduncularis*.

Materials and methods

Chemicals

All the solvents and reagents used in this research work, were of analytical-grade and procured from reputable importer (Bright Scientific Co. Bangladesh; DaeJung, Korea; Merck, Germany). 1,1-diphenyl-2- picryl-hydrazyl (DPPH), butylated hydroxy toluene and Folin-Ciocalteu reagent was procured from Sigma Chemicals Co. (St. Louis, MO, USA). Lyophilized streptokinase was procured from Incepta Pharmaceuticals Ltd., and normal saline solution from Beximco Pharmaceuticals Ltd., Bangladesh during this research work.

Collection and identification of the plant materials

The fresh leaves of *V. peduncularis* Wall ex. Schauer (Locally: Goda, Horina) were collected from Kamalganj, Moulvibazar, Bangladesh during the month of March 2021 and identified by taxonomist from Bangladesh National Herbarium, Dhaka (Accession number DACB-65392). A voucher specimen was deposited there for further reference.

Extraction

The collected leaves were isolated from the stalks, cleaned properly and subjected to shade drying for two weeks. The dried leaves were pulverized by high-capacity grinding machine and approximately 1.2 kg of coarse powder was obtained.

This powder was taken in an amber-colored clean bottle and soaked into appropriate amount of methanol. The bottle with its content was preserved for a period of 20 days with occasional shaking and stirring. After cold extraction, the mixture was filtered through a fresh cotton plug placed inside a large funnel and then through Whatman No.1 filter paper.

The filtrate was then dried up using a vacuum rotary evaporator (Heidolph, UK) at low temperature (50 degree) and pressure. After which the filtrate was kept for complete drying to convert into a dry crude extract. The weight of the dried extract was 52 g.

Fractionation of the crude extract

The protocol was employed for solvent-solvent partitioning of the methanolic crude extract of *V. peduncularis* (VanWagenen *et al*. 1993). First, 10% aqueous methanol was used to dissolve the crude extract (5 g) and then extracted consecutively using n-hexane, chloroform, and ethyl acetate based on the polarity.

Antioxidant activity

Analysis of total phenolic content

The assessment of entire phenolic content of the methanolic extract and different solvent fractions of *V. peduncularis* was done by using the (Skerget *et al*. 2005) technique, which included an oxidizing agent (Folin-Ciocalteu reagent) and a reference (Gallic acid) (Majhenic *et al*. 2007). Folin-Ciocalteu reagent is normally yellow in hue, however following oxidation, the result turns blue. For this, 2.0 ml Na_2CO_3 (7.5 percent w/v) solution and ten times diluted 2.5 ml Folin-Ciocalteu reagent and were added to 0.5 ml extracted solution (2 mg/ml). The mixture was incubated for 30 minutes at room temperature (RT), and after that UV-spectrophotometer was used to find out the absorbance at 765 nm which reflect the compound's full phenolic content (Harbertson and Spayd, 2006). The total phenolic content of the sample was calculated using the equation obtained from a quality curve made from gallic acid solutions of various concentrations and the results were described as mg gallic acid equivalents per g of the crude extract (mg GAE/g crude extracts).

DPPH Assay: free radical scavenging activity

The plant extracts' free radical scavenging activities (antioxidant capacity) on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) were calculated using a modified Brand-Williams technique (Ahmed *et al*. 2006; Brand-Williams *et al*. 1995) where BHT (butylated hydroxy toluene) was considered as a positive control. Briefly, the mother solution (2000 µg/ml) of different solvent extracts was serially diluted to produce concentrations ranging from 400.0 to 0.781 µg/ml, which were stored in the designated flasks. Then, 2.0 ml methanol solutions of sample (extractives/control) at various concentrations (400.0 to 0.781 µg/ml) were combined with 2.0 ml DPPH methanol solution (0.004%). The absorbance was quantified at 517 nm using a UV spectrophotometer against methanol as a blank after a half-hour reaction time at RT in a dark environment. The free radical DPPH percent inhibition was estimated as follows:

$$(1\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \text{ X } 100\%$$

Where A_{blank} and A_{sample} denotes the control reactions and the absorbance of test sample respectively.

From the graph plotting inhibition percentage versus extract concentration, the extract concentration providing 50% inhibition (IC_{50}) was computed using excel 2019 Office software.

Evaluation of thrombolytic activity

The assessment of thrombolytic activity of different fractions as well as methanolic extracts of *V. peduncularis* was carried out by using 10 mg of extracts which mixed with 1 ml distilled water into different vials according to the slightly modified method developed by Prasad (2006) (Azad *et al*. 2015; Prasad *et al*. 2006). Healthy volunteers (not taking anticoagulant therapy or oral birth control pill) provided aliquots (5 ml) of venous blood, which were kept at 37°C for 45 minutes after splitting into five pre-weighed sterilized vials (1.0 ml/tube). Following clot formation, the serum was withdrawn completely without disrupting the clot, and each vial containing clot was weighed again to calculate the clot weight

Clot weight = weight of clot containing tube – weight of tube alone.

100 µl aqueous solutions of prepared fractions were added separately to every single vial comprising pre-weighed clot.

Distilled water (100 µl) and streptokinase (100 µl) were also added to the control vial as negative and positive non-thrombolytic controls, respectively. After that, all of these vials were reserved at 37°C for 90 minutes to check for clot lysis. After the time period, the discharged fluid was withdrawn, and the vials were weighed again to see if the weight difference after clot disruption was significant. The percentage difference in weight acquired before and after the clot lysis was calculated as shown below:

% Clot lysis = Weight of the lysis clot / Weight of clot before lysis × 100

Determination of cytotoxic activity

The possible cytotoxic activity of the various extracts of *V. peduncularis* was evaluated through brine shrimp lethality bioassay according to the slightly modified method explained by Meyer *et al* (1982) (Ahmed *et al*. 2008; Meyer *et al*. 1982). The test organism of Artemia salina leach (brine shrimp eggs) was obtained from pet stores and were allowed to hatch in simulated seawater with continuous oxygen supply till mature as nauplii for 24 hours. All the test samples (4 mg) were dissolved in 200 µl of pure dimethyl sulfoxide (DMSO) in vials to make the stock solution. From which every time, 100 µl solution was moved to a test tube filled with 10 shrimp nauplii in 5 ml simulated sea water and new 100 µl DMSO was added to stock vials. Therefore, using the serial dilution procedure, a series of solutions with varied concentrations (400 to 0.781 µg/ml) were made from the stock solution. In this cytotoxicity studies, a commonly

accepted cytotoxic agent Vincristine sulfate was employed as a positive control which was dissolved in DMSO to produce an initial concentration of 20 µg/ml and serially diluted to prepare a range of concentrations. To be used as negative control groups, 100 µl of DMSO was added to each of three pre-marked test tubes containing 10 shrimp nauplii in 5 ml simulated sea water. All the vials were left for twenty-four hours after that, a visual assessment using a magnifying glass was performed to calculate the percentage survivors of the nauplii. The concentration-mortality data were analyzed, and the median lethal concentration (LC_{50}) was calculated by windows Microsoft Excel 2019 software.

Assessment of antibacterial activity

The *in-vitro* inquiry for primary screening of antibacterial property of the various extracts of *V. peduncularis* was tested by using the widely established disc diffusion method (Ahmed *et al.* 2006; Barry, 1976). Five gram (+)ve bacteria (*Bacillus megaterium, Staphylococcus aureus, Bacillus cereus, Sarcina lutea, Bacillus subtilis*) and seven gram (-)ve bacteria (*Salmonella paratyphi, Shigella boydii, Vibrio mimicus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Shigella dysenteriae*) were chosen for the analysis. In this traditional approach, dried and sterile 6 mm diameter filter paper discs containing pre-weighted test samples (400 µg/disc) were positioned on nutrient agar medium which was uniformly seeded with the test microorganisms. Antibiotics permeate into the agar gel from a restricted source, creating a degree gradient. Positive and negative controls were standard ciprofloxacin antibiotic (30 µg/disc) discs and blank discs. These plates were placed in a low temperature (4°C) for 16 to 24 hours to allow maximum dispersion of the test ingredients to the surrounding milieu (Barry, 1976). The plates were then inverted and incubated for 24 hours at 37°C for optimal growth of the organisms. Antimicrobial test materials prevented microbial growth in the media surrounding the discs, resulting in a transparent, distinct zone known as the zone of inhibition. The diameter of the zone of inhibition was then measured in millimeters to determine the antibacterial activity of the test agent (Barry, 1976).

Results and discussion

Antioxidant activity

Analysis of total phenolic content

Total phenolic content was measured for the methanolic crude extract of *V. peduncularis* along with four different solvent fractions including aqueous fraction (VPAF), n-hexane fraction (VPHF), chloroform fraction (VPCF), ethyl acetate fraction (VPEF). The colorimetric measurement of the total phenolic content of different extracts was obtained according to the absorbance values of these extracts and compared to standard solutions of Gallic Acid equivalents which presented in Table I.

Table I. Total phenolic content of different fractions of *V. peduncularis*

Plant parts	Sample	Absorbance	Total phenolic content (mg of GAE/g of extractive)	Regression equation
Leaves of *V. peduncularis*	VPHF	0.146	14.71	$y = 0.0098x + 0.0018$ $R^2 = 0.9996$
	VPCF	0.721	73.39	
	VPEF	0.473	48.08	
	VPAF	0.267	27.06	
	VPMF	0.531	54.00	

VPHF: n-hexane fraction, VPCF: chloroform fraction, VPEF: ethyl acetate fraction, VPAF: aqueous fraction, VPMF: crude methanolic fraction

From the test result, it was found that the total amount of phenolic content differs in various fractions and ranged from 14.71 mg of GAE/g of extractives to 73.39 mg of GAE/g of extractives. The highest amount of phenolic content was found 73.39 mg of GAE/g of extractives in chloroform soluble fraction (VPCF) which supports its significant free radical scavenging and lipid peroxidation inhibitory action. On the other hand, the lowest phenolic content was found 14.71 mg of GAE/g of extractives in n-hexane soluble fraction (VPHF). This may be happened due to the changes in solvent polarity which significantly affects the solubility of phenolic compounds.

DPPH Assay: free radical scavenging activity

The outcomes of the DPPH free radical scavenging activity of the methanolic extract of *V. peduncularis* and its different solvent fractions were represented in the Table II and these results were compared to that of butylated hydroxy toluene (used as positive control). These results revealed that the 50% inhibitory concentration (IC_{50}) differed in different fractions and ranged from 26.69 µg/ml of extractives to 163.96 µg/ml of extractives. Among all extractives, the highest DPPH free radical scavenging activity was detected in VPCF (26.69 µg/ml) and lowest was found in VPHF (163.96 µg/ml) with respect to the butylated hydroxy toluene (BHT) 15.89 µg/ml.

Table II. DPPH free radical scavenging activity of *V. peduncularis*

Sample	IC_{50} (µg/ml)	Regression equation
BHT	15.89	$y = 16.302\ln(x) + 4.9101$; $R^2 = 0.9887$
VPHF	163.96	$y = 11.035\ln(x) - 6.2742$; $R^2 = 0.9475$
VPCF	26.69	$y = 15.776\ln(x) - 1.8147$; $R^2 = 0.9513$
VPEF	47.41	$y = 12.489\ln(x) + 1.8075$; $R^2 = 0.9865$
VPAF	49.38	$y = 11.898\ln(x) + 3.6027$; $R^2 = 0.9953$
VPMF	36.69	$y = 14.541\ln(x) - 2.3859$; $R^2 = 0.9766$

VPHF: n-hexane fraction, VPCF: chloroform fraction,
VPEF: ethyl acetate fraction,
VPAF: aqueous fraction, VPMF: crude methanolic fraction

Previous phytochemical screening for various parts of *V. peduncularis* showed the presence of many flavonoids, triterpenoid, iridoids, alkaloids, steroids, cardiac glycosides, tannins, phenols and free amino acids. So, the present study demonstrated that the leaves of *V. peduncularis* has the significance to be a worthy source of natural antioxidants.

Evaluation of thrombolytic activity

The effective thrombolytic percentage by methanolic crude extract along with four different solvent fractions of the leaves of *V. peduncularis* was assessed and the results were presented in Table III. In this study, Streptokinase (SK) was used for positive thrombolytic control and water as negative

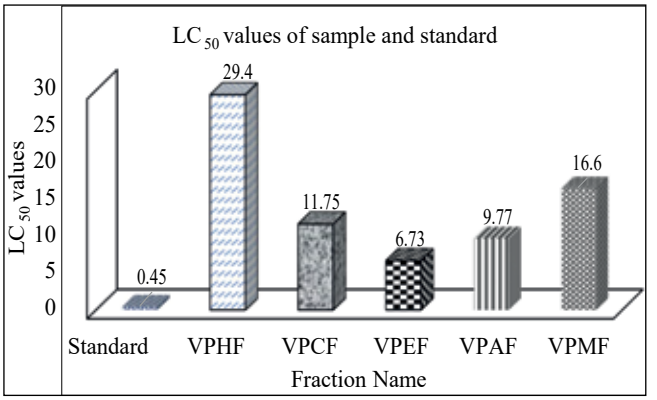

Fig. 1. Comparison of cytotoxicity results (LC_{50} values) of standard and different extracts of *V. peduncularis*

Standard: Vincristine sulphate, VPHF: n-hexane fraction, VPCF: chloroform fraction, VPEF: ethyl acetate fraction, VPAF: aqueous fraction, VPMF: crude methanolic fraction

Table III. Thrombolytic activity of different fractions of *V. peduncularis*

Fractions	Wt. of empty Eppendorf tube W_1 g	Wt. of (clot + tube) before clot disruption, W_2 g	Wt. of (clot + tube) after clot disruption, W_3 g	Wt. of clot before lysis $W_4 = W\text{-}_2\ W_1$ g	Wt. of lysis clot $W_5 = W_2 - W_3$ g	% of lysis (W_5/W_4) x 100
VPHF	0.7800	1.2756	1.1695	0.4956	0.1061	21.41
VPCF	0.8229	1.3134	1.1348	0.4905	0.1786	37.35
VPEF	0.8191	1.3120	1.2397	0.4929	0.0723	14.67
VPAF	0.8171	1.3060	1.2393	0.4889	0.0667	13.64
VPMF	0.7937	1.2938	1.1645	0.5001	0.1293	25.85
Blank	0.8179	1.3268	1.3088	0.5089	0.0180	3.53
SK	0.8314	1.3195	1.0152	0.4881	0.3043	62.34

VPHF: n-hexane fraction, VPCF: chloroform fraction, VPEF: ethyl acetate fraction,
VPAF: aqueous fraction, VPMF: crude methanolic fraction

control (blank). The following results showed that the various fractions of *V. peduncularis* leaves extract possessed mild to moderate clot lysis activity in the range between 13.64% to 37.35% while the standard streptokinase (30,000 I.U.) exhibited 62.34% lysis of clot. Among these, the chloroform soluble fractions (VPCF) demonstrated the highest thrombolytic activity of 37.35% and supposed that the isolation of pure compound from this extract might have potential thrombolytic activity.

Determination of cytotoxic activity

The Artemia salina (Brine Shrimp) nauplii, which can be utilized as a simple, quick, and advantageous method for screening and fractionation of various bioactive compounds (McLaughlin *et al*. 1998). The brine shrimp fatality bioassay was performed using the methanolic extract of *V. peduncularis* along with the different solvent fractions. After 24 hours, the 50% lethal concentration (LC_{50}) of the experimental samples was attained by a plot of percentage of brine shrimps died against the log concentration of the sample (toxicant concentration). Vincristine sulphate (VS) was used as positive control and 0.45 µg/ml LC_{50} value was found for VS. The best-fitted line was observed from the curve data by means of regression analysis. The LC_{50} values of above-mentioned fractions were compared to positive control (Figure 1). The LC_{50} values of VPHF, VPCF, VPEF, VPAF and VPMF were measured to be 29.40 µg/ml, 11.75 µg/ml, 6.73 µg/ml and 9.77 µg/ml and 16.60 µg/ml respectively.

This bioassay test is very effective in establishing different biological activities such as pesticidal, trypanocidal, ion regulation, cytotoxic, enzyme inhibition, and phototoxic activities (Ramamoorthy *et al*. 2012). So, the outcomes obtained from various tested extracts indicated that they might have one or more potential phytochemicals which is responsible for this specific kind of biological activities.

Assessment of antibacterial activity

The methanolic extract of *V. peduncularis* as well as different solvent fractions were subjected to antibacterial activity screening. 400 µg/disc concentration was used in every case. As a positive control, Ciprofloxacin antibiotic (30 µg/disc) discs were used to confirm that the standard antibiotic was active against the tested organisms, as well as to compare the reaction produced by the known antibacterial agent to the response generated by the test sample. For negative control, blank discs were utilized to make sure that the residual solvents (which remained on the discs after air drying) and hence the paper did not become active. The chloroform soluble fraction (VPCF) and methanolic crude extract (VPMF) showed better result against both gram (+) ve and gram (-) ve bacteria ranging from 8 to 15 mm zone of inhibition (Table IV). Though the ethyl acetate soluble fraction (VPEF) possessed antibacterial activity against some bacteria, the n-hexane soluble fraction (VPHF) and aqueous fraction (VPAF) did no possesses any significant antibacterial activity. Therefore, the methanolic and chloroform soluble

Table IV. Antimicrobial activity of test sample of *V. peduncularis*

Test organism	Zone of Inhibition (mm)					
	VPHF	VPCF	VPEF	VPAF	VPMF	Ciprofloxacin (30 µg/disc)
Gram Positive bacteria						
Bacillus cereus	10	9	-	-	8	30
Bacillus subtilis	-	10	-	-	8	28
Bacillus megaterium	-	15	-	-	10	38
Sarcina lutea	-	10	8	-	12	25
Staphylococcus aureus	-	9	-	-	15	25
Gram Negative bacteria						
Escherichia coli	-	8	-	-	10	50
Salmonella paratyphi	-	-	-	-	-	29
Salmonella typhi	-	10	10	-	9	30
Pseudomonas aeruginosa	-	9	-	-	-	30
Vibrio mimicus	-	-	8	-	12	45
Shigella dysenteriae	-	8	-	-	-	47
Shigella boydii	-	10	8	-	8	25

VPHF: n-hexane fraction, VPCF: chloroform fraction, VPEF: ethyl acetate fraction,
VPAF: aqueous fraction, VPMF: crude methanolic fraction

portions might have some chemicals that possess potential antibacterial activity.

Conclusion

The biological activity of different fractions (chloroform, ethyl acetate, n-hexane and aqueous soluble fraction) and methanolic crude extract of *V. peduncularis* was performed and evaluated their antioxidant, thrombolytic, brine shrimp lethality and antibacterial activity. This study suggested that among the entire extractives, the maximum phenolic content and the amount of DPPH free radical scavenging activity was found in chloroform soluble fraction. Besides, this fraction also exhibited most promising thrombolytic effect than all other extracts of *V. peduncularis.* Notably, the ethyl acetate soluble fraction exhibited significant cytotoxic activity. In case of antibacterial assay, results found that the chloroform and methanolic fraction possesses moderate to mild antibacterial activity against both gram-negative and gram-positive bacteria. Therefore, based upon the data from this biological activity of different fractions, it is concluded that the leaves of *V. peduncularis* can be a prospective source for various biological activity and can be researched in greater depth to identify their previously unknown efficacy, legitimize their usage as traditional medicines and ideally, aid in the identification of a new lead compound. However, further investigations are continued to isolate the specific compounds from the extracts of its leaves.

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