

Hypoglycemic Activity of *Curculigo latifolia* on Insulin and Glucose Uptake Activities in *in vitro*

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Abstract: *Curculigo latifolia* (*C. latifolia*) has been shown to potentiate the antidiabetic and hypolipidemic effects on induced diabetic rats by altering the lipid and glucose metabolisms. In the present study, we evaluated the effect of extracted *C. latifolia* on *in vitro* glucose uptake and insulin secretion by adipocyte and investigated muscle and pancreatic cells. The aqueous extracted samples underwent polyphenol assay such as Total Phenolic Content (TPC) and the antioxidant assay to screen the antioxidant activity of extracted samples by inhibition of free radical from DPPH radical. With regard to *in vitro*, glucose uptake assay was analysed using L6 myotube and 3T3-L1 adipocyte cell lines and insulin secretion was assayed using BRIN-BD11 pancreatic cell in which both assays were examined under different extracted parts of samples investigated. The results indicated that *C. latifolia* fruit possessed the highest amount of TPC (95 mg GAE/100 g *C. latifolia* fruit extract) and antioxidant activity as it achieved the lowest IC₅₀ for DPPH assay (1.0±0.1 mg/mL). Cells treated with *C. latifolia* fruit extract under insulin secretion assay has the highest stimulation compared to others in the following descending order fruit>root>leaf. In addition, *C. latifolia* fruit extract also showed the highest result in glucose uptake activity by 13 fold at 0.05 mg/mL with insulin presence in 3T3-L1 adipocyte cells. However, in L6 myotube cells, *C. latifolia* fruit extract increased basal and insulin-mediated glucose uptake activity. The results indicated the presence of antihyperglycaemic from insulin-releasing and glucose uptake activity in *C. latifolia*.

Key words: Antidiabetic, antihyperglycaemic, *Curculigo latifolia*, glucose uptake, phenolic compounds, pancreatic cell

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a chronic disorder characterized by hyperglycemia and it causes disturbance on fat, protein and carbohydrate due to the defects in insulin secretion, insulin action or both (Inzucchi and Sherwin, 2011). In addition, insulin resistance and impaired insulin secretion remain the core defects in T2DM (DeFronzo *et al.*, 2015). Insulin resistance is mainly caused by impaired β -cell functioning which is usually regulated by abnormal insulin secretion or defective action. A report by the International Diabetes Federation indicated that this type of diabetes is seen in about 90-95% diabetic patients. In fact, the number of diabetic patients was estimated at 424.9 million in 2017 and it is estimated to double by 2030 (International Diabetes Federation, 2017). Currently,

T2DM's management treatment is from mechanism of action by pharmaceutical drugs whereby, these drugs are classified based on the different mechanism actions that act on the different therapeutic agents. However, as all of these drugs focused on reducing and controlling the glycemia level, they have their own complications and produce adverse effects when patients consume these drugs over a long period of time. An alternative treatment of diabetes may be from plant-based products which are rich in antioxidants including various bioactive molecules with diverse structures.

C. latifolia, locally known as *lemba* or *lumbah*, belongs to the family of Hypoxidaceae. It is claimed to have medicinal values in treating several diseases such as asthma, hemorrhoids, jaundice and diarrhea apart from having properties that affect antidiabetic activity by ameliorating insulin resistance *in vivo* studies

(Ishak *et al.*, 2013). Besides, *C. latifolia* fruits have been used by the natives as sweetener. Interestingly, the sweet taste comes from the isolated protein, curculin, that is presence in *C. latifolia* fruits. The sweet taste from the isolated protein, curculin can be one of the potential approaches in treating diabetes (Farzinebrahimi *et al.*, 2016). Previous studies reported that the fruit and root extracts of *C. latifolia* have been shown to modulate glucose and lipid metabolism in experimental diabetic rats and the presence of curculigoside may have bestowed its anti-diabetic efficacy (Ishak *et al.*, 2013). Moreover, several studies have also shown that phenolic compounds exhibit antidiabetic activities and thus, they contribute highly towards achieving high antioxidant activities (Rodriguez-Mateos *et al.*, 2013). Consumption of phenolic rich compound protects the body against free radical and thereby reduces the risk of diabetes.

Our current researcher determines the phenolic content and antioxidant activity in *C. latifolia* fruits, roots and leaves. The obtained extracts underwent selected assays by insulin secretion and glucose uptake to test the antidiabetic activity through *in vitro* way.

MATERIALS AND METHODS

Chemicals and reagents: The following items were purchased from Sigma Chemicals Co. (St. Louse, Missouri, USA): ammonium hydroxide (NH₄OH), bovine insulin, Calcium Chloride (CaCl₂), Dimethylsulphoxide (DMSO), Dulbecco's Modified Eagle Medium (DMEM), D-glucose, ethanol, glibenclamide, HEPES, Isobutylmethylxanthine (IBMX), Magnesium Sulphate (MgSO₄), methanol, potassium dihydrogen phosphate (KH₂PO₄), potassium chloride (KCl), Roswell Park Memorial Institute (RPMI) 1640, sodium chloride (NaCl), sodium hydrogen Carbonate (NaHCO₃) and Sodium Dodecyl Sulphate (SDS). The 2-Deoxy-[1-³H]-glucose was purchased from GE healthcare (Massachusetts, USA). Ultima Gold LLT was purchased from Perkin Elmer (Massachusetts, USA) while tetrazolium compound [3-(4,5-dimethylthiazol-2-yl) 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) was purchased from Promega (Southampton, UK).

Plant materials: Plant materials were collected from Beranang, Selangor, Malaysia. The plants were authenticated by a taxonomist at the Biodiversity Unit in the Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen (Voucher No. SK 1709/09) for future correspondence was deposited into the Biodiversity Unit in the Institute of Bioscience, Universiti Putra Malaysia.

Preparation of *C. latifolia* extract: The leaves and roots were cleaned with tap water and immediately dried in an air oven (Memmert, Schwabach, Germany) at 40°C overnight. The dried leaves and roots were then ground to fine powder using an electric grinder (Philips, Malaysia). Powdered leaves and roots were sealed in plastic bags and kept at 4°C until further use. Meanwhile, the fruits were plucked directly at the apex of *C. latifolia* stems, cleaned with tap water and blotted with tissue papers. Fruits were stored at -20°C until further use.

Different parts of *C. latifolia* plant, i.e., the fruits, roots and leaves were extracted with 2000 mL of distilled water at 25°C for 24 h with continuous stirring at room temperature. This extract was filtered through Whatman No. 1 filter paper, collected and lyophilized. The lyophilized sample was kept at -80°C until further use.

Determination of the Total Phenolic Compound (TPC) in *C. latifolia*: The total phenolic compound was determined in accordance with the protocol previously described by Limmongkon *et al.* (2017) with slight modifications. Briefly, different concentrations of *C. latifolia* extracts were prepared and 1 mL of each concentration was mixed with 5 mL of Folin-Ciocalteu reagent in a test tube. Then, 4 mL of 2% sodium carbonate was added to each tube and incubated at room temperature for 2 h in darkness. The absorbance of the sample was measured at 725 nm with a spectrophotometer (Pharmaspec UV-1700, Shimadzu, Kyoto, Japan). Gallic acid was used as the standard and the results were expressed as milligram of gallic acid/100 g of extract (GAE). The analyses were done in triplicates.

2-Diphenyl-1-Picrylhydrazyl (DPPH) free radical-scavenging activity in *C. latifolia*: DPPH free radical-scavenging activity was carried out according to Hua *et al.* (2014) with a few modifications. Different concentrations of *C. latifolia* extracts were added to 0.06 mM DPPH solution in pure methanol and the mixture was shaken vigorously. After 60 min of incubation at room temperature and in darkness, the absorbance was measured at 517 nm using a spectrophotometer (Pharmaspec UV-1700, Shimadzu, Kyoto, Japan). Pure methanol was used as blank and a negative control while ascorbic acid was used as a reference. The DPPH analysis was done in triplicate. The IC₅₀ value was defined as the concentration of the compound required to scavenge the DPPH radical by 50%.

Cell culture: L6 myotube and 3T3-L1 adipocyte cell lines were provided by Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia while

BRIN BD11 cell line was provided by the Animal Cell Culture Laboratory, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. BRIN-BD11 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) Foetal Bovine Serum (FBS) and 1% (v/v) penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO₂. The cells were subcultured every 2-3 days at approximately 80% confluency. 3T3-L1 adipocytes and L6 myotubes cells were maintained in high glucose DMEM containing 1% (v/v) penicillin-streptomycin and 10% (v/v) FBS at 37°C in a humidified atmosphere of 5% CO₂ cells were subcultured after approximately 90% confluence and plated onto 24 or 96 well plates for 3 days. After 3 days, the cultured medium was switched to a Different Medium (DMEM, 10% (v/v) FBS, 0.25 µm dexamethasone, 0.25 mM IBMX and 1 µg/mL insulin) for 3 days. On day 3, the cells were maintained in a different medium but without IBMX and dexamethasone for another 4 days and the medium was changed every 2 days. Plates where cells were >90% differentiated were then maintained in the original propagation DMEM media and were ready to be used for the experiments.

Insulin secretion assay: Insulin secretion was determined in accordance with Chen *et al.* (2016) study. Cells were seeded at 2×10^5 cells/well in RPMI 1640 containing 10% foetal bovine serum, 1% penicillin and 1% glutamine and allowed for attachment overnight. The cells were then washed thrice with Kreb's-Ringer Bicarbonate buffer (KRB; 115 mM NaCl, 4.7 mM KCL, 1.28 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 24 mM NaHCO₃, 10 mM Hepes-free acid, 1% (w/v) Bovine Serum Albumin (BSA), 1.1 mM glucose; pH 7.4) and preincubated for 40 min in KRB buffer at 37°C. The cells were then incubated for 1h with 1 mL KRB buffer in the absence or presence of *C. latifolia* fruit, root and leaf extracts. Glibenclamide was used as a positive control. Aliquots were removed from each well and stored at -20°C for insulin assay.

Aliquots from insulin secretion assay were used following the Mercodia Rat Insulin ELISA (Uppsala, Sweden) protocol. The 25 µL of aliquots and calibrators were added into each well which was coated with mouse monoclonal antibody. Then, 50 µL of peroxidase conjugated mouse monoclonal anti-insulin was added. A simple washing step removed unbound enzyme labeled antibody. The bound conjugate was detected by reaction with 3, 3', 5, 5'-tetramethylbenzidine. The absorbance of each well was measured at 450 nm using a microplate reader (Opsys MR, Thermolabsystems) to quantify insulin concentration.

Deoxy-D-Glucose (2DOG) uptake: Glucose uptake was measured in fully differentiated L6 myotubes and 3T3-L1 adipocytes. Cells were rinsed thrice with Krebs-Ringer HEPES buffer (pH 7.4) treated with *C. latifolia* extracts in the presence or absence of insulin (100 nm). Rosiglitazone was used as control. This treatment was allowed to proceed for 30 min. Then 1 µCi/mL of 2-deoxy-D-[1-³H] glucose was added and incubated for 1 h. Plates were washed thrice with ice-cold KRH buffer and the cells were digested with 0.1% SDS. An aliquot was used to measure the radioactivity using a scintillation counter (Tri-Carb 2300TR, Perkin Elmer Life and Analytical Services, Boston, MA, USA) and Ultima Gold™ LLT as the scintillation cocktail (Perkin Elmer, Boston, MA, USA). Glucose uptake was expressed as disintegrations per minute (dpm).

Statistical analysis: All results are expressed as means±standard deviation and all assay conditions were performed in triplicate. Data were analyzed using a one-way Analysis of Variance (ANOVA), followed by Turkey's post hoc test. Level of significance was set at p<0.05. Linear correlation was determined using Pearson correlation and p<0.05 was considered significant.

RESULTS AND DISCUSSION

Total phenolic compounds in *C. latifolia*: Phenolic compounds are a major component in plants resulted from secondary metabolites. It is one of the constituents that is known to possess antioxidant properties (Esmaili *et al.*, 2015). Thus, they have credible effects on the prevention of various oxidative stress associated diseases (Al-Qassabi *et al.*, 2018). In the present study, the total phenolic content in *C. latifolia* fruit, root and leaf extracts were determined using the Folin-Ciocalteu reagent (FCR) method. The total phenolic compounds of *C. latifolia* are shown in Table 1. Results indicate that *C. latifolia* fruit extract (95 mg GAE/100 g *C. latifolia* fruit extract) contains the highest amount of TPC, followed by the root (90 mg GAE/100 g *C. latifolia* root extract). Several studies have shown that more than 110 compounds have been isolated from Curculigo genus including the following types of chemicals: phenols and phenolic glycosides (lignans and lignan glycosides (Wang *et al.*, 2013, 2014), triterpenes

Table 1: Total phenolic compounds of *C. latifolia*

Extract	TPC (mg GAE/100 g extract)
Fruit	95±0.4*
Root	90±0.3*
Leaf	74±0.5*

Data are expressed as means±SD (n = 3); Data with*are considered significant at p<0.05

and triterpenoid glycosides, flavones, eudesmanes (Chen *et al.*, 2017) and other constituents. Norlignans, triterpenoids and phenol glycosides are regarded as the major constituents and are most likely responsible for most of the activities found in plants of this plant genus (Nie *et al.*, 2013).

Antioxidant activity of *C. latifolia*: A stable free radical from DPPH and hydroxyl was used to determine the scavenging activity of the plant in this study. The results are shown in Table 2. There is a significant difference ($p < 0.05$) in antioxidant activities among the different parts of *C. latifolia* plant extracts. *C. latifolia* fruit has the highest antioxidant activities (1.0 ± 0.1 mg/mL) while the leaf has the lowest antioxidant activities (2.0 ± 0.3 mg/mL). The present study reports that *C. latifolia* fruit and root extracts have a better reduction of DPPH radical than the leaf extract. This indicates that *C. latifolia* fruit and root extracts have high scavenging activity and can be assumed to contain antioxidant agent that might be from the total phenolic compound presence in *C. latifolia* extracts. A similar observation and trend were also seen in the study of antioxidant activity of the *C. orchioides* done by Murali and Kuttan (2016). They found that a natural phenolic derivative from the plant curculigo orchioides, curculigoside, exhibits antioxidant effects both in the *in vitro* and *in vivo* systems. In the case of hydroxyl radical scavenging and inhibition of lipid peroxidation, curculigoside gave better results than vitamin C while in the case of DPPH and super oxide radicals, the scavenging effect was more prominent in vitamin C.

The correlation between the total phenolic content and scavenging activity of *C. latifolia* fruit, root and leaf extracts is shown in Table 3. There is a positive correlation between the total phenolic content and antioxidant activity. The root extract shows a strong positive correlation 100% ($r = 1.0$), followed by fruit extract 65.5% ($r = 0.655$) and leaf extract 57.3% ($r = 0.573$). The

Table 2: Antioxidant activity of *C. latifolia*

<i>C. latifolia</i> extract	IC ₅₀ (mg/mL)
Fruit	$1.0 \pm 0.1^*$
Root	$1.0 \pm 0.4^*$
Leaf	$2.0 \pm 0.3^*$
Ascorbic acid	0.02 ± 0.01

Data are expressed as means \pm SD (n = 3); Data with *are considered significant at $p < 0.05$

Table 3: Linear correlation coefficient (r) between antioxidant activity and Total Phenolic Content (TPC) of *C. latifolia* extracts

Total phenolic contents	Correlation
DPPH _{Total} vs. TPC _{Total}	$r = 0.683$
DPPH _{Fruit} vs. TPC _{Fruit}	$r = 0.655$
DPPH _{Root} vs TPC _{Root}	$r = 1.000$
DPPH _{Leaf} vs. TPC _{Leaf}	$r = 0.573$

Data are expressed as means \pm SD (n = 3)

correlation between the phenolic content and antioxidant activity is well-documented by Wang *et al.* (2018). Wang found a high correlation between the total phenolic content and antioxidant activity in Curculigo genus. The methanol extract of *C. orchioides* rhizomes was found to be extremely effective in scavenging superoxide radicals and was moderately effective in scavenging DPPH radicals, nitric oxide radicals and the inhibition of lipid peroxidation. The antioxidant activities of *C. orchioides* were further confirmed by ABTS, DPPH and FRAP assays (Hejazi *et al.*, 2018). It has been reported that phenolic compounds are a major contributor to the antioxidant activity of *C. orchioides*. The study done by Wang *et al.* (2014) showed that curculigoside prevented H₂O₂-induced damage of human umbilical vein endothelial cells and reduced cell apoptosis. It also decreased the activity of caspase-3 and p53 mRNA expression which is known to play a key role in H₂O₂-induced cell apoptosis (Wang *et al.*, 2014). However, other secondary metabolites such as vitamins, terpinoides and caratinoides might be also be the responsible compounds for the higher scavenging activity in *C. latifolia* fruits and leaves. Thus, the high capability of *C. latifolia* extract due to its ability to scavenge free radicals have provided preliminary indication on its effects in preventing and treating diseases caused by free radical such as diabetes.

Insulin secreting activity of *C. latifolia* extract in BRIN

BD11 cell line: Insulin is a hormone that is synthesized and secreted by pancreatic β -cells (Table 4 and 5). It is being secreted in response to the increase in glucose concentration in body system, especially, after taking a meal which then triggers insulin signaling in insulin-responsive tissues to facilitate glucose disposal (Ferdaoussi and MacDonald, 2017; Nicholls, 2016). However, in T2DM, insulin secretion is defective and is insufficient. Besides, insulin-responsive tissues such as muscle, adipose and liver are being impaired. The present study was conducted to determine the ability of *C. latifolia* extracts to stimulate insulin secretion. Results show that cells treated with *C. latifolia* fruit extract have the highest stimulation compared to others in the following descending order fruit>root>leaf. At 0.1 mg/mL, *C. latifolia* fruit extract increased insulin secretion significantly by 3.6 folds compared to control. Meanwhile *C. latifolia* root extract significantly increased basal insulin secretion at 0.05 mg/mL (2.1 fold). These findings suggest that *C. latifolia* fruit and root extracts may contain insulin-secreting compound which could trigger insulin being secreted in pancreatic β -cells. Kalwat and Cobb (2017) found that phenolic plays a role as insulin secreting compound due to its ability to trigger insulin signaling pathway through PI3-kinase. The finding of this study is similar to the finding made by Kalwat and

Table 4: The effect of *C. latifolia* extract on BRIN-BD11 cells in insulin secretion after 1h of treatment

Extract/drug concentration (mg/mL)	Fold of insulin secretion against control			
	Fruit	Root	Leaf	Glibenclamide
0.01	1.8	1.5	0.4	2.6*
0.025	2.2*	1.7	0.7	3.0*
0.05	2.8*	2.1*	0.6	3.5**
0.1	3.6**	1.9*	0.5	5.0***

Data were derived from the triplicates of three experiments and expressed as means \pm SD; *p<0.05, **p<0.01, ***p<0.001 compared with control (KRH)

Table 5: Effect of *C. latifolia* extracts on 2 DOG uptake activity in the presence or absence of insulin in differentiated 3T3 adipocytes

Extract/drug concentration (mg/mL)	Fold of 2-deoxy-glucose uptake against control with insulin and without insulin presence							
	Fruit		Root		Leaf		Rosiglitazon	
	Without insulin presence	With insulin presence	Without insulin presence	With insulin presence	Without insulin presence	With insulin presence	Without insulin presence	With insulin presence
0.01	3.3**	9.6***	1.1	0.8	1.3	1.2	1.6	1.8
0.025	1.6	11.6***	1.5	1.2	1.9*	1.5	1.9	2.2
0.05	1.2	13.0***	2.0*	1.3*	2.1*	1.8	2.2*	2.5
0.1	1.2	2.0	1.6	1.4*	2.5*	2.0*	2.0	3.0*

Data are means \pm SD of three replicate experiments. Significant difference between untreated cells and treated cells was tested within groups based on insulin presence or absence; *p<0.05, **p<0.01, ***p<0.001 compared with control (KRH)

Table 6: Effect of *C. latifolia* extract on 2 DOG uptake activity in the presence or absence of insulin in L6 myotubes

Extract/drug concentration (mg/mL)	Fold of 2-deoxy-glucose uptake against control with insulin and without insulin presence							
	Fruit		Root		Leaf		Rosiglitazon	
	Without insulin presence	With insulin presence	Without insulin presence	With insulin presence	Without insulin presence	With insulin presence	Without insulin presence	With insulin presence
0.01	3.3**	3.5**	1.0	0.8	0.6	0.7	2.0*	2.7*
0.025	2.8**	3.0**	1.4	1.4	0.7	0.9	2.4*	3.5*
0.05	14.0***	14.5***	1.9*	1.5	0.7	1.3	3.0*	4.4*
0.1	14.6***	15.6***	2.0*	1.8*	0.8	1.6*	2.7**	3.8*

Data are means \pm SD of three replicate experiments. Significant difference between untreated cells and treated cells was tested within groups based on insulin presence or absence; *p<0.05, **p<0.01, ***p<0.001 compared with control (KRH)

Cobb (2017) where *C. latifolia* extracts phenolic compounds were abundantly present. However, in normal water extract of *C. latifolia* leaf, insulin secreted was below than the basal insulin secretion. BRIN BD11 cells treated with glibenclamide had significantly stimulated insulin secretion at all concentrations with magnitudes of secretion at 2.6, 3.0, 3.5 and 5 folds, respectively. Thus, our *in vitro* insulin secretory activity of *C. latifolia* is in good agreement with our *in vivo* results as the blood glucose levels were significantly decreased and improved glucose tolerance as was found in *C. latifolia*-treated diabetic rats.

Glucose uptake activity of *C. latifolia* extract in 3T3-L1 adipocyte and on L6 myotube cell line: Type 2 diabetes mellitus is usually accompanied by a defect in glucose uptake activity in adipose and muscle tissues. In the present study, the ability of *C. latifolia* extracts to enhance glucose uptake activity in 3T3-L1 adipocytes and L6 myotubes was investigated (Table 6). Glucose uptake activity was significantly enhanced by *C. latifolia* fruit extracts by 13 fold at 0.05 mg/mL with insulin presence in 3T3-L1 adipocyte cells. This indicates that *C. latifolia*

fruit extracts have synergistic effects with the presence of insulin and it stimulates GLUT4 translocation through phosphatidylinositol 3-kinase (PI3K) pathway (Chen *et al.*, 2018). However, in L6 myotube cells, *C. latifolia* fruit extracts increased the basal and insulin-mediated glucose uptake activity. This indicates that *C. latifolia* fruit extracts possess both sensitize and insulin mimicking actions. Meanwhile *C. latifolia* root and leaf extracts enhanced basal glucose uptake activity in both 3T3-L1 adipocyte and L6 myotube cells. It shows that *C. latifolia* root and leaf extracts mimic insulin action as they activate a part of insulin signaling cascade and stimulate glucose uptake activity. However, the activity decreased with the increase in extract concentration. These findings suggest that glucose uptake activity was not influenced by a high concentration of *C. latifolia* extracts. In this study, rosiglitazone was used as a positive control and it has been shown to enhance glucose uptake in 3T3-L1 adipocytes and L6 myotubes. However, *C. latifolia* fruit and root extracts enhanced glucose uptake activity higher than rosiglitazone. This finding suggests that *C. latifolia* fruit and root extracts have the potential to be developed as an alternative antidiabetic agent.

CONCLUSION

In this study, it can be concluded that the high scavenging activity of *C. latifolia* is due to its phenolic compound. Besides, the different parts of *C. latifolia* plant extracts have different mode of antidiabetic actions and *C. latifolia* fruit and root extracts have shown significant results in all tests done. This finding suggests that *C. latifolia* could be used as a potential functional food ingredient for the prevention of type 2 diabetes. This is a preliminary study to prove the hypoglycemic effects of *C. latifolia* in in vitro study. Further, research is needed to elucidate the detailed mechanism of active compounds on glucose uptake activity.

ACKNOWLEDGEMENT

The researchers acknowledge the financial support of the Malaysian Ministry of Higher Education for Fundamental Research Grant Scheme; FRGS (Project No. 04-01-16- 1861FR).

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