

Isolation and Characterization of Two New Polyphenols from the Anti-diabetic Fraction of Pod Extract of *Caesalpinia pulcherrima* Swartz

ABSTRACT

This study was planned and executed to validate the anti-diabetic ethno-medicinal claim of *Caesalpinia pulcherrima* pods and the isolation of potential chemical compounds responsible for the activity.

The anti-diabetic activity of the extract was assayed using oral glucose tolerance test and streptozotocin induced hyperglycaemic rats. The partition fractions of the extract were evaluated for their anti-hyperglycaemic activity using oral glucose tolerance test. The most active fraction was subjected to chromatographic separations that led to the isolation and characterisation of two new polyphenolic compounds. The structures of these compounds were elucidated and characterized using IR, 1D- and 2D-NMR and MS techniques

The extract gave comparable ($p > 0.05$) activity to glibenclamide (5 mg/kg) at 100, 200 and 400 mg/kg at 4 h in oral glucose tolerance test and streptozotocin induced diabetic model on day 21. The most active ethyl acetate fraction (200 mg/kg) elicited comparable activity to the positive control at 0.5-4 h with blood glucose reduction of 52.9 % as compared with glibenclamide (5 mg/kg) of 38.9 % at 4 h. The isolate compounds were identified to be 5-(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol and 3-(4-methanetriol-2,6-dihydroxyphenyl)-3',4',5',5,7-pentahydroxyflavanonol.

The significant anti-diabetic property shown by the pods of *C. pulcherrima* justified its anti-diabetic ethno-medicinal use and the two new polyphenolic compounds isolated from its most active fraction could have contributed to the observed activity.

Keywords: *Caesalpinia pulcherrima*, anti-diabetes, hyperglycaemia, chromatography, polyphenols

1. INTRODUCTION

Diabetes mellitus is a chronic endocrine and metabolic disorder with deleterious effects on every organ system in the body. It is underlined predominantly by either insulin insensitivity or insulin deficiency or both which results in impaired metabolism of carbohydrates [1,2]. About 451 million people are diabetic globally and this figure is presumed to increase to 693 million by 2045, with low or middle income countries having the largest percentage of this diabetic population [3]. Although a wide array of options are now available for treating diabetes, including several new pharmacological classes of drugs many patients fail to achieve adequate glycemic control due to several factors among which is poor medication adherence [4]. Poor medication adherence may be due to; the complexity and inconveniences of treatment, cost of treatment, medication beliefs and side effects such as hypoglycemia, heart failure, liver disease, dropsy, and weight gain [4,5,6]. Therefore there is need for novel medications with little or no side effects.

Caesalpinia pulcherrima Swartz (Fabaceae) commonly called Peacock flower or Pride of Barbados is an ornamental plant that is widely grown in tropical gardens [7]. Various parts of

the plant are used ethno-medicinally in the treatment of liver disorder, inflammation, bronchitis, ulcer, asthma, tumors, diabetes, malaria and fungal infections [8,9,10,11]. Studies done on the plant have reported its antiulcer [12], anticancer [13], antimicrobial [14] anti-inflammatory and antinociceptive [15], anthelmintic [16], anti-diabetic [17] and antioxidant activities [18]. Isolated compounds from *C. pulcherrima* include; terpenoids, bonducellin and isobonducellin, flavanones, chalcone, benzoquinone, isoliquiritigenin, homoisoflavonoids, pulcherrimins [11,19,20,21,22,23,24]. In spite of the array of biological activities, its activity guided isolation has not been studied. This work was therefore aimed at investigating the anti-diabetic effect of the pods of *Caesalpinia pulcherrima* and the isolation of bioactive compounds from the most active fraction through activity guided isolation.

2. MATERIALS AND METHODS

2.1 Plant materials and extraction

The pods of *C. pulcherrima* were collected at Obafemi Awolowo University campus, Ile-Ife, Osun State, Nigeria. It was identified and authenticated by Mr. Ademorriyo at the herbarium of the Department of Botany, Obafemi Awolowo University, Ile-Ife. Its voucher specimen (IFE 17513) was prepared and deposited at the same herbarium. The pods were peeled to remove the seeds and air dried for 30 days. The air dried pods were ground to powder and 6 kg of the powdered pods was exhaustively extracted with methanol and concentrated *in vacuo* to obtain a methanolic extract with 9.6 % w/w yield.

The extract (160.0 g) was suspended in 500 mL of methanol/water (1:1) and successively extracted with *n*-hexane (9×500 mL), DCM (3×500 mL) and ethyl acetate (16×500 mL) to obtain their corresponding *n*-hexane (28.1 g), DCM (47.8 g), ethyl acetate (19.7 g) and aqueous methanol (58.6 g) fractions.

2.2 Animal study

Healthy albino rats (120–160 g) of both sexes bred under standard conditions (temperature 25 ± 3 °C) at the animal house, Department of Pharmacology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria were used for the experiment. They were fed on a standard pellet diet (Vital Feeds, Nigeria) and water was given *ad libitum*. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Academies Press [25].

2.2.1 Glucose lowering activity of the crude extract and the fractions

A glucose tolerance test was performed by giving glucose (10 g/kg, *p.o.*) to 24 h fasted rats. Those that were hyperglycaemic (blood glucose level ≥ 7 mmol/L (126 mg/dL) after 0.5 h (time point 0 h, *To*) were divided into groups of five and administered (*p.o.*) with 1 % tween 80 in normal saline (negative control), extract (100, 200 and 400 mg/kg) and glibenclamide (5 mg/kg) to determine their hyperglycaemic lowering activities. A drop of blood, taken from the tip of the tail of each rat, was dropped onto a glucometer strip and the blood glucose (bg) level read off directly. The blood glucose levels at 0.0 h (*To*) were normalised to 100 % while those at other times were percentages of these values. Their blood glucose (bg) levels were determined and recorded at 0 h, 0.5, 1, 2 and 4 h after administration of the normal saline/extract/drug [26].

2.2.2 Anti-diabetic activity of the crude extract using streptozotocin-induced diabetic rats

Rats used for this study were fasted overnight before the induction of diabetes with streptozotocin. The fasted rats were injected intraperitoneally with freshly prepared solution of

STZ (65 mg/kg) in 0.1 M sodium citrate buffer (pH 4.5). Hyperglycemia was confirmed in induced rats by the elevated fasting blood glucose levels determined at 72 h. Rats with fasting blood glucose levels ≥ 14 mmol/L after 72 h were considered diabetic and divided into three groups of 10 rats each for the study. They were administered with 1 % tween 80 in normal saline, extract (200 mg/kg) and glibenclamide (5 mg/kg) for 21 days. Their fasting blood glucose levels were determined on days 1, 4, 7, 10, 14 and 21 respectively [27].

2.3 Statistical analysis

Data obtained from this study were expressed as the mean \pm SEM for the number (N) of animals in the group. Analysis of variance (ANOVA) was first used followed by Bonferroni t-test to determine the source of significant differences for all determinations and $p < 0.05$ was considered to be statistically significant.

2.4 Isolation and purification of compounds

Ethyl acetate fraction (112 g) was adsorbed on 112 g of silica gel (60–200 mesh) and was left overnight to dry. The adsorbed fraction was subjected to column chromatography over silica gel (60–200 mesh) using gradient elution using *n*-hexane, ethyl acetate and methanol. About 30 mL of the eluent was collected into each test tube and 202 column fractions were collected. The fractions were combined into five sub fractions according to their TLC profile. Fractions collected at 100 % EtOAc to EtOAc : MeOH (8:2) was coded as CPEB and was further subjected to column chromatography which yielded a brown solid (1.19 g) at EtOAc:MeOH (8:2). The brown solid obtained was further subjected to column chromatography and eluted with EtOAc:MeOH (9.5:0.5, 9:1, 8.5:1.5, 8:2) to yield 16 sub-fractions. Compound **1** (10.2 mg) was afforded from sub-fraction 15 by purification with Sephadex LH-20 CC (CH_2Cl_2 :MeOH, 7:3). Subsequent elution of impure brown solid obtained with EtOAc:MeOH (9:1) followed by purification with Sephadex LH-20 (CH_2Cl_2 : MeOH, 7:3) yielded compound **2** (13.7 mg).

2.4.1 5[4-hydroxyphenyl]-3-hydroxy-2-methoxyphenol (compound 1)

Brown solid, IR (neat): 3253 cm^{-1} (phenolic OH), 1196 cm^{-1} (aromatic C-O-C). ^1H and ^{13}C NMR: Table 4. HRMS-ESI (positive mode): m/z $[\text{M-H}]^+$ calcd for $\text{C}_{13}\text{H}_{13}\text{O}_4$, 233.0814; found 233.1141.

2.4.2 3[4-methanetriol-2,6-dihydroxyphenyl]3',4',5',5,7-pentahydroxyflavonol (compound 2)

Brown solid, IR (neat): 3282 cm^{-1} (phenolic OH), 1678 cm^{-1} (C=O), 1604 and 1515 cm^{-1} (aromatic C=C), 1234 and 1165 cm^{-1} (C-O-C). ^1H and ^{13}C NMR: Table 5. HRMS-ESI (positive mode): m/z $[\text{M-OH}]^+$ calcd for $\text{C}_{22}\text{H}_{27}\text{O}_{12}$, 473.0720; found 473.0715.

3. RESULTS AND DISCUSSION

3.1 Glucose lowering activity of the extract

Table 1: Dose related glucose lowering effect of the pods of *C. pulcherrima* extract

Dose of extract/drug (mg/kg)	Blood glucose level as percentage of To (reduction in blood glucose relative to negative control at Tt)				
	0.0 h	0.5 h	1 h	2 h	4 h
GLU	100	83.79±3.81 ^a	85.89±0.50 ^b	76.45±1.71 ^b	74.18±1.97 ^b
(10 g/kg)					
CPA (100)	100	92.32±3.60 ^a (-10.17 %)	85.26 ± 3.51 ^{a,b} (0.73 %)	69.51 ± 4.64 ^{a,b} (-1.28%)	55.18±5.00 ^a (25.61 %)
CPA (200)	100	93.69±2.92 ^a (-11.81 %)	81.91±6.85 ^a (4.56 %)	75.46±6.37 ^{a,b} (0.99 %)	45.31±3.00 ^a (38.92 %)
CPA (400)	100	86.24±3.38 ^a (-2.92 %)	84.27±3.80 ^a (1.89 %)	76.30±3.02 ^{a,b} (0.22 %)	43.05±5.56 ^a (41.97 %)
GLI (5)	100	75.64±6.73 ^a (9.73%)	70.68±6.86 ^{a,b} (17.71%)	58.32±6.44 ^a (23.72%)	45.27±6.88 ^a (38.97%)

Data show the mean ± SEM blood glucose levels at the different time points expressed as percentages of levels at 0h (To), n=5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (p < 0.05). GLU (negative control); CPA: Extract of *C. pulcherrima* pods; GLI: Glibenclamide (positive control).

Glibenclamide, a sulphonylurea compound, has been reported to work through major insulin stimulation [28]. It was reported that the glucose-loaded rat model in hyperglycaemia lowering study of plant materials and the use of glibenclamide or other insulin stimulatory drugs as reference could be extrapolated to be type-2 diabetes [29]. Therefore activity profile similar to that of glibenclamide by plant extracts/fractions in glucose-loaded rat model allowed a guess of insulin stimulating mechanisms of action.

In the glucose-loaded rat model (Table 1), there was a time-dependent reduction in blood glucose in the negative control group that lasted up to the fourth hour. This confirmed homeostatic regulatory mechanism of the animals and indicated a healthy state of their pancreas [30]. Glibenclamide also gave a time dependent blood glucose lowering effect from 0.5 – 4 h which established its insulin secretion mechanism of action. The extract at 100, 200 and 400 mg/kg was devoid of activity at 0.5-2 h but at 4 h it showed hyperglycaemia lowering effect comparable to glibenclamide (5 mg/kg) thus indicating only insulin stimulation as its mechanism of action (Table 1). Similar effects have been reported for the extracts of *Parquetina nigrescens* and *Uvaria afzelii* [26,31].

3.2 Anti-diabetic activity of the extract on streptozotocin-induced diabetic rats

Since there was no significant difference between the activity of the extract at 200 and 400 mg/kg at 4 h in glucose-loaded rats (Table 1), 200 mg/kg was therefore used to evaluate its anti-diabetic effect in streptozotocin induced rats. Rats in the negative control group showed consistent hyperglycaemia throughout the duration (21 days) of the experiment which showed that the induced hyperglycaemia was permanent (Table 2). The extract demonstrated a time dependent anti-diabetic activity that was significantly less active than glibenclamide on days 1-14 but gave comparable (p<0.05) effect on day 21 (Fig. 2). This result further confirmed insulin release as the major mechanism of action of the extract, as was earlier suggested from its anti-hyperglycaemic action with the glucose-loaded rat model (Table 1). Furthermore, the results were in agreement with the reported anti-diabetic activity of the pod extract on alloxan-induced diabetic rats [17].

Table 2: Anti-diabetic activity of the extract of *C. pulcherrima* pods in streptozotocin induced-diabetic rats.

Dose of extract/drug (mg/kg)	Blood glucose level as percentage of T ₀ (reduction in blood glucose relative to negative control at T _t)					
	Day 1	Day 4	Day 7	Day 10	Day 14	Day 21
Normal saline	100.00	92.53 ± 5.71 ^c	97.22 ± 4.41 ^c	93.52 ± 3.42 ^c	94.65 ± 5.37 ^c	104.21 ± 4.46 ^c
CPA (200)	100.00	43.38 ± 3.98 ^b (53.11 %)	33.20 ± 4.74 ^b (65.85 %)	29.05 ± 4.08 ^b (68.94 %)	25.82 ± 0.82 ^b (72.72 %)	22.60 ± 1.62 ^a (78.31 %)
GLI (5)	100.00	17.27 ± 2.74 ^a (81.34 %)	15.52 ± 2.23 ^a (84.03 %)	15.11 ± 1.62 ^a (83.84 %)	15.21 ± 1.04 ^a (83.93 %)	17.00 ± 1.91 ^a (83.68 %)

Data show the mean ± SEM blood glucose levels at the different time interval expressed as percentages of levels at 0 h (T₀), n=5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (p<0.05). GLI: glibenclamide, CPA: Extract of *C. pulcherrima* pods.

Table 3: Hyperglycaemia lowering effect of the partition fractions of the crude extract of the pod of *C. pulcherima*

Dose of Fraction/drug (mg/kg)	Blood glucose levels as percentages of T ₀ (% percentage reduction in blood glucose relative to negative control at T _i)				
	0 h	0.5 h	1 h	2 h	4 h
GLU (10g/kg)	100.00	83.79 ± 3.81 ^a	85.89 ± 0.50 ^b	76.45 ± 1.71 ^c	74.18 ± 1.97 ^c
<i>n</i> -hexane (200)	100.00	82.11 ± 4.09 ^a (2.00 %)	79.32 ± 3.98 ^b (7.65 %)	73.71 ± 4.01 ^{b,c} (3.58 %)	62.50 ± 4.58 ^b (15.75 %)
DCM (200)	100.00	80.13 ± 5.12 ^a (4.37 %)	78.26 ± 5.12 ^b (8.88 %)	62.63 ± 3.46 ^b (18.08 %)	46.57 ± 6.71 ^{a,b} (37.22 %)
EtOAc (200)	100.00	77.20 ± 5.53 ^a (7.87 %)	58.22 ± 3.01 ^a (32.21 %)	49.28 ± 2.07 ^a (35.54 %)	34.92 ± 2.02 ^a (52.92 %)
Aqueous MeOH (200)	100.00	78.82 ± 4.82 ^a (5.93 %)	76.93 ± 4.62 ^b (10.43 %)	66.33 ± 5.37 ^{b,c} (13.24 %)	58.30 ± 2.87 ^b (21.41 %)
GLI (5)	100.00	75.64 ± 6.73 ^a (9.72 %)	70.68 ± 6.86 ^{a,b} (17.71 %)	58.32 ± 6.44 ^a (23.71 %)	45.27 ± 6.88 ^a (38.97 %)

Data show the mean ± SEM blood glucose levels at the different time points expressed as percentages of levels at 0 h (T₀), n=5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (P<0.05). GLU (negative control); CPA: Extract of *C. pulcherrima* pods; GLI: Glibenclamide (positive control).

The result of the hyperglycaemia lowering activity of the partition fractions of the extract showed that solvent partitioning of the crude extract increased the activity in which ethyl acetate fraction showed 52.2 % blood glucose reduction at 4 h compared to glibenclamide of 38.97 % suggesting the constituents responsible for the activity were concentrated in this fraction (Table 3).

3.3 Structure elucidation of isolated compounds

3.3.1 Compound 1

The HRESIMS (positive mode) of the compound showed a peak of (M+H) at m/z 233.1141 which is consistent with a molecular formula C₁₃H₁₃O₄. The ¹H NMR of the compound showed resonances for aromatic protons at δ 6.84 (d, J = 8.7), δ 7.06(s) and δ 7.90 (d, J = 8.7) each integrated for two protons with signals at δ 6.84 and δ 7.90 suggesting protons of AA'BB' system of an aromatic ring. And signal at δ 7.06 suggesting protons of AA' system of an aromatic ring. The only non-aromatic signal at δ 3.84 (s) integrated for three protons suggested methoxy protons. The ¹³C NMR showed very distinct signals for nine carbon atoms at δ 50.8, 108.7, 115.4, 120.1, 131.6, 138.4, 145.1, 161.8 and 167.6. The HSQC experiment showed cross peaks between carbon atoms at 50.8, 108.7, 115.4 and 131.6 ppm with protons at 3.84, 7.06, 6.84 and 7.90 ppm respectively. Thus the compound was identified as 5[4-hydroxyphenyl]-3-hydroxy-2-methoxyphenol (Fig. 1).

3.3.2 Compound 2

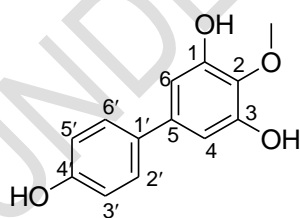
The HRESIMS (positive mode) of the compound showed a pseudo-molecular ion peak at m/z 473.0715 of the molecular ion peak calculated to be 490.0797 which is consistent with a molecular formula $C_{22}H_{18}O_{13}$. The pseudo-molecular ion observed with pseudo-molecular ion formula of $C_{22}H_{17}O_{12}$ at $m/z = 473.0715$ was as a result of loss of water molecule from the protonated molecular ion $[M+H]^+$ as shown in Fig. 2. The 1H NMR of the compound showed resonances for aromatic protons at δ 5.98 (s), 6.54 (s) and 7.07 each integrated for two protons. Signals at δ 5.34 (d, $J = 11.5$ Hz) and δ 5.88 (d, $J = 11.5$) each integrated to be one proton suggesting protons on oxygenated methine groups. The ^{13}C NMR showed very distinct signals for eighteen carbon atoms at δ 192.0, 167.7, 165.4, 164.1, 162.8, 145.6, 145.0, 138.8, 133.8, 126.6, 119.1, 109.1, 106.4, 100.8, 96.3, 95.2, 81.5 and 72.8. The HSQC experiment showed peaks correlations between carbon atoms at 72.8, 81.5, 95.2, 96.3, 106.4 and 109.1 ppm with protons at 5.88, 5.34, 5.98, 5.98, 6.54 and 7.07 ppm respectively suggesting protons on positions 6 and 8 of ring A of a typical flavonoid for cross peaks between carbon atoms at 95.2, 96.3 ppm and protons at 5.98, 5.98 ppm respectively.. The cross peaks between carbon atoms at 106.4, 109.1 and protons at 6.54, 7.07 ppm suggested two distinct AA' systems. The aforementioned analysis together with HMBC analysis, Table 3, made the compound to be identified as 3[4-methanetriol-2,6-dihydroxyphenyl]3',4',5',5,7-pentahydroxyflavonol (Fig. 1).

Table 4: NMR data of compound 1 in MeOD (multiplicities and J values are given in Hz in parenthesis).

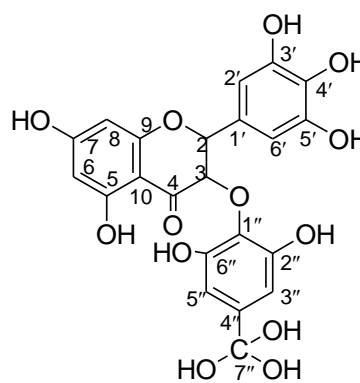
Position	Proton (multiplicity)	Carbon-13	Hmbc (1H to ^{13}C)
1	-	145.1	
2	-	167.6	
3	-	145.1	
4 & 6	7.06 (s)	108.7	108.7, 120.1, 138.4, 145.1, 167.6
5	-	138.4	
1'	-	120.1	
2' & 6'	7.90 (d, $J=8.7$)	131.6	131.6, 161.8
3' & 5'	6.84 (d, $J=8.7$)	115.4	115.4, 120.1, 161.8
4'	-	161.8	
2-OCH ₃	3.84 (s)	50.8	167.6

Table 5: NMR data of compound 2 in MeOD (multiplicities and J values are given in Hz in parenthesis).

Position	Proton (multiplicity)	Carbon-13	Hmbc (^1H to ^{13}C)
1	-	-	
2	5.34 (d, J=11.5 Hz)	81.5	72.8, 106.4, 126.6, 192.0
3	5.88 (d, J=11 Hz)	72.8	81.5, 126.6, 165.4, 192.0
4	-	192.0	-
5	-	164.1	-
6	5.98 (s)	95.2	96.3, 100.8, 164.1, 167.7
7	-	167.7	-
8	5.98 (s)	96.3	95.2, 100.8, 167.7
9	-	165.4	-
10	-	100.8	-
1'	-	126.6	-
2' & 6'	6.54 (s)	106.4	81.5, 106.4, 126.6, 133.8, 145.6
3' & 5'	-	145.6	-
4'	-	133.8	-
1''	-	138.8	-
2'' & 6''	-	145.0	-
3'' & 5''	7.07 (s)	109.1	109.1, 119.1, 138.8, 145.0, 162.8
4''	-	162.8	-
7''	-	119.1	-



Compound 1



Compound 2

Fig. 1: The structures of compounds 1 and 2

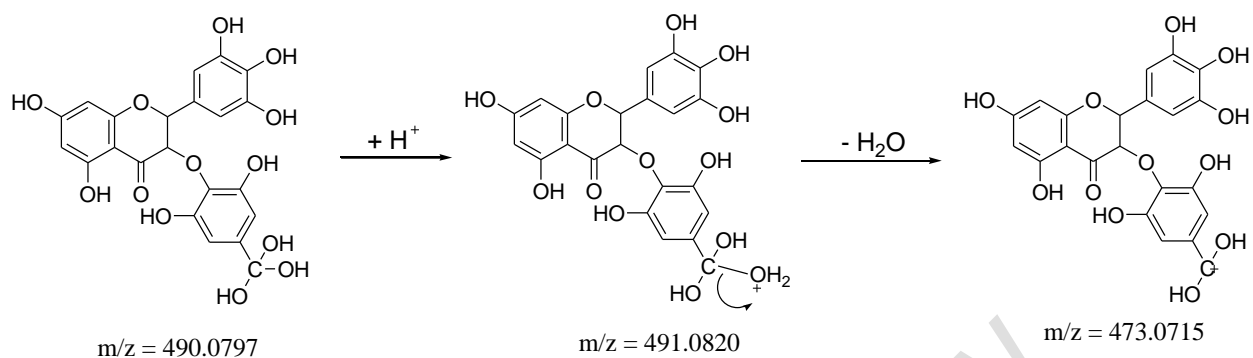


Fig. 2: The proposed fragmentation of $[M+H]^+$ of compound 2 to form the pseudo-molecular ion.

4. CONCLUSION

Purification of the extract led to increase in the activity of the fractions in which two new polyphenols were isolated from the most active fraction. These compounds are expected to contribute to the spectrum of potential compounds that could be drugs and/or lead drugs in the treatment of diabetes.

CONSENT (WHERE EVER APPLICABLE)

Not applicable.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

All animal experiments conformed to the guide for the care and use of Laboratory Animals published by the National Academies Press [25].

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