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Rajinder Kaur Gill
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar-143005, Punjab, India.

Saroj Arora
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar-143005, Punjab, India.

A.K. Thukral
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar-143005, Punjab, India.

Correspondence:
Rajinder Kaur Gill
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar-143005, Punjab, India.

Evaluation of protective effects (antioxidant and antimutagenic) of methanol extract of seeds of *Chlorophytum borivilianum* Sant. et Fernand

Rajinder Kaur Gill, Saroj Arora and A.K. Thukral

Abstract
Chlorophytum borivilianum Sant. et Fernand. is known in the world market as 'White Gold' and tubers of this plant are used as an aphrodisiac in Ayurvedic medicines. In the present investigation, experiments were designed to evaluate the antioxidant and antimutagenic potential of methanol extract of seeds of *C. borivilianum* through various standard *in vitro* assays. Antioxidant activities of the seed methanol extract were determined through DPPH free radical scavenging assay, lipid peroxidation, deoxyribose degradation (site-specific and non-site specific), reducing power, and chelating power assays. The antimutagenic activity of the seed methanol extract was evaluated by employing plate incorporation assay. The extract showed significant free radical scavenging activity in 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay, while low to moderate inhibitory potential in all the other antioxidant assays. The methanol extract showed significant antimutagenic effect in Ames assay in both co-incubation and pre-incubation modes in the presence as well as absence of S9. The results of the present study indicate that seed of *C. borivilianum* can be used as a good source of antioxidant and antimutagenic compounds.

Keywords: Free radicals, antioxidants, Ames assay, carcinogen, mutagen.

1. Introduction
Increasing environmental pollution and daily stresses of life is deteriorating human health. It may be because of excessive generation of free radicals in the body, leading to oxidative stress that can damage the vital cell organelles and bio molecules of the cells. Increased cell oxidation contributes to various diseases and antioxidants delay or prevent the oxidation of cellular oxidizable substrates [1]. Normally free radicals produced in the body can be removed by body's own natural antioxidant defence system [2]. Human beings are exploring plants and their different parts for therapeutic values for centuries because plants contain different natural compounds *viz.*, polyphenols, alkaloids, flavonoids, saponins and other secondary metabolites that have protective effects and can alleviate the oxidative stress in human beings responsible for various disorders like cardiovascular diseases, cancer and neurodegenerative diseases etc. Recently, plant based therapeutic compounds have received great attention as a source of biological active substances for the cure of oxidative stress related diseases. No doubt human body contains several endogenous antioxidant systems that normally restrict the reactivity of free radicals, but additional antioxidant supplements are also required to restrict the activities of free radicals. There are many plants, vegetables and fruits that are rich in antioxidants such as vitamin A, vitamin C, vitamin E etc that prevent free radical damage and reduce the risk of chronic diseases especially cardiovascular diseases. Similarly seeds of many plants like grapes, sesame, tomato etc. are known to have free radical scavenging properties. Therefore, there is a considerable interest in finding new and safe antioxidants from plants to replace the synthetic oxidants [3]. The antioxidant and antimutagenic potential of the tuber peels of this species have been explored in our previous studies [4, 5]. Therefore, in the present study the antioxidant and antimutagenic potential of methanol extract of seeds of *Chlorophytum borivilianum* was investigated.

2. Materials and Methods
2.1 Plant material and preparation of seed methanol extract
2.1.1 Procurement of study material: Tubers of *Chlorophytum borivilianum* were purchased from Maa Umiya Safed Musli Farm, Indore, India. Botanical Identification was made by Dr. Amit Chawla, IHBT, Palampur by referring to Herbarium of Institute of Himalayan Bioresource Technology, Palampur.

A voucher specimen (Accession No. 6772/dated April 4, 2012) was submitted in the Herbarium of Department of Botanical and Environmental Sciences, G.N.D.U., Amritsar. Plants of *C. borivilianum* were propagated from the tubers in the experimental plot of the Botanical Garden of G.N.D.U., Amritsar and mature fruits were collected and seeds were removed from the dried fruits.

2.1.2 Preparation of seed methanol extract: Dried seeds of *Chlorophytum borivilianum* were extracted thrice with methanol at room temperature to obtain the seed methanol extract. The extract was evaporated under reduced pressure and the residue was lyophilized and used for the assessment of antioxidant and antimutagenic activities.

2.2 Estimation of total phenolics content

The total phenolic content of the seed methanol extract was estimated using the Folin - Ciocalteu method [6]. Total phenolic content was expressed as mg of gallic acid/g of extract.

2.3 Antioxidant activity assays

The antioxidant activity of seed methanol extract was determined by using standard *in vitro* assays like DPPH free radical scavenging, lipid peroxidation, deoxyribose degradation, reducing power and chelating power. The extract was checked by taking 50-1000 µg/ml concentrations. Gallic acid was used as a positive control in all the assays except chelating power assay where ethylene diamine tetra acetic acid (EDTA) was used as a standard.

2.3.1 DPPH free radical scavenging assay

The radical scavenging activity of seed methanol extract was evaluated as per method described by Blois [7]. The radical scavenging activity was determined by using the following formula.

% DPPH radical scavenging = (1- Absorbance of sample / Absorbance of control) ×100

The samples were measured against methanol as blank.

2.3.2 Lipid peroxidation assay

In this experiment, lipid peroxidation was induced by ascorbate-Fe^{2+} in rat liver homogenate and the inhibitory effect of extracts was measured according to the method given by Halliwell and Gutteridge [8].

2.3.4 Deoxyribose degradation assay

The method of Halliwell *et al.* [9] and Aruoma *et al*. [10] was used to determine the hydroxyl radicals scavenging effect of the extract. Hydroxyl radical is the most reactive radical among reactive oxygen species and induces severe damage to biomolecules of the cells. In this method, when deoxyribose sugar is exposed to free radicals generated through the Fenton reaction system, degrades into fragments and generate pink chromogen on heating with TBA at low pH. This experiment was conducted in two modes i.e. site-specific and non-site specific.

2.3.5 Reducing power assay

The reducing ability of seed methanol extract was determined by the method described by Oyaizu [11]. The reductone present in the extract donate a hydrogen atom and break the free radical chain reactions. In this method, reductons present in the plant extract cause reduction of Fe^{3+}/ ferricyanide complex to ferrous form and concentration of Fe^{2+} is measured spectrophotometrically.

2.3.6 Chelating power assay

The chelating ability of ferrous ions by seed methanol extract of *C. borivilianum* was checked as per method given by Dinis *et al*. [12]. This assay measures the ability of antioxidants (present in plant extract) to compete with ferrozine in chelation of ferrous ion. Ferrozine quantitatively form complex with Fe^{2+} and the presence of chelating agents in the extract decrease the formation of ferrozine-Fe^{2+} complex by chelating Fe^{2+}. Therefore, measurement of decrease in colour helps in estimating the chelating capacity of the plant extract. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated as follows:

$$\% \text{ inhibition} = [(\text{absorbance of control})-(\text{absorbance of sample})/(\text{absorbance of control})] \times 100$$

EDTA was used as a positive control.

2.4 Antimutagenic activity assay

Seed methanol extract of *C. borivilianum* was evaluated for antimutagenic activity by employing method of Maron and Ames [13]. This assay is used all over the world for the preliminary screening of plants for their antimutagenic potential. In the present study, two strains of *Salmonella typhimurium* i.e. TA98 and TA100 were used. The strains of *Salmonella typhimurium* TA98 and TA100 were procured from the Institute of Microbial Technology (CSIR), Chandigarh, India. In all the experiments, spontaneous reversion was checked. Toxicity of extract (taken as negative control)/standard was also checked. The experiments with metabolic activation (S9 mix) were carried out with 2-AF in both the tester strains, while in experiments without metabolic activation NPD was used for TA98 and sodium azide was used for TA100. Gallic acid was used as standard. The experiments were conducted in two modes i.e. co-incubation and pre-incubation mode. All the experiments were conducted in triplicates and percent inhibition of mutagenic activity was calculated as follows:

The inhibitory activity of the extract was expressed as:

$$\text{Inhibitory activity } (\%) = [(a-b) / (a-c)]^* 100$$

Where, 'a' is the number of histidine revertants induced by mutagen alone (positive control), 'b' is the number of histidine revertants induced by mutagen in the presence of extract, and 'c' is the number of histidine revertants induced in the presence of extract alone and solvent (negative control).

2.5 Statistical analysis

The statistical analysis was carried for mean, standard deviation (SD), linear regression, one-way and two-way analysis of variance (ANOVA). The differences ($p \leq 0.001$, $p \leq 0.01$) among means were compared by honestly significant difference (HSD) using Tukey's test.

3. Results

3.1 Total phenolic content

Phenols are known for their antioxidant nature and efficiently scavenge free radicals. In the present study, the total phenolic content was found to be 122.33±1.18 (Mean±SD) mg/g GAE. The antioxidant and antimutagenic activities of the seed

methanol extract may be because of the presence of phenolic compounds.

3.2 Antioxidant activity of the extract

The results of antioxidant properties of seed methanol extract in terms of percent inhibition and IC_{50} values are given in Table 1. Statistical analysis of results is given in Table 2. The results are explained briefly as follows:

Table 1: Antioxidant activities of seed methanol extract of *C. borivilianum* in various *in vitro* antioxidant assays.

S.No.	Concentration (µg/ml)	DPPH Assay	Lipid Peroxidation Assay	Site-Specific Deoxyribose Assay	Non-Site Specific Deoxyribose Assay	Reducing Power Assay	Chelating Power Assay
		Inhibition (%) Mean ± SD					
1	50	42.83±0.10	20.55±0.10	26.25±0.11	20.20±0.27	3.86±0.04	3.41±0.53
2	250	49.50±0.20	21.46±0.05	28.00±0.07	30.52±0.48	11.28±0.06	13.57±0.51
3	450	56.67±0.80	29.71±0.07	30.91±0.03	39.95±0.24	20.18±0.07	18.10±0.46
4	650	67.20±0.23	32.88±0.06	37.03±0.08	50.57±0.22	21.03±0.01	23.76±0.15
5	850	68.42±0.31	39.89±4.24	27.87±0.12	54.74±0.94	24.29±0.06	25.62±0.31
6	1000	71.57±0.09	35.25±0.09	23.61±0.04	57.78±0.07	22.67±0.08	26.98±0.41
7	IC_{50} Value	255.05	1554.798	1455.73	635.34	2184.02	1859.89

Data shown is Mean±SD of experiments performed in triplicate.

Table 2: Statistical analysis of all the results of *in vitro* antioxidant assays.

Statistical Analysis	DPPH Assay	Lipid Peroxidation Assay	Site-Specific Deoxyribose Assay	Non-Site Specific Deoxyribose Assay	Reducing Power Assay	Chelating Power Assay
F-ratio (5,12)	2827.90^{***}	59.27^{***}	9471.36^{***}	3042.56^{***}	58121.74^{***}	1390.56^{***}
HSD	1.03	4.75	0.23	1.27	0.16	1.14
Regression Equation	y = 0.0314x + 42.346	y = 0.0198x + 19.215	y = 0.0176x + 24.379	y = 0.0404x + 20.41	y = 6.9623Ln(x) - 24.176	y = 7.9746Ln(x) - 28.839
R-value	$R=0.9800^{***}$	$R =0.9308^{***}$	$R =0.9621^{***}$	$R =0.9856^{***}$	$R =0.9733^{***}$	$R =0.9890^{***}$

Significant at $^{***}p\leq0.001$

a) DPPH radical scavenging assay

The extract showed significant DPPH radical scavenging activity at all the tested concentrations. The maximum percent inhibition was found to be 71.57% at 1000 µg/ml extract concentration as compared to gallic acid where 91.31% inhibition was found at the same concentration. This assay is based on the measurement of the reducing ability of the antioxidants toward DPPH radical. This test is commonly used in most of the antioxidant studies and gives reliable information regarding the antioxidant ability of the tested compounds in a short time span [2].

b) Lipid peroxidation assay

The seed methanol extract showed moderate inhibition of lipid peroxidation. The maximum percent inhibition was found to be 39.89% at 850 µg/ml extract concentration comparable to gallic acid where 87.63% inhibition was found at the same concentration. In the present study, the inhibitory effect of seed methanol extract of *C. borivilianum* on lipid peroxidation may be due to hydrogen donation or due to chelation of Fe^{3+} ions. These metal ions, otherwise interact with phospholipids of membrane and form peroxyl radical and initiate the chain reaction and damage the membrane of the cell.

c) Deoxyribose assay

In site-specific deoxyribose assay maximum percent inhibition was found to be 37.03% at 650 µg/ml extract concentration as compared to 54.35% inhibition with gallic acid found at the same concentration. The maximum percent inhibition in deoxyribose non-site specific assay was found to be 57.78% at 1000 µg/ml extract comparable to gallic acid where 80.89% inhibition was found at the same concentration. In deoxyribose assay, extract exhibited a stronger concentration-dependent inhibition of deoxyribose oxidation in non-site specific assay as compared to site-specific assay.

d) Reducing power assay

The reducing ability of the extract was found to be 22.67% at 1000 µg/ml concentration as compared to gallic acid that showed 99.90% inhibition at the same concentration.

e) Chelating power assay

The extract showed 26.98% inhibition at 1000 µg/ml concentration as compared to EDTA that showed 91.85% inhibition at the same concentration.

3.3 Antimutagenic activities

In antimutagenic assay, seed methanol extract of *C. borivilianum* exhibited 58.40% and 64.43% inhibitory activity at the maximum dose tested (2500 µg/0.1ml/plate) against NPD in TA98 strain, both in co-incubation and pre-incubation modes of treatment respectively (Table 3). The seed methanol extract exhibited 58.85% and 64.62% inhibitory activity in S9 dependent mutagen in TA98 strain in both co-incubation and pre-incubation modes of treatment respectively. Inhibitory activity in TA100 strain against sodium azide was found to be 54.23% and 59.50%, while in S9 dependent mutagen inhibitory activity was found to be higher in co-incubation (72.04%) than pre-incubation modes (59.50) respectively (Table 4). At the same concentration, gallic acid exhibited 74.56% and 76.45% inhibitory activity against NPD in TA98 strain, while it exhibited 95.27% and 95.81% inhibitory activity against 2-AF in both the modes respectively. It was found that in both TA98 and TA100 strains, seed methanol extract showed almost similar inhibitory activity against all the mutagen. All the results were found to be statistically significant in both one-way and two-way ANOVA.

Table 3: Antimutagenic activities of seed methanol extract without (-S9) and with (+S9) in TA98 tester strain of *S. typhimurium*.

Treatment	Concentration (µg/100 µl/plate)	TA98			
		Without S9 (-S9)		With S9 (+S9)	
		Revertants/plate	Percent inhibition	Revertants/plate	Percent inhibition
Spontaneous		26.00±2.00		27.33±2.31	
Positive control					
NPD	**20**	1053.00±13.45			
2-AF	**20**			2277.00±32.14	
Negative control	**100**	23.33±0.58		22.33±1.15	
	400	24.33±0.58		23.33±0.58	
	800	22.33±1.15		24.33±0.58	
	1000	26.00±1.00		22.67±0.58	
	1500	24.00±1.00		24.67±0.58	
	2000	24.67±2.89		25.33±2.08	
	2500	25.00±1.73		23.33±1.53	
Co-incubation	**100**	1843.00±12.17	12.30±1.06	926.33±10.97	18.15±0.13
	400	1695.00±13.53	21.68±1.13	830.00±11.79	23.86±0.87
	800	1636.00±7.55	25.06±0.98	794.67±10.41	27.26±0.59
	1000	1584.00±9.54	32.16±1.23	722.67±12.90	34.82±0.62
	1500	1256.00±7.55	38.84±0.38	653.33±4.16	46.28±0.42
	2000	1060.00±16.52	44.93±0.54	591.00±6.56	50.61±2.99
	2500	934.00±13.08	58.40±1.08	452.67±12.10	58.85±0.57
Pre-incubation	**100**	1720.67±13.58	14.08±0.52	908.00±5.29	28.03±0.42
	400	1586.00±9.64	27.41±1.95	771.00±19.97	29.03±0.12
	800	1545.33±13.20	32.21±1.44	721.00±14.73	40.69±0.32
	1000	1458.33±8.08	36.29±1.94	680.33±19.76	45.69±0.58
	1500	1182.00±14.53	42.05±0.69	620.33±7.51	46.93±0.79
	2000	945.33±9.87	57.51±2.76	461.67±26.76	55.17±0.35
	2500	830.00±16.37	64.43±1.19	390.67±13.32	64.62±0.33
One-way ANOVA					
Positive control and co-incubation		F(7,16)=948.62***; HSD=30.37		F(7,16)=670.78***; HSD=81.85	
Positive control and pre-incubation		F(7,16)=526.59***; HSD=46.54		F(7,16)=2728.98***; HSD=42.02	
Two-way ANOVA					
Co-incubation and pre-incubation					
Treatment		F(1,28)=192.05***		F(1,28)=603.52***	
Concentration		F(6,28)=870.83***		F(6,28)=1315.25***	
Treatment x Concentration		F(6,28)=9.99***		F(6,28)=32.10***	
		HSD=41.748		HSD=63.961	

Data shown is Mean±SD of experiments performed in triplicate. Significant at $^{***}p \leq 0.001$

Table 4: Antimutagenic activities of seed methanol extract without (-S9) and with (+S9) in TA100 tester strain of *S. typhimurium*

Treatment	Concentration (µg/100µl/plate)	TA100			
		Without S9 (-S9)		With S9 (+S9)	
		Revertants/plate	Percent inhibition	Revertants/plate	Percent inhibition
Spontaneous		249.33±11.02		247.33±10.02	
Positive control					
Sodium azide	**2.5**	2550.00±17.69			
2-AF	**20**			2702.00±10.58	
Negative control	**100**	245.33±5.69		239.00±9.85	
	400	241.00±10.15		241.67±3.06	
	800	231.00±6.00		251.33±7.09	
	1000	234.00±2.65		250.33±3.06	
	1500	253.00±6.24		243.67±11.37	
	2000	236.00±9.64		235.33±4.62	
	2500	253.00±9.85		247.67±7.77	
Co-incubation	**100**	1963.67±20.60	25.44±0.87	1843.00±12.17	34.88±0.37
	400	1938.00±7.94	26.51±0.45	1695.00±13.53	40.93±0.58
	800	1850.67±14.15	30.16±0.62	1636.00±7.55	43.50±0.27
	1000	1817.67±12.86	31.62±0.54	1584.00±9.54	45.60±0.43
	1500	1635.67±10.97	39.81±0.54	1256.00±7.55	58.82±0.38
	2000	1468.33±32.59	46.75±1.44	1060.00±16.52	66.57±0.69
	2500	1304.33±14.74	54.23±0.70	934.00±13.08	72.04±0.61
Pre-incubation	**100**	1847.00±17.78	30.50±0.78	1720.67±13.58	30.50±0.78
	400	1827.67±4.04	31.28±0.06	1586.00±9.64	31.28±0.06
	800	1636.00±11.53	39.41±0.58	1545.33±13.20	39.41±0.58

	1000	1570.67±40.07	42.29±1.78	1458.33±8.08	42.29±1.78
	1500	1528.33±4.04	44.48±0.30	1182.00±14.53	44.48±0.30
	2000	1332.00±15.13	52.64±0.87	945.33±9.87	52.64±0.87
	2500	1183.33±12.34	59.50±0.59	830.00±16.37	59.50±0.59
One-way ANOVA					
Positive control and co-incubation		F(7,16)=1325.26***; HSD=50.67		F(7,16)=6708.04***; HSD=33.05	
Positive control and pre-incubation		F(7,16)=1492.95***; HSD=52.75		F(7,16)=6719.42 ***; HSD=34.73	
Two-way ANOVA					
Co-incubation and pre-incubation					
Treatment		F(1,28)=705.00***		F(1,28)=793.06***	
Concentration		F(6,28)=1069.57***		F(6,28)=4793.36***	
Treatment x Concentration		F(6,28)=14.18***		F(6,28)=3.38*	
		HSD=54.953		HSD=36.416	

Data shown are Mean ± SD of experiments performed in triplicate. Significant at *** $p \leq 0.001$, * $p \leq 0.05$

4. Discussion

A lot of research work is going on throughout the world for exploring the plants for the presence of antioxidant and antimutagenic compounds because these compounds have protective effects against free radical mediated pathogenesis. *Chlorophytum borivilianum* is a wonder drug and is used to cure many ailments like diabetes, fever, bronchitis, gonorrhea, leucorrhea etc. It is anti-inflammatory so used to cure arthritis and rheumatism. Keeping all these in mind, the seeds of this plant were evaluated for antioxidant and antimutagenic effects. The high value of percent inhibition in DPPH radical scavenging assay, suggests that the extract is able to donate electrons and therefore can scavenge free radicals. The present observations are in agreement with studies of Maltas and Yildiz [3] who reported 85% inhibition of DPPH radical in the methanolic extract of leaves *Gingko biloba*. The presence of phenols in the extract support the antioxidative effect of seed methanol extract as phenols are known for their free radical scavenging and metal chelating activity [14]. Phenolic compounds present in the extract may act as electron donors thus assist in conversion of H_2O_2 to H_2O. As they are good electron donors so they also exhibit good reducing power as evidenced in reducing power assay. In the present study, the lipid peroxidation assay was used to determine the peroxyl radical scavenging capacity of the extract. Peroxyl radicals are involved in lipid peroxidation that destroy the cell membrane and cause tissue damage. Antioxidants of plant origin can effectively scavenge the peroxyl radicals and inhibit lipid peroxidation that is considered as the main cause of cancer, atherosclerosis and arthritis. Results of lipid peroxidation assay indicate that seed methanol extract has low to moderate inhibitory effect on lipid peroxidation. Thus, protective effect of methanol seed extract might be due to its electron or hydrogen donating ability. In deoxyribose degradation assay, the effect of $^{\cdot}OH$ radicals on the degradation of deoxyribose sugar was checked in the presence (non-site specific) and absence of EDTA (site-specific) [15]. In site-specific mode, Fe^{2+} ions directly attack on deoxyribose. In non-site specific assay, EDTA form complex with metal ion and damage occur only by the free $^{\cdot}OH$ radicals present in free form in solution. In this assay, the effect was found more in non-site specific mode indicating the hydroxyl radical scavenging ability of the extract instead of metal chelation. In reducing power assay, the effect of plant extracts may be attributed to the presence of reductones that terminated the radical induced chain reactions by donating electron [16] and a strong correlation also exist between antioxidant activity and total phenolic content that scavenge free radicals by donating electron [17]. Chelating power assay determines the amount of freely available Fe^{2+} in the solution. In the presence of chelating agent, Fe^{2+} ion could not form the complex with the ferrozine and stop the generation of hydroxyl radical production. These radicals initiate lipid peroxidation in cell membranes and cause oxidative stress in the living organisms [18]. In the present study, seed methanol extract of *C. borivilianum* showed less potential in chelation of metal ions. Therefore, it is suggested that antioxidant potential of extract may be due to electrons or hydrogen donation rather than metal chelation. It is pertinent to mention here that in addition to free radical scavenging activity, the seed methanol extract also showed significant antimutagenic potential in Ames assay in TA98 and TA100 strains of *S. typhimurium* and in presence as well as absence of metabolic system. A similar study was done on pistachio green hull extract that showed potent antioxidant and antimutagenic effects in DPPH and *Salmonella typhimurium* TA100 strain against 2-nitrofluorene respectively [19]. Extract of *Mimosa tenuiflora showed* significant decrease in mutagenicity index for tested mutagen (NQO) in tester TA97, TA98, TA100 and TA102 tester strains of *S. typhimurium*. The antimutagenic potential was attributed to the presence of saponins and tannins [20]. Extract of *Picrorhiza kurroa* showed antimutagenic effect in five tester strains of *S. typhimurium* (TA1537, TA1535, TA98, TA100 and TA102) in absence and presence of S9 [21].

In conclusions, to the best of our knowledge, this report provides the first experimental evidence of the antioxidant and antimutagenic potential of seeds of *C. borivilianum* that can have the potential to cure diseases induced by free radicals and mutagens. However, these results are bases on *in vitro* experiments and further research is needed to evaluate the effects of extract under *in vivo* conditions. In spite of this fact, the preliminary results of this work can help the researchers in future studies.

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