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Research Article

Phytochemical Investigation and In Vitro Antioxidant Activities of *Tetracarpidium conophorum* (African Walnut) Seeds

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Abstract

Background: *Tetracarpidium conophorum* (African walnut) is an African plant with ethnobotanical uses.
Objectives: The purpose of this study was to evaluate the phytochemical screening and in vitro antioxidant activities of methanol extract and fractions (F) [n-hexane (HEX-F), dichloromethane (DCM-F) and, ethyl acetate (EA-F)] of *T. conophorum* seeds.
Methods: Phytochemical screening and in vitro antioxidant activity study were carried out using DPPH, ABTs radical scavenging assays, nitric oxide inhibitory and reducing potential assays.
Results: Methanol extract and its fractions contain phenols, flavonoids, saponins, tannins, terpenoids, and alkaloids. The concentrations of total phenols and flavonoids content were significantly higher in EA-F and crude methanol extract compared to other fractions. Crude methanol and EA-F contain higher concentrations of tannin while hexane fraction had the lowest tannin content but relatively higher proanthocyanidin content compared to other fractions. The antioxidant activity study showed that both methanol crude extract and fractions of *T. conophorum* seeds have significant activities for DPPH radical scavenging, reducing power, ferric reducing antioxidant potential, nitric oxide inhibitory activities, ABTS and hydroxyl radical scavenging for. DPPH radical scavenging activities of EA-F showed the lowest IC_{50} of 33.11 µg/mL, followed by Hex-F, DCM-F and crude methanol extract with IC_{50} of 33.43, 42.09 and 45.44 µg/mL, respectively, when compared to ascorbic acid with IC_{50} of 17.08 µg/mL.
Conclusion: The study showed that *T. conophorum* seed is a rich source of secondary metabolites, which may be responsible for its antioxidant activities.
Keywords: Phytochemicals, Antioxidants, *Tetracarpidium conophorum*, African walnut

Background

Free radicals are highly reactive chemical species with unpaired electrons (1). They are important to normal metabolism of living cell but harmful unless tightly controlled (2). Free radical especially reactive oxygen species (ROS), such as superoxide anions ($\cdot O_2^-$), hydroxyl radical ($OH^\cdot$) and hydrogen peroxide (H_2O_2) are capable of causing deleterious effects in biological systems. However if the body fails to sequester these ROS, they interact with biomolecules such as DNA, lipids and proteins, thereby causing chronic diseases, such as cancer, arteriosclerosis, diabetes mellitus, autoimmune diseases and neurodegenerative disorders such as Parkinson's disease (3). However to prevent this toxic insult, antioxidants are generated to mop up these free radicals before they cause damage to cells. Antioxidants are essential for maintaining the health status of an individual (4) and are classified into two type; enzymatic and non-enzymatic. The enzymatic antioxidants include superoxide dismutase, catalase and peroxidases while the non-enzymatic antioxidants include ascorbic acid, α-tocopherol, carotenoids, glutathione and polyphenols (5). Various studies have shown that antioxidants from plant based (natural) products or present in diet play an essential role in the prevention of diseases and very recently, there has been an increased interest in the therapeutic potential of medicinal plants as antioxidants in the mitigation of oxidative damage to biological cells (6).

One of such plants is *Tetracarpidium conophora* seed from the family Euphorbiaceae (7). It is found in African countries like Nigeria, Cameroon, Sudan and Ivory Coast. *T. conophorum* is called ukpa by the Igbo and awusa or asala by the Yoruba. It is climber and is cultivated principally for the nut (8, 9). *T. conophorum* has been found to be useful in the treatment of cardiovascular diseases (10), male infertility (11) because of its rich phytochemicals (12) and hepatoprotective properties (13). Isolation and

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structural identification of phytochemicals such as steroidal terpenoids, flavonoids and phenols from *T. conophorum* seed have already been accomplished by our research group. This study aims to evaluate the phytochemical and *in vitro* antioxidant activities in the extract of *T. conophorum* seeds.

Materials and Methods

Plant Materials

The seeds of *T. conophorum* used in this study were obtained according to method described by Oriakhi et al (13).

Extract Preparation

The seeds were collected from an open forest in May/June and were rinsed properly, de-shelled, cut into pieces, and shade dried until fully dried. The dried seeds were then pulverized. The powder sample (1 kg) was macerated in 5000 mL of absolute methanol for 72 hours. The samples were filtered with Whatman filter paper no. 50 and the filtrate evaporated to dryness in a Rotary evaporator (RE 300, Bibby Scientific, UK) at 40°C to give a percentage yield of 32%. The concentrated extract was stored in a sample container in the refrigerator at 4°C.

Fractionation by Vacuum Liquid Chromatography

The crude methanol extract (CME) of *T. conophorum* seeds (320 g) was loaded onto a Merck grade silica gel (70–230 mesh) in a vacuum liquid chromatographic (VLC). Elution of the column was carried out step wisely using 100 % n-hexane, dichloromethane and ethyl acetate solvents polarity to obtain the respective fractions (Hex-F, DCM-F and EA-F). Fractions collected were evaporated to dryness and their percentage yields determined.

Chemicals and Reagents

The chemicals/reagents used were purchased from Sigma (St Louis, MO, USA) and were of analytical grade.

Phytochemical Investigation

Qualitative phytochemical constituents of CME/fractions were determined according the method described by Sofowora (14) and Trease and Evans (15).

Quantitative Phytochemical Determination

Total phenolic and flavonoid contents were determined according to Folin-Ciocalteu method (16) and Ebrahimzadeh et al (17) slightly modified by Oriakhi et al (18). Total tannin content in both methanol extract and fractions of *T. conophoru*m were determined according to the method described by Broadhurst and Jones (19) with some modifications, while proanthocyanidin content was determined by the method described by Sun et al (20).

Antioxidant Activity

Diphenyl-2-picryl-hydrazyl (DPPH) scavenging effects of CME/fractions of *T. conophorum* seed were determined by the method described by Jain et al (21). The reducing potential activity of extract was determined according to the method described by Lai et al (22). ferric reducing antioxidant potential (FRAP) and Trolox equivalent antioxidant capacity (TEAC) were determined by the method described by Benzie and Strain (23) and Re et al (24), respectively.

Nitric oxide (NO) inhibitory activities of CME/fractions of *T. conophorum* seeds were determined by the method described by Garrat (25), while hydroxyl radical scavenging activity was determined according to the method described by Chen et al (26).

Statistical Analysis

Data were expressed as the mean ± SEM of the 3 measurements using the GraphPad Prism software. Statistical significance was investigated by one-way analysis of variance. Differences between mean values were investigated by Duncan multiple range test.

Results

Phytochemical Analysis

Phytochemical investigation of CME of *T. conophorum* seeds and its fractions is shown in Tables 1 and 2. Qualitative phytochemical investigation of the CME of *T. conophorum* seeds and its fractions demonstrate the presence of six secondary metabolite (alkaloids, saponins, tannins, phenol, flavonoids and terpenoids) and two primary metabolites namely carbohydrates and reducing sugars. However, no anthraquinones were observed in the crude extract and fractions (Table 1). Results from the quantitative phytochemical investigation showed significantly higher total phenolic and flavonoid contents in ethyl acetate fraction (71.25 ± 4.13 mg GAE/g extract and 97.13 ± 0.13 mg quercetin equivalent, respectively) when compared to the CME (49.18 ± 0.38 mg GAE/g extract and 80.69 ± 3.56 mg quercetin equivalent/g extract, respectively) and other fractions (Table 2). Similarly, ethyl acetate fraction showed the highest tannin content, followed by HEX-F, while CME had the lowest tannin content, but relatively higher proanthocyanidin content compared to other fractions (Table 2) with DCM-F showing the lowest proanthocyanidin content.

In Vitro Antioxidant Activity

DPPH Radical Scavenging Activity

DPPH radical scavenging activities are shown in Figure 1. The methanol extract and its fractions demonstrated remarkable and dose dependent increase in radical scavenging activity. The ethyl acetate fraction showed the highest inhibition percentage (92.84%) at 200 µg/mL concentration. The ethyl acetate fraction competed favourably with standard ascorbic acid with inhibition percentage of 95.5%. Estimation of the 50% inhibitory concentration (IC_{50}) (Table 3) showed that the ethyl acetate

Table 1. Qualitative Phytochemical Investigation of Crude Methanol Extract of *Tetracarpidium conophorum* seeds and its Fractions

Test	Constituent	CME	HEX-F	DCM-F	EA-F
Molish	Carbohydrate	+	+	+	+
Benedict	Reducing Sugar	+	+	+	++
Dragendoff's	Alkaloid	++	+	++	+
Borntrager's	Anthraquinone	–	–	–	–
Gelatin	Tannin	+++	+	++	+
Lead Acetate	Flavonoid	+	+	+	+
Froth	Saponin	+	+	+	+
Folin-Ciocalteu	Phenol	+	+	+	+
Liberman-burchard	Terpenoid	+	+	+	+

– Absence of constituents; + Presence of constituents; ++ Highly present.
CME, crude methanol extract; HEX-F, hexane fraction; EA-F, ethyl acetate fraction; DCM-F, dichloromethane fraction.

Table 2. Quantitative Determination of Polyphenolic Compounds

Sample	Total Phenol (mg GAE/g extract)	Total Flavonoid (mg QE/g extract)	Total Tannin (mg TAE/g extract)	Proanthocyanidin (mg CE/g extract)
Crude methanol	49.13±0.38[a]	80.69±3.56[a]	946.5±30.50[a]	29.43±0.51[a]
Hex- fraction	36.00±0.25[b]	69.76±0.01[b]	407.0±17.00[b]	26.60±0.25[a]
DCM-fraction	26.50±0.14[c]	55.13±0.13[c]	595.0±39.00[c]	21.43±0.15[b]
EA- fraction	71.25±4.13[d]	97.13±0.13[d]	607.0± 10.50[d]	27.27±0.22[a]

Values are expressed as mean ±SEM (n = 3/group). Values in a column with superscripts are not significantly different ($P < 0.05$).

fraction showed the highest radical scavenging activity with IC_{50} value of 33.11 µg/mL, followed by hexane fraction with IC_{50} value of 33.43 µg/mL. This estimated IC_{50} value for ethyl acetate fraction was significantly higher compared to that of ascorbic acid ($P < 0.05$) suggesting a higher antioxidant activity of ascorbic acid with IC_{50} value of 17.08 µg/mL.

Nitric Oxide Inhibitory Activities

NO inhibitory activities of CME and its fractions are shown in Figure 2. The NO inhibitory ability was higher in HEX-F with an IC_{50} value of 2.07µg/mL (Table 3) compared to dichloromethane (76.26 µg/mL) and ethyl acetate fraction (53.85 µg/mL). The reference antioxidant compound, quercetin, and CME showed good inhibiting ability of NO at all concentrations with IC_{50} value of 14.8 and 4.49 µg/mL, respectively (Table 3).

Reducing Potential Activity

The reducing potential activities of CME and its fractions are shown in Figure 3. The CME as well as its fractions exhibited marked reducing ability as revealed by the increased absorbance value ranging from 0.137 ±0.006–1.270±0.150 at 50-1000 µg/mL but showed lower potential when compared with ascorbic acid at the same concentrations (Figure 3). However, it was observed that the reducing ability of EA-F was higher in a dose dependent manner as compared to CME which was also dose dependent, followed by the HEX-F.

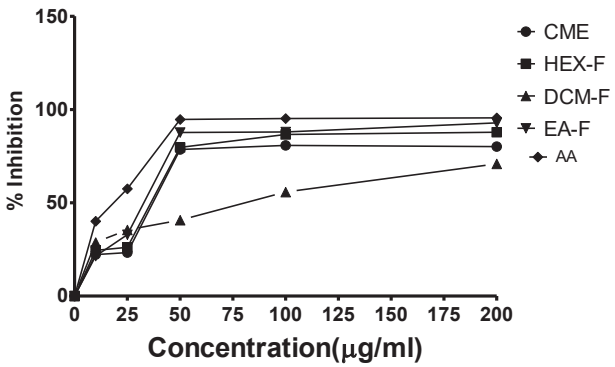

Figure 1. DPPH Radical Scavenging Activity of Different Concentrations of Vitamin C and Extracts From the Seeds of *Tetracarpidium conophorum*. Values are expressed as mean ± SEM, n = 3/group

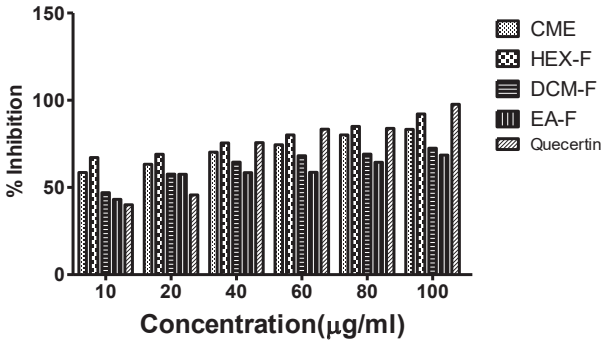

Figure 2. Nitric Oxide Radical Scavenging Activity of Crude Methanol Extract and its Fractions From *Tetracarpidium conophorum* Seeds. Values are expressed as mean ± SEM, n =3/group.

Table 3. DPPH Radical Scavenging and Nitric Oxide Inhibitory Activities IC_{50} Values of Crude Methanol Extract of *Tetracarpidium conophorum* Seeds and its Fractions

Plant extract	DPPH, IC_{50} (µg/mL)	NO, IC_{50} (µg/mL)
Ascorbic acid	17.08	-
Quercetin	-	14.86
CME	42.09*	4.49*
HEX-F	33.43*	2.07*
DCM-F	45.44*	76.26*
EA-F	33.11*	53.85*

Values in a column with asterisk (*) are significantly different from the standard ($P < 0.05$).

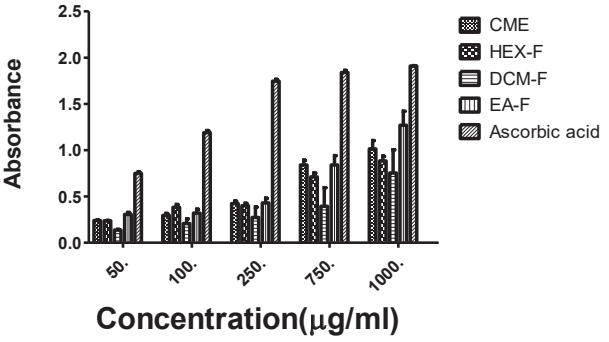

Figure 3. Reducing Potential Activity of Seed Extracts of *Tetracarpidium conophorum* and Standard Ascorbic Acid. Values are expressed as mean ±SEM, n = 3/group.

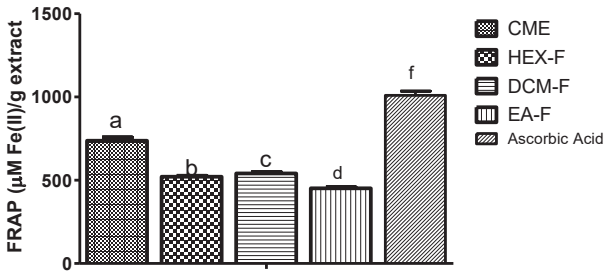

Figure 4. Ferric Reducing Antioxidant Potential of Crude Extract and Fractions of *Tetracarpidium conophorum* Seeds. Different lower case letters represent significant difference between means at $P < 0.05$

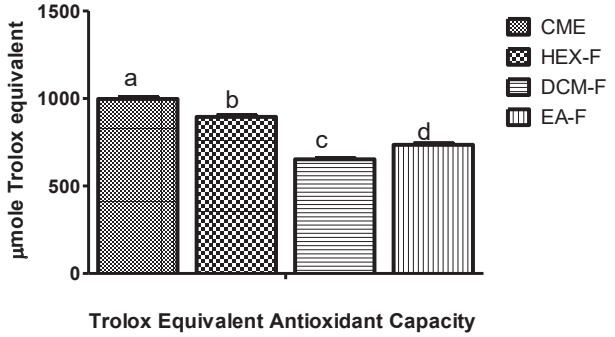

Figure 5. Trolox Equivalent Antioxidant Capacity of Extracts of *Tetracarpidium conophorum* Seeds and Ascorbic Acid. Values are expressed as mean ±SEM, n = 3/group

Ferric Reducing Antioxidant Potential

The FRAP of CME and fractions of *T. conophorum* seeds are shown in Figure 4. Methanol extract of *T. conophorum* seed exhibited high Ferric reducing antioxidant potential (737.25µM Fe (II)/g extract) which was significantly higher ($p < 0.05$) than that of HEX-F (521.0 µM Fe (II)/g extract), DCM-F (541.0 µM Fe (II)/g extract) and ethyl acetate fraction (452.0 µM Fe (II)/g extract). However, the FRAP values of both extract and fractions were significantly ($p < 0.05$) lower than those observed for the standard ascorbic acid (1009.5 µM Fe (II)/g extract) (Figure 4).

Trolox Equivalent Antioxidant Capacity

TEAC of CME and fractions of *T. conophorum* seeds are shown in Figure 5. It was observed that the CME of *T. conophorum* seeds showed the highest TEAC, followed by the HEX-F, while DCM-F showed the lowest TEAC.

Hydroxyl radical scavenging activities of CME and its fractions are shown in Figure 6. Results in the present study showed that hydroxyl radical scavenging activities of CME and its fractions demonstrated remarkable and dose dependent increase in hydroxyl radical scavenging effect, except for at concentration of 100 µg/mL, n-hexane and dichloromethane fractions had negative hydroxyl radical.

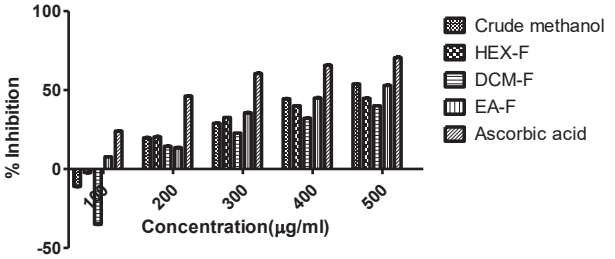

Figure 6. Hydroxyl Radical Scavenging Activity of Different Concentrations of Extracts and Ascorbic Acid From the Seeds of *Tetracarpidium conophorum*. Values are expressed as mean±SEM, n = 3/group.

Crude methanol extract also exhibited negative hydroxyl radical.

Discussion

The result of phytochemical investigation test of methanol extract/fractions (Hex-F, DCM-F, EA-F) of *T. conophorum* seeds showed the presence of six secondary metabolites (alkaloids, saponins, tannins, phenol, flavonoids and terpenoids) and two primary metabolites namely carbohydrates and reducing sugars. However, anthraquinone was below the detectable levels in the extract/fractions (Table 1). There was a marked increase

in the concentration of total phenolic and flavonoid contents in ethylacetate-fraction (71.25 ± 4.13 mg GAE/g and 97.13 ± 0.13 mg QE/g of extract, respectively) when compared to the CME (49.18 ± 0.38 mg GAE/g of extract and 80.69 ± 3.56 mg QE /g of extract, respectively) and other fractions (Table 2). Similarly, EA fraction showed higher concentration in tannin content, followed by Hex-fraction, while CME had the least tannin content but higher proanthocyanidin content compared to its fractions (Table 2). The high polyphenolic content of the EA-F is attributed to its polar nature of the solvent ethylacetate as compared to other fractions used in this study. This is because ethylacetate is more polar than hexane and dichloromethane but less polar than methanol. The antioxidant activity of *T. conophorum* seeds is attributed to the high concentrations of phenols and favonoids present in the plant. This result agrees with our previous work on the phytochemical constituents of methanol extract of *T. conophorum* seeds (12).

In this study *T. conophorum* seeds exhibited high antioxidant capacity. The DPPH radical assay is employed as a substrate to evaluate the antioxidant activity of methanol extract and fractions of *T. conophorum* seeds. The methanol extracts and fractions exhibited high percentage of inhibition in a concentration dependent manner, with EA-F having the highest percentage of inhibition followed by HEX-F and CME compared to ascorbic acid. The higher the percentage of inhibition of a plant extract/sample, the lower the IC_{50} value. IC_{50} is the amount of antioxidant required to scavenge DPPH radical concentration by 50% (27). The estimation of the 50% inhibitory concentration (IC_{50}) showed that the ethyl acetate fraction exhibited the highest radical scavenging activity with IC_{50} value of 33.11 µg/mL, followed by HEX-F (33.43 µg/mL). The IC_{50} value of EA-F was significantly higher compared to that of ascorbic acid ($P < 0.05$) suggesting a higher antioxidant activity of the standard (ascorbic acid) with IC_{50} value of 17.08 µg/mL.

The reducing potential of *T. conophorum* extracts was also evaluated. The result showed that the plant extracts exhibited higher reducing potential at all concentrations. It was observed that the extract contains high concentrations of polyphenolic content which may be responsible for the plant's inherent reducing ability (oxidation reaction) (27). This was in agreement with Uadia et al (12) who reported that the methanol extract of *T. conophorum* seeds is capable of reducing Fe^{3+} to Fe^{2+} thereby acting as an electron donor. The CME and fractions showed a marked reducing ability as indicated by the increase in absorbance value ranging from 0.137 ±0.006- 1.270±0.150 at 50-1000 µg/mL but lower potency when compared with the standard ascorbic acid at the same concentration (Figure 3). However, it was observed that the reducing ability of EA-F was the highest compared to other extracts but lower compared to ascorbic acid. Another method to study reducing ability used in this study was FRAP. In the present study, the extracts of *T. conophorum* seeds were able to reduce the ferric tripyridyltriazine (Fe (III)-TPTZ) complex to ferrous tripyridyltriazine (Fe(II)-TPTZ). The present study showed that the FRAP of the CME of *T. conophorum* seeds was highest ($P < 0.05$) followed by that of HEX-F. The antioxidant capacity of methanol extract and its fractions against $ABTS^{+}$ radical was also investigated in the TEAC (Figure 5). It was observed that the CME of *T. conophorum* seeds was highest in TEAC (997.5 µmole TE), followed by that of HEX-F (895.5 µmole TE), while DCM-F (652.5 µmole TE) was the lowest in TEAC. $ABTS^{+}$ is a better radical than DPPH and is easily stabilized by antioxidants that can supply electrons (28).

Hydroxyl radical scavenging activities of the plant extracts were evaluated. The extract/fractions of *T. conophorum* seeds showed moderate inhibition potential at all concentrations as compared to ascorbic acid (standard), but at the lowest concentration (100 µg/mL), three of the plant extracts (HEX-F, DCM-F and CME) acted as a pro-oxidant that were capable of inducing oxidative stress either by generating ROS or by inhibiting antioxidant systems as displayed by the hydroxyl radical scavenging system. Hydroxyl radical has been reported to be the most reactive radical known capable of causing damage to biological membrane (29-31). The high inhibition percentage exhibited by the EA-F at the lowest concentration may suggest that the fraction of the plant extract is responsible for the anti-oxidative properties of the plant. However, at concentrations ranging from 200-500 µg/mL, the crude extract and its fractions showed moderate to relatively high hydroxyl radical scavenging abilities.

NO is important with respect to inflammatory processes and signaling, especially in signaling of programmed cell death (apoptosis) of cells that suffer from DNA damage and other types of damage. The extract/fractions exhibited nitric oxide inhibitory activity compared to the standard quercetin. However the evaluation of the IC_{50} showed that the HEX fraction had the highest radical scavenging activity with IC_{50} value of 2.07 µg/mL, followed by CME (4.49 µg/mL). The IC_{50} value of HEX-F was significantly lower compared to that of quercetin ($\mathbf{P} < 0.05$) suggesting that HEX-F has higher NO scavenging abilities. Therefore, the ability of *T. conophorum* seed extracts to scavenge $NO^{\bullet}$ against tissue damage is implicated in this study (32,33).

Conclusion

In conclusion, this study demonstrated that extracts of *T. conophorum* seeds are rich sources of secondary metabolite and strong antioxidant properties. All extracts exhibited good scavenging activity for DPPH, FRAP, hydroxyl radical ($OH^{\bullet}$), NO, $ABTS^{+}$ radical scavenging activity in a dose-dependent manner. The activity may be strongly associated with the presence and concentration of secondary metabolites present in *T. conophorum* seed

extracts. This argument could be further investigated for the treatment and management of diseases.

Authors' Contributions

POU designed the experiments and supervised the work. KO and KOO carried out the experimental bench work and analysed and interpreted the data.

Conflict of Interest Disclosures

The authors declare no potential conflicts of interest relevant to this article.

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