ANTI OXIDANT, ANTI INFLAMMATORY AND ANTINOCICEPTIVE STUDY ON ARECA NUT

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ABSTRACT

The objective of study is to evaluate the anti oxidant activity and the total phenolic and falvanoid content of areca nut aqueous extract. Areca nut extract scavenged super oxide, DPPH, hydroxyl radicals and inhibited tissue lipid peroxidation *in vitro*. Oral administration of areca nut extract for one month, significantly increased super oxide dismutase, glutathione and glutathione reductase enzymes level($p<0.001$) in blood of rat and glutathione -s-transferase, glutathione peroxidise and superoxide dismutase enzyme in liver. The result showed mild scavenging ability against stable free radicals DPPH and ABTS and good antioxidant activity through enzymatic system possessing 68±0.007mg GAE/g and 7.03±CE mg/g phenolic and flavanoid content respectively.

Key words: *Areca catechu,* Antioxidant activity, Total phenolic and flavanoid compounds.

INTRODUCTION

In many Asian cultures such as India, Taiwan, and Southeast Asia, betel nut , *Areca catechu* L. (family Palmaceae), is traditionally masticated either alone or as a quid along with a large variety of ingredients, such as betel leaf (*Piper betel*; family Piperaceae), slaked lime, different types of tobacco besides additives, perfumes and stimulants (1). In herbal medicine, the areca nut has been used medicinally as a drug against parasitic worms (2). In old Indian scripts such as Vagbhata (4th century), and Bhavamista (13th century), betel nut was described as a therapeutic agent. Its use was recommended in many diseases such as leucoderma, leprosy, anaemia and obesity. It was also reported to have de-worming properties (3).The present study is to evaluate the total phenolic and flavanoid contents in areca nut and in order to determine their health-promoting antioxidant property.

MATERIALS AND METHODS

Drugs

Sample of areca nut (*Areca catechu*) obtained locally from areca nut farmers of Angamaly, Kerala were used for the study. The nuts were dehusked and nut portion was collected. This was further crushed to get coarse powder with help of mortar and pestle. The powder was weighed and soaked in the methanol in a ratio of 1gm in 2.5 ml. It was covered with aluminium foil and kept overnight. After soaking overnight, sample was again stirred and filtered through Whatsman No.4 filter paper. Filtrate was then collect separately and evaporated to dryness. Red colored flakes were obtained which were crushed and powdered. In order to get a uniform suspension of areca extract, the extract was dissolved in hexane (100 mg/10 ml) and 10µ of Triton X 100 (Sigma-Aldrich) was added and

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further evaporated to dryness and finally made up to 10ml with distilled water. The extract was dissolved in paraffin oil for *in vivo* studies.

Animals

Wilstar albino rats (20-25 g) were used in the study. They were purchased from Little Flower Medical Research Centre (LFMRC) Animal Breeding Station, Angamaly, Kerala,India and were housed in well ventilated cages under controlled conditions of light and humidity and provided with normal mouse chow and water ad libitum. All the animal experiments were done as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, and implemented through the Institutional Animal Ethical Committee of the Research Centre.

Chemicals and reagents

Nitroblue tetrazolium (NBT), glutathione, glutathione oxidized (GSSG), nicotinamide adenine dinucleotide phosphate reduced (NADPH) and 5-5'dithiobis 2-niotrobenzoic acid (DTNB) were purchased from Sisco Research Laboratories Pvt. Ltd., (Mumbai, India), catechin, gallic acid, 2,2-Diphenyl-1-picryl hydrazyl (DPPH) and 2,2-azobis-3-ethylbenzthiozoline-6-sulphonic acid (ABTS) were purchased from Sigma Aldrich (St.Louis, USA). All other chemicals and reagents used were of analytical reagent grade.

Estimation of total phenolic and flavanoid content of areca nut

Total phenolic content (TPC) of plant extracts was determined using the *Folin Ciocalteu* assay by Kahkone *et al* (1999) with some modifications. *Folin-Ciocalteu* reagent (1.5 ml; diluted 10 times) and sodium carbonate (1.2 ml; 7.5% w/v) were added to the extracts (300 µl; triplicate). The tubes were vortexed and allowed to stand for 30 min at 40°C for color development. Absorbance was then measured at 765 nm using UV-VS spectrophotometer. Total phenolic content were expressed as mg gallic acid equivalents/g dry weight (GAE).

The total flavanoid content was determined using colorimetric method. 0.25ml of sample (catechine for standard or extract)was mixed with 1.25 ml of distilled water in a test tube, followed by addition of 0.075ml of 5% sodium nitrate solution. After 6 minutes, 0.15ml of 10% aluminium chloride solution was added and the mixture was allowed to stand for 5 minutes before the addition of 0.5 ml of 1M sodium hydroxide solution. 2.5 ml of distilled water was added and the absorbance was measured immediately at 510 nm.

Determination of antioxidant activity of areca nut extract by in vitro method

Superoxide radical scavenging activity

Superoxide radical scavenging activity was determined by the NBT reduction method (7). Different concentrations of areca nut extract (10-200 µg) were added to the reaction medium containing 3 µg NaCN in 6 mM EDTA, riboflavin (2 µM), NBT (50 µM) and phosphate buffer (67 mM, pH 7.8) in a final volume of 3 ml. The tubes containing reaction mixture were uniformly illuminated with an incandescent lamp for 15 minutes. The optical density was measured at 560 nm before and after illumination. The percentage inhibition of superoxide generation was evaluated by comparing the absorbance value of the control and experimental tubes.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe_3+/ascorbate/EDTA/H_2O_2 system (Fenton reaction). The reaction mixture (1.0 ml final volume) contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O (1 mM), ascorbate (0.1 mM), KH_2PO_4 - KOH buffer (20 mM, pH 7.4) and different concentrations of areca nut extract (10-200 µg). The reaction mixture was incubated for 1 hr at 37°C. Deoxyribose degradation was measured as TBARS and percentage inhibition calculated (8). The percentage inhibition was calculated according to the formula: Inhibition percentage (Ip) = [(AB-AA)/AB] x 100 where A and B are absorbance values of the blank sample and of areca extract solution, respectively.

Determination of inhibition of lipid peroxidation

Different concentrations of the areca nut extract (10- 200 µg) were added to rat liver homogenate (0.1 ml, 25% w/v), ascorbic acid (0.06 mM), 30 mM KCl, 0.16 mM $FeSO_4$ solution and final volume made up with Tris buffer (40 mM, pH-7) to 0.5 ml. Tubes were incubated for one hour at 37°C. Aliquots of the incubation mixture (0.4 ml) were treated with sodium dodecyl sulphate (SDS-8%, 0.2 ml), thiobarbituric acid (TBA-0.8%, 1.5 ml), and acetic acid 20%, pH-3.5, 1.5 ml). The total volume was then made up to 4 ml with distilled water and incubated for 1 hr at 100 4°C in a water bath. After cooling, 1 ml of distilled water and 5 ml of mixture of n-butanol and pyridine (15:1, v/v) were added and vortexed. After centrifugation the absorbance of the organic layer was measured at 532 nm. Percentage inhibition of lipid peroxidation was determined by comparing the results of the test compound with those of the control (8).

Determination of DPPH radical scavenging activity

Different concentrations of areca nut extract (0.1-1 mg) were mixed with 0.375 ml methanolic solution of DPPH (1, 1-diphenyl- 2-picrylhydrazyl- 0.25 g/L). Total volume was made up to 2 ml with methanol. The disappearance of DPPH was read spectrophotometrically at 515 nm after 20 min of incubation at room temperature in dark (9).

Determination of ABTS radical scavenging activity

ABTS radical scavenging activity of the extract was determined using ferryl myoglobin/ABTS protocol (10). The stock solutions and 500 µM ABTS diammonium salt, 400 µM myoglobin (MbIII), 740 µM potassium ferricyanide and 450 µM H_2O_2 was prepared in phosphate-buffered saline (PBS; pH 7.4). Metmyoglobin was prepared by mixing equal volumes of myoglobin and potassium ferricyanide solutions. The test tubes contained ABTS (150 µM), MbIII (2.25 µM), varying concentrations of areca nut extract (10-200 µg) and PBS (pH-7.4) in volume of 2 ml. The reaction was initiated by adding 75 µM H_2O_2 and lag time was recorded in seconds before absorbance of ABTS at 734 nm began to increase.

Ferric reducing antioxidant power (FRAP) assay.

The ferric reducing ability was measured at low pH. FRAP reagent contained 25 ml 0.3 M acetate buffer, 2.5 ml 4,6-Tris-2-pyridyl- (s)-Triazine (TPTZ) and 2.5 ml ferric chloride. Different concentration of areca nut extract (10-200 µg) was made up to one ml with freshly

prepared FRAP reagent. The mixture was incubated at 37°C for 15 minutes and absorbance read at 595 nm against distilled water at 595 nm. Standard graphs were constructed using known concentrations of ferrous salt in water/methanol to replace $FeCl_3$ and expressed as EC1 values. EC1 is defined as concentration of an antioxidant having a ferric reducing ability equivalent to that of 1 mM ferrous salt (11).

Determination of effect of Areca extracts on antioxidant enzyme levels in vivo.

Wilstar albino rats (5-6 weeks)weighing 20-25 g were divided into 4 groups of five animals and they were fed orally with areca nut extract dissolved in paraffin oil at different doses for 30 days. Group I was kept as normal. Group II was kept as control treated with paraffin oil only. Group III and Group IV were treated respectively with 100 mg and 500 mg areca nut extract/kg body weight.

At the end of the feeding trial, animals were sacrificed by ether anesthesia, and blood was collected by heart puncture, liver was excised and washed in ice-cold Tris Hcl buffer (0.1 M, pH-7.4), and cytosolic samples of liver homogenate were prepared by centrifuging at 10,000 rpm for 30 mins at 4°C. Whole blood was used for determination of superoxide dismutase, catalase and glutathione levels. Serum was used for glutathione reductase estimation. Liver tissue homogenate was used for super oxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione -S-transferase and glutathione assays.

Superoxide dismutase activity was measured by the NBT reduction method (7). Catalase activity was estimated by the method of Aebi, 1974(12) by measuring the rate of decomposition of hydrogen peroxide at 240 nm. Glutathione activity was assayed by the method of Moron, Depierre and Manner, 1979(13) based on the reaction with DTNB. The assay of glutathione peroxidase was done by the method of Hafeman, Sundae and Houestra, 1974(14) based on the degradation of H_2O_2 in the presence of GSH. Glutathione reductase activity was measured by the method of Racker, 1955 (15). The method of Habig, Pabst and Jakoby, 1974 (16) was followed assay of glutathione -S-transferase (GST) activity which is based on the rate of increase in conjugate formation between GSH and 1-chloro-2, 4- nitrobenzene (CDNB).

Statistical analysis

The values were expressed as mean ±standard deviation (SD). Statistical evaluation of data was done by one way ANOVA followed by Dunnet's test.

RESULTS

Determination of total flavanoid and phenol content

The total phenolic and flavanoid content values in the areca nut seeds were 7.03±0.12 CE mg/g extract and 68±0.007GAE mg/g extract estimated respectively (Table. I)

TABLE I: ESTIMATION OF TOTAL FLAVANOID AND PHENOL CONTENTS OF AREACA NUT

Total flavanoids(CE)mg/g	Total phenol content(GAE)mg/g
7.03±0.12	68±0.007

Values are the mean ±SD of different determinations

Antioxidant activities of areca nut extract in vitro

Areca nut extract was found to scavenge superoxide, hydroxyl radicals and inhibit tissue lipid peroxidation (Fig. 1). Areca nut extract gave an IC50 of 36 µg/ml for scavenging superoxide radicals but IC50 for inhibition of hydroxyl radicals and lipid peroxidation was found to be greater than 200 µg/ml and 400 µg/ml respectively and so could not be calculated. The ferric reducing activity for 50 µg of areca nut extract was found to be 1.8 mM for areca nut extract. Areca nut extract possessed only mild DPPH radical scavenging ability as IC50 was more than 1000 µg/ml. It showed only a mild capacity to scavenge ABTS radical.

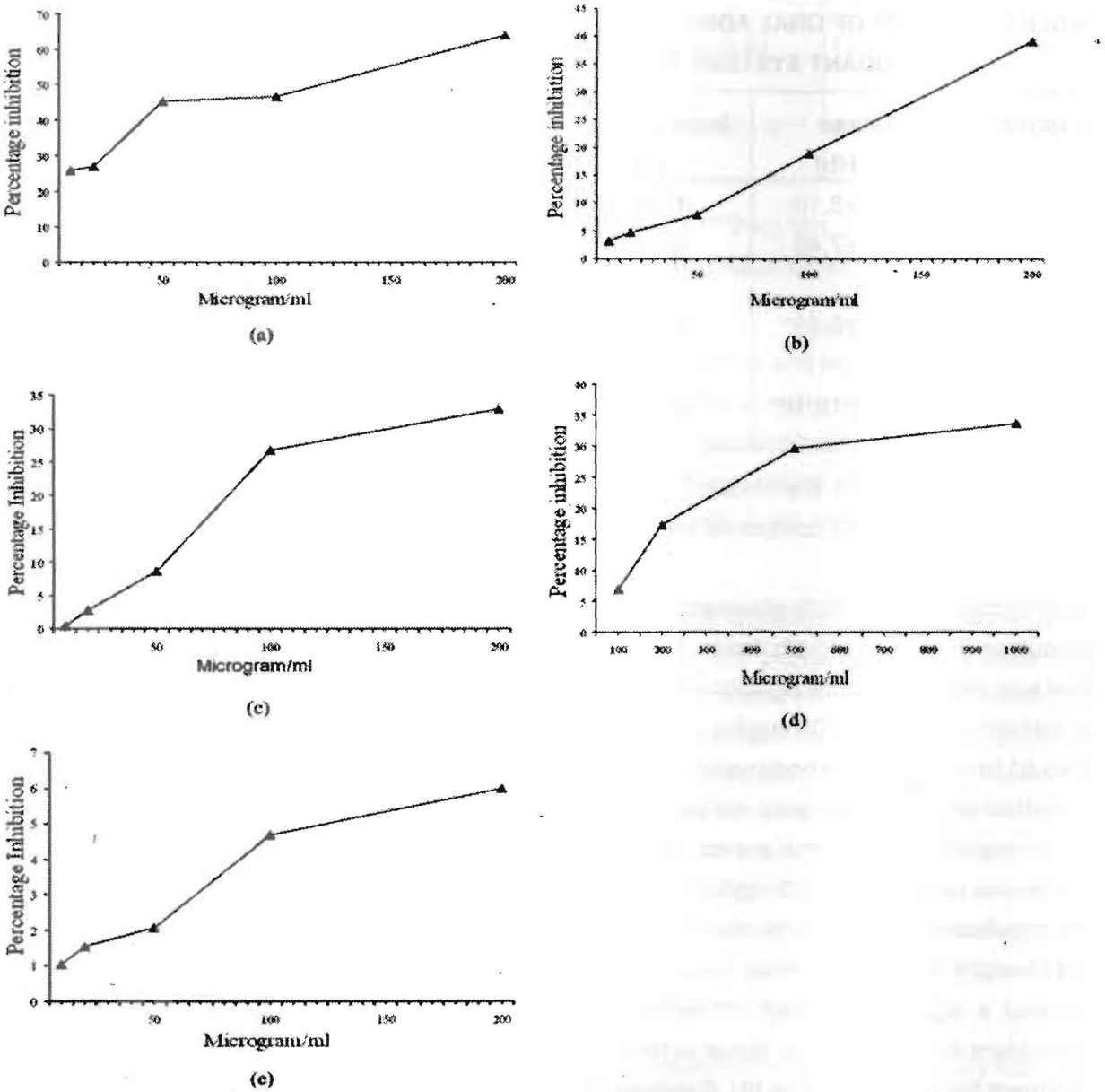

Fig. 1: In vitro free radical scavenging activity of areca nut extract. (A) Superoxide radical scavenging activity. (B) Hydroxyl radical scavenging activity. (C) Inhibition of lipid peroxidation.(D) DPPH radical scavenging activity.(E) ABTS radical scavenging activity

Effect of administration of areca nut extract on antioxidant enzymes and glutathione

Antioxidant enzymes in blood and serum of mice were increased after administration of areca nut extract for a period of 30 days (Table II). Catalase was found to be increased in all animals treated with 100 and 500 mg/kg body weight of areca nut extract (P<0.05). The essential oil administration significantly elevated superoxide dismutase (P<0.001) at both doses. Glutathione level was also found to be significantly increased in treated groups at 100 mg/kg body weight (P<0.01) and 500 mg/kg body weight (P<0.001). Glutathione reductase was elevated by the administration of areca nut extract (P<0.05) in all animals treated with 100 mg/kg body weight and significant activity was shown at 500 mg/kg body weight (P<0.001). Areca nut extract also showed a significant effect on some of the antioxidant enzymes in liver tissue of mice after treatment for 30 days (Table III). Catalase level did not change significantly in any of the treated groups. Superoxide dismutase activity was elevated in the group treated with 500 mg/kg body weight (P<0.01) areca nut extract. The level of glutathione peroxidase enzyme was significantly increased by areca nut extract at 500 mg/kg body weight. (P<0.01). Even though glutathione was found to be increased, it was not found significant. The level of glutathione reductase was found to be unaltered in both the treated groups. Glutathione-S-transferase (GST) level was found to be elevated in animals and significant activity was shown at 500 mg/k body weight (P<0.01) treated group.

TABLE I I: EFFECT OF ORAL ADMINISTRATION OF ARECA NUT EXTRACT FOR ONE MONTH ON ANTIOXIDANT SYSTEMS IN BLOOD.

Treatment	Catalase (k/g Hb)	Super oxide dismutase (U/g Hb)	Glutathione reductase (U/g Hb)	Glutathione (nmol/ml)
Normal	34.74±8.18	473±42.65	2.88±0.54	46.3±4.7
Control	32.57±7.43	444±38.89	2.75±0.26	44.2±7.2
100mg/kg body weight	56.91±8.43*	605±31.00***	6.64±0.83*	68.7±6.5**
500mg/kg body weight	50.36±19.18*	865±23.76***	8.08±1.57***	77.9±5.6***

Each value represents the mean±S.D (n=5). *P<0.05 compared with control, **P<0.01 compared with control,***P<0.001 compared with control.

DISCUSSION

Reactive Oxygen Species (ROS) are involved in the cell growth, differentiation, progression and death. Low concentrations of ROS may be beneficial or even indispensable in processes such as intracellular signalling and defence against micro-organisms. Nevertheless, higher amounts of ROS are indicated in the aging process as well as in a variety of human diseases including cardiovascular and inflammatory disease, cataract, cancer, ischemia and failures in immunity and endocrine functions.

TABLE I II: EFFECT OF ORAL ADMINISTRATION OF ARECA NUT EXTRACT FOR ONE MONTH ON ANTIOXIDANT SYSTEMS IN LIVER.

Treatment	Catalase (U/mg protein)	Super oxide dismutase (U/mg protein)	Glutathione peroxidase (U/mg protein)	Glutathione -s-transferase (nmol/mg protein)	Glutathione reductase^	Glutathion (nmol/ml)
Normal	5.29±0.66	0.84±0.12	8.16±0.65	42.57±1.66	88.83±12.03	13.8±1.7
Control	4.97±0.7	0.82±0.04	8.98±1.54	39.46±3.43	79.0±20.16	11.4±1.7
100mg/kg body weight	5.68±0.98	0.90±0.16	13.12±5.65	32.46±3.43	19.79±17.48	12.3±2.2
500mg/kg body weight	8.36±0.45	1.10±0.13*	19.2±7.53**	102.09±11.9***	69.56±9.53	13.9±3.3

*P<0.05 compared with control, **P<0.01 compared with control, ***P<0.001 compared with control. Each value represents the mean ±S.D (n=5), ^ (nmol of NADPH consumed/min/mg Protein).

As a safeguard against the accumulation of ROS, several non enzymatic and enzymatic antioxidant mechanisms exist naturally in each cell. Latter include antioxidant enzymes and anti oxidant systems. Major antioxidant enzymes are superoxide dismutase (SOD), catalase and peroxidases, of which glutathione peroxidises is thought to be most important. Glutathione (GSH) and numerous GSH dependent enzymes, metal binding proteins and vitamins are the important antioxidant enzyme systems.

Result of this study shows lower amount of phenolic (68±0.007mg GAE/g) and flavanoid (7.03±CE mg/g) in the areca nut seed aqueous extract compared to earlier report (17, 18). Study reveals the ability of areca nut seed to inhibit oxygen radicals as seen from the inhibition of lipid peroxidation, scavenging of superoxide and hydroxyl radical in vitro. However showed only a mild scavenging activity against stable free radicals as DPPH and ABTS making confusion with earlier studies and reports. Even in the low concentration of phenolic and flavanoid constituents, administration of areca nut seed extract increase serum antioxidant status for protecting cell from intracellular oxidative damage.

Various literature and research findings suggest that the polyphenolic and flavanoid constituents of areca seed varies depending up on the geographical region where it is grown or cultivated, species and its maturity(19). Sufficient research data about its phenolic and flavanoid contents are not available to compare this result. However, little variation was observed in the amount of constituents when compared with the available data. This variation may be due to the above mentioned reasons.

Antioxidant activity of this study highlight the mild scavenging activity of areca seed aqueous extract act against stable free radicals such as DPPH and ABTS. DPPH is a free

radical compound that has been widely used to determine the free radical scavanging ability of various sample (20,21). DPPH decreases significantly up on exposure to proton radical scavengers(22).2,2'-Azinobis-3 ethylbenzothiazoline-6-sulfonic acid radical (ABTS+) is a stable organic radical that has gained general acceptance as the organic radical for use in measuring radical scavenging activity as an expression of hydrogenating antioxidative activity in plant crude extract(23). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. A higher absorbance indicates a higher ferric reducing power (24, 25). Therefore DPPH scavanging,ABTS scavenging and reducing power assay were used to analyse the antioxidant activity of the areca seeds. Many studies clearly reported the high scavenging activity of areca seed against stable free radicals DPPH and ABTS(18,26). This strong scavenging activity of areca seed extract was possibly due to its high content of phenolic and flavanoid compounds which could act as a hydrogen donor antioxidant(19). This point confusing our result that areca seed aqueous extract shows mild scavenging activity with the lower amount of phenolic and flavanoid constituents but the administration of same areca seed aqueous extract shows good antioxidant activity by increasing serum antioxidant status. It implies the capacity of areca seed to act as an antioxidant through enzymatic system independent with the concentration of phenolic and flavanoid constituents.

Numerous publications report excellent linear correlations between "total phenolic profile" and "antioxidant capacity" (27). It should be noted that the correlation of IC50 radical scavenging activity of enzymes to total phenolic and flavanoid was not studied. So it is possible that the relation between the antioxidant activity through enzymatic system to total phenolic and flavanoids of areca seed extract is still need in our further study. All these experimental evidence suggests that the areca seed aqueous extract is a potential source of natural antioxidant through enzymatic and non enzymatic antioxidant mechanisms. However further investigation require to identify the actual component responsible for its enzymatic and non enzymatic antioxidant activity prior to the development in to the drug.

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