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EVALUATION OF ANTIOXIDANT ACTIVITY OF CHLOROPHYLL

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ABSTRACT:
Antioxidant play an important role to protect damage caused oxidative stress (OS).Chlorophyll having phenolic contents are reported to possess antioxidant properties. The present study was designed to investigate the antioxidant properties of methanolic solution from Super Chlorophyll.
Objective: The main objective of the study was to evaluate the antioxidant activity of the chlorophyll in different validated *in vitro* models.
Methods: The antioxidant activities of methanolic solution were evaluated by *in vitro* standard method using UV-Spectrophotometer. The antioxidant activity were determined bt total antioxidant capacity, DPPH (1-1-diphennyl-2-picrylhydrzine) radical scavenging assay, Nitric Oxide scavenging assay and Hydrogen Peroxide scavenging assay methods.
Result: The solution of Chlorophyll was studied for antioxidant potential. Ascorbic acid was used as standard. This method is simple and activity of the solution is reported in term of IC_{50} value. Antioxidant properties depend on the IC_{50} value. Lesser the IC_{50} value, more is the antioxidant activity. Chlorophyll showed good antioxidant activity.
Conclusion: In case of in vitro antioxidant activity, Chlorophyll scavenged DPPH, hydrogen peroxide, nitric oxide radicals significantly, showed IC_{50} values near to standard ascorbic acid, thus proving to have good antioxidant potential.
Keywords: Oxidative stress, DPPH, Antioxidant, Super chlorophyll.

INTRODUCTION

Antioxidant: A substance that reduces damage due to oxygen, such as that caused by free radicals. Well-known antioxidants include enzymes and other substances, such as vitamin C, vitamin E, and beta carotene, which are capable of counteracting the damaging effects of oxidation. Antioxidants are also commonly added to food products such as vegetable oils and prepared foods to prevent or delay their deterioration from the action of air. Antioxidants may possibly reduce the risks of cancer. Antioxidants clearly slow the progression of age-related macular degeneration.[1]Living cells generate free radicals and other reactive oxygen species (ROS) by-products as a result of physiological and biochemical processes. Antioxidant dietary supplements have been not shown to improve health in humans, or to be effective at preventing disease.[2] Supplements of beta-carotene, vitamin A, and vitamin E have no positive effect on mortality rate[3][4] or cancer risk.[5][6] Additionally, supplementation with selenium or vitamin E do not reduce the risk of cardiovascular disease.[7][8]

1.2. TYPES OF ANTIOXIDANTS

1.2.1 Antioxidant Enzymes

Enzymes are types of antioxidants that come from the protein and minerals we eat as part of our daily diets. These enzymes are synthesized in the human body, and include superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, and catalases.

1.2.2 Antioxidant Vitamins

The human body does not produce antioxidant vitamins naturally, so it is essential to include

dietary sources of them in our daily intake of food, be it through foods or supplements. Common antioxidant vitamins include vitamins A, C, E, folic acid, and beta-carotene.

Vitamin A is particularly important for improving the immune system, eye health, tissue repair, and cholesterol levels. Hawaiian Spirulina is rich in vitamin A. Vitamin C helps to protect the skin from UV damage, promotes better iron absorption, provides greater resistance to infections, and helps to regulate blood cholesterol. Vitamin E is important for maintaining healthy blood vessels, improving skin conditions, and protecting the body's membrane. Meanwhile, folic acid is important to women of childbearing years, particularly in preventing the development of neural tube defects in the fetus.Beta-carotene is a powerful carotenoid (which is a type of phytochemical) that is considered to offer the best protection against singlet oxygen and free radicals.Coenzyme Q10 (or CoQ10), is a vitamin-like substance produced by the body that has been shown to be a necessary component in the basic functioning of cells. The production of this substance does decrease naturally as we age, and its reduction has been linked to the development of various age-related diseases and conditions.

1.2.3 Antioxidant Phytochemicals

Phytochemicals are the antioxidants that are naturally used by plants to protect themselves against free radicals. Studies show that humans who eat sources of phytochemical also benefit from the antioxidant properties of the plant. Phytochemicals are broken down into the following categories:

- Carotenoids
- Flavonoids
- Allyl sulfides
- Polyphenols

1.3 REACTIVE OXYGEN SPECIES

Primary ROS plays an important role in the host defence mechanism against microorganism, but the increased production of ROS is associated with the onset of a variety of disease including cancers,[9] inflammation,[10] neurodegeneration,[11] Parkinson's disease,[12] atherosclerosis[13] and premature ageing.[14] A number of different free radicals and non radicals are produced during normal aerobic metabolism.[15][16] A collective term, "reactive oxygen species"(ROS), is used for oxygen derived species including oxygen bearing free radicals, as well as certain non radicals. Some non oxygenated radicals are also generated in biological system, such as carbon-centered free radicals and sulphur- centered radicals which are produced by the attack of free radicals on hydrocarbons and the oxidation of glutathione, respectively.[17][18]

1.4 USED IN TECHNOLOGY

- Food preservatives
- Industrial use

1.5 COMPOUND INTRODUCTION

Chlorophyll is a natural, fat soluble molecule found in plant that gives plant their green color. Chlorophyllin, the form of chlorophyll used in UNICITY SUPER CHLOROPHYLL is a water soluble version of chlorophyll that contains copper instead of Mg as its central room. Plants use Chlorophyll to trap light needed for photosynthesis, the process which creates the energy needed to separate water to make sugar and oxygen. A key ingredient in Super Chlorophyll is chlorophyll from the **Alfalfa plant**. That has been used for centuries by various cultures as a vitalizing and cleaning agent, and science has recently confirmed the potential health benefits of chlorophyll as supplementation. Protecting the skin for freeradicals.[19] **Chlorophyll** (also **chlorophyll**) is any of several related green pigments found in mesosomes of cyanobacteria, as well as in the chloroplasts of algae and plants.[20] Chlorophyll is essential in photosynthesis, allowing plants to absorb energy from light.[21] Chlorophylls absorb light most strongly in the blue portion of the electromagnetic spectrum as well as the red portion.[22]Conversely, it is a poor absorber of green and near-green portions of the spectrum, which it reflects, producing the green color of chlorophyll-containing tissues. Two types of chlorophyll exist in the photosystems of green plants: chlorophyll a and b. [23]

2. OBJECTIVIES

The main objective of the study was to evaluate the antioxidant activity of the chlorophyll in different validated *in vitro* models.

Phase-1:

Preliminary phytochemical screening

The preliminary phytochemical investigations of Chlorophyll were carried out for qualitative identification of the phytochemical constituents present like alkaloids, steroids, carbohydrates, triterpenes and saponins etc. Tests were carried out using standard methods. All the chemical and reagents to be used in the tests were of analytical grade.

Phase-2:

Evaluation of Antioxidant activity of **Chlorophyll** using:-

In vitro models

1. DPPH Assay
2. H_2O_2 Scavenging Assay
3. NO Scavenging Assay

3. REVIEW OF LITERATURE

1. Takeastsu, (1998) reported the traditional uses of plant *Viscum Articulatum.* It has been used in the treatment of rheumatic arthritis, urinary tract infection, leucorrhoea, epistaxis, lumber muscle strain, low back pain, bacillary desentry, uterine bleeding, pyodermas, psoriasis, weakness, lactation deficiency.[24]

2. Amabeoku.G.J. et.al, (1998) reported that dichloromethane, methanol and water extracts of *Viscum capense* L.f., of the Loranthaceae family, were tested for antimicrobial activities against Staphylococcus aureus, Pseudomonasaeruginosa and Candida albicans. Methanol extract was also tested for activity against seizures in albino mice induced by pentylenetetrazole (PTZ), bicuculline and N-methyl-DL-aspartic acid (NMDLA). Methanol extract of V.capense inhibited the growth of S. aureus. Methanol extract also protected the mice against PTZ- and bicuculline-induced tonic seizures but did not significantly alter NMDLA-induced tonic seizures. The data indicate that the extract of V. capense has antibacterial activity against S. aureus and also anticonvulsant activity.[25]

3. Viscum album L. is extensively used for treatment in traditional medicine. This plant was collected in the region of Artvin, northern Turkey. Different concentrations of n-hexane extract were tested using the agar diffusion technique against 6 bacteria (Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomogas aeruginosa, Enterobacter cloacae and Proteus vulgaris), and fungus (Candida albicans). *Viscum album L*. showed antibacterial activity against the micro organisms tested.[26]

4. Tenorio.F.A. et.al, (2005) reported that the aqueous extract of *Viscum album* leaves showed a significant coronary vasodilator activity on the Langendorff‟s isolated and perfused heart model. The data obtained suggest that the aqueous extract of V. album contains some biologically active principles that may act as inducers of the nitric oxide/soluble guanylate cyclase pathway.[27]

5. Ursula M, Lanfer-Marquez et.al, (2005) reported the antioxidant activity of six natural isolated chlorophyll derivatives and Cu-chlorophyllin was investigated by measuring their protective action against lipid oxidation. For this beta-carotene bleaching method and the stable radical DPPH scavenging assay were employed.[27]

6. Evren Onay Uçar et.al, (2005) reported that methanolic extracts of *Viscum album* ssp. album (mistletoe) grown on different host trees were investigated for their potential antioxidant activity. Scavenging activity was tested by 1,1diphenyl2picrylhydrazyl (DPPH) method and the inhibitory effect on lipid peroxidation was examined by ferric thiocyanate and thiobarbituric acid methods. The extract from mistletoe grown on lime tree in summer showed the highest activity. It was found that antioxidant capacity of the plant differed according to the harvesting time as well as the host tree.[28]

7. Didem Deliorman Orhanet.al, (2005) reported that the acute hypoglycemic effect of water and ethanolic extracts of three *Viscum album* subspecies, ssp. album, ssp. *austriacum*, ssp. abietis, were investigated in normal glycaemic and streptozotozocin-induced diabetic rats. All results were compared with the diabetic control groups. The findings obtained in the experiments demonstrated that European mistletoe (Viscum album L.) subspecies

possess potent antihyperglycaemic and antioxidant activity depending on host plant.[29]

8. Gurpreet Kaur, (2006) has reported that free radicals are the main cause of oxidative damage of biomolecules, which is crucial eitiological factor of chronic human diseases and also reports regarding the use of natural antioxidants. The study was aimed at evaluating the antioxidant activity of alcoholic extract of *Cassia siamea Lam*. (Fabaceae) flowers. The extract was found to contain a large amount of polyphenols and also exhibited an immense reducing ability. Oral administration of the extract at a dose of 50–150 mg/kg of body weight significantly protected from CCl4 induced elevation in AST and ALT in the serum, elevation in hepatic LPO, depletion of hepatic GSH and decrease in the activities of hepatic antioxidant enzymes: SOD, CAT and GPX. The extract also protected against histopathological changes produced by CCl4 such as necrosis, fatty changes, ballooning degeneration, etc. The data obtained in the present study suggests that the alcoholic extract of C. siamea flowers have potent antioxidant activity against free radicals, prevent oxidative damage to major biomolecules and afford significant protection against oxidative damage in the liver.[30]

9. Grah. C et.al, (2007) reported that base on the results obtained from preliminary observation of *Viscum album,* Viscum therapy can used effectively to treat chronic sarcoidosis.[31]

10. Seifert G, (2008) reported the use of *Viscum Album*, one of the most widely used alternative for cancer therapies. Aqueous mistletoe extracts (MT) contain the three mistletoe lectins I, II and III as one predominant group of biologically active agents. Although MT is widely used, there is a lack of scientifically sound preclinical and clinical data. This paper, described for the first time the in vivo efficacy and mechanism of action of MT in lymphoblastic leukemia.[32]]

11. Mojiminiyi F.B.O.et.al., (2008) reported that leaf extract of *Viscum album* showed Vasorelaxant effect mediated by calcium – depended mechanisms.[33]

12. Li Y et al. (2008) have reported two new phenolic glycosides, 1-O-benzyl-[5-O-benzoyl-beta-D-apiofuranosyl (1-->2)]-beta-D-glucopyranoside (1), and 4;-hydroxy-7,3;-dimethoxyflavan-5-O-beta-D-gluco-pyranoside (2), together with nine known flavanones 3-11, isolated from the dried whole plants of *Viscum articulatum*. Their structures were identified by extensive spectral analysis, especially 2D NMR techniques.[34]

13. Balakrishna N et.al. (2009) Reported that the ethanol and aqueous extracts of *Acalypha indica* (Euphorbiaceous) were screened for antioxidant activity using Nitric Oxide Scavenging activity method, which showed significant percentage of inhibition in dose dependent manner.[35]

14. Shreedhara.C.S et.al., (2009) reported that aqueous extract of *Argyreia nervosa* was studied for its in vitro scavenging activity by different methods viz. DPPH radical scavenging, ABTS radical scavenging, lipid peroxidation, iron chelating activity, superoxide scavenging, total antioxidant capacity. The results indicate that Argyreia nervosa has significant antioxidant activity.[36]

15. Harish Singh et.al, (2010) reported that whole plant *Viscum articulatum* Burm.f is grounded with water and the paste is applied on the part affected by gout for two days by the tribals of Mayurbhanj district of Orissa, India.[37]

4. METHODOLOGY

IN VITRO ANTIOXIDANT STUDIES

1) DPPH Assay (2,2-diphenyl-1-picrylhydrazyl[38]

Procedure:

1. To 1 ml of various conc. of chlorophyll, 1 ml solution of DPPH 0.1 mM (0.39mg in 10 ml methanol) was added.

2. An equal amount of water and DPPH was added and used as control.

3. Ascorbic acid was used as standard for comparison.

4. After incubation for 10 minutes in dark, absorbance was recorded at 517nm.

5. % scavenging was calculated using the formula.

%Scavenging = OD of Control – OD of Test / OD of Control x 100

6. Graph was plotted with conc. (µg/ml) on X axis and % scavenging on y axis and IC_{50} value was calculated.

2) Hydrogen Peroxide Scavenging Assay [39]

Procedure:

1. A solution of hydrogen peroxide 20Mm was prepared in phosphate buffer.

2. To 1ml of various conc. of chlorophyll, 2 ml solution of hydrogen peroxide was added.

3. To 1 ml of methanol, 2ml of hydrogen peroxide solution was added, and used as control.

4. Methanol was used as blank.

5. Ascorbic acid was used as standard for comparison.

6. After incubation for 10 minutes in dark, absorbance was recorded at 230nm.

7. % scavenging was calculated using the formula.

%Scavenging = OD of Control – OD of Test / OD of Control x 100

8. Graph was plotted with conc. (µg/ml) on X axis and % scavenging on y axis and IC_{50} value was calculated.

3) Nitric oxide Scavenging Assay [40]

Method:

1) To 0.5ml of various conc. of chlorophyll, 2ml of Sodium nitropruside solution and 0.5 ml of phosphate buffer was added.

2) To 2ml of Sodium nitropruside solution 0.5 ml of phosphate buffer was added and used as control.

3) The reaction mixture was incubated at 25^0 for 180 minutes.

4) To 0.5 ml of incubated reaction mixture 1ml of sulphanilic acid was added and allowed to stand for 5 min. then 1ml NEED was added and incubated for 30 min.

5) Ascorbic acid was used as standard for comparison.

6) After incubation for 10 minutes in dark, absorbance was recorded at 230nm.

7) % scavenging was calculated using the formula.

%Scavenging = OD of Control – OD of Test / OD of Control x 100

8) Graph was plotted with conc. (µg/ml) on X axis and % scavenging on y axis and IC_{50} value wascalculated

5. RESULTS

5.*2 In vitro* antioxidant activity:

The solution of *Chlorophyll* was studied for antioxidant potential. Three different in vitro methods namely DPPH Assay, H2O2 Scavenging and NO Scavenging methods were employed. Ascorbic acid was used as standard. This method is simple and activity of the solution is reported in terms of IC_{50} value. Antioxidant properties depend on the IC_{50} value. Lesser the IC_{50} value, more is the antioxidant activity. Chlorophyll showed good antioxidant activity.

1) DPPH method:

Chlorophyll showed increase in DPPH scavenging activity with corresponding increase in its concentration (Table no.1). Chlorophyll showed good antioxidant activity with IC_{50} value of 8.8 which was comparable to IC_{50} value of ascorbic acid (Table no.2).

Table 1: Antioxidant activity determined by DPPH method

CONCENTRATION (µg/ml)	% INHIBITION	
	Chlorophyll	Ascorbic acid
2	32.48± 0.33	33.75±0.27
4	46.46±0.65	48.02±1.85
8	48.44±0.85**	95.18±0.55
16	47.03±1.26**	95.32±0.987
32	85.84±0.56**	95.33±1.25
64	79.94±0.75**	96.18±1.52
128	88.55±1.36**	96.89±0.91
256	81.77±1.25**	97.03±0.75
512	88.27±0.87**	95.62±0.64

Value are mean ± SEM (n=3) unpaired t test, where, * represents significant atp<0.05, ** represents significant at p<0.01.

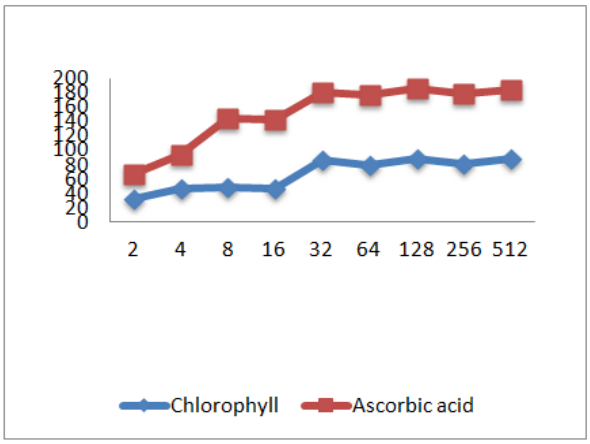

Table 2: IC_{50} values of chlorophyll and Ascorbic acid in DPPH method.

Sl. No.	Solution	IC_{50} values(µg/ml)
1.	Chlorophyll	8.8
2.	Ascorbic acid	4.0

2) Hydrogen Peroxide scavenging method:

Chlorophyll showed increase in Hydrogen Peroxide scavenging activity with Corresponding increase in its concentration (Table no.3). Chlorophyll showed good antioxidant activity with IC50 value of 2.2 which was comparable to the IC50 value of ascorbic acid (Table no.4).

Table 3: Antioxidant activity determined by H_2O_2 Method

CONCENTRATION (µg/ml)	% INHIBITION	
	Chlorophyll	Ascorbic acid
2	48.96±0.566**	8.21±0.55
4	58.09±0.28**	23.02±0.48
8	61.81±0.36**	45.28±0.43
16	64.05±0.56**	95.03±0.85
32	96.42±0.89*	95.06±0.56
64	96.48±1.36	95.07±1.69
128	96.73±0.52	95.09±0.58
256	96.48±1.25	96.0±1.58
512	96.05±0.69	96.04±0.54

Values are mean ± SEM (n=3) unpaired t test, where, * represents significant at p<0.05, ** represents highly significant at p< 0.01.

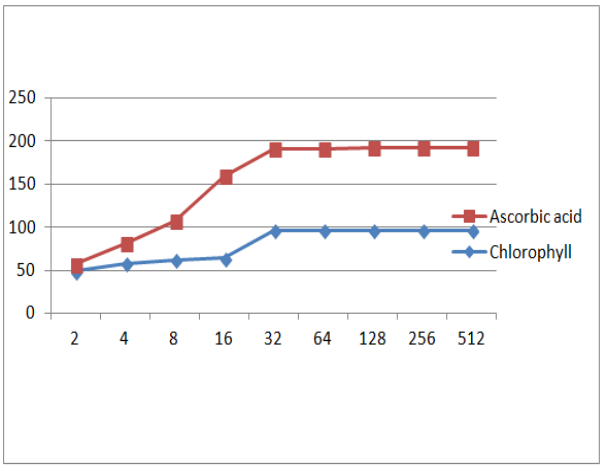

Table 4: IC50 values of Chlorophyll and Ascorbic acid in Hydrogen Peroxide

Sl.No.	Solution	Ascorbic acid
1.	Chlorophyll	2.2
2.	Ascorbic acid	8.8

3) Nitric oxide scavenging method

Chlorophyll showed increase in nitric oxide scavenging activity with corresponding increase in its conc. (Table no.5). Chlorophyll showed moderate antioxidant activity with IC_{50} value of 16 which was comparable to the IC_{50} value of ascorbic acid (Table no.6)

Table 5: Antioxidant activity determined by Nitric Oxide scavenging method

CONCENTRATION (µg/ml)	%INHIBITION	
	Chlorophyll	Ascorbic acid
2	41.16±0.56*	45.31±0.69
4	46.76±0.52**	73.59±1.62
8	49.46±0.412**	72.41±0.659
16	50.21±1.53**	77.26±0.587
32	50.75±1.85**	79.52±0.258
64	50.75±2.1**	81.03±0.159
128	50.00±1.85**	82.32±0.458
256	49.24±0.64	86.53±0.758
512	61.42±0.95**	89.00±0.687

Values are mean ± SEM (n=3) unpaired t test, where, * represents significant at p<0.05, ** represents highly significant at p< 0.01.

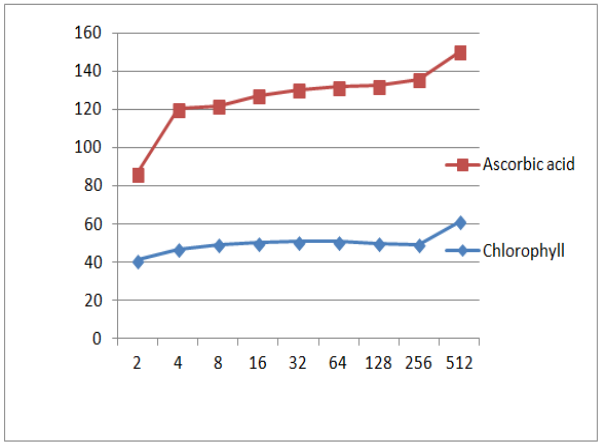

Table No.6: IC_{50} values of Chlorophyll and Ascorbic acid in Nitric oxide Scavenging

1.	Chlorophyll	16
2.	Ascorbic acid	2.2

6. DISCUSSION

Antioxidant activity:

Antioxidants are a class of vitamins and nutritional ingredients that help fight and rid the body of free radicals- the gremlins that can cause untold damage to your body. When the cells and body carry on with their daily functions, oxygen is used in the process and oxidation takes place - and although these are normal functions, they do cause free radicals, the waste material of these processes that can have an influence on the forming of cancer, arterial damage, inflammation, and accelerated aging through oxidative damage. There are restrictions on the use of synthetic antioxidants, such as BHT, as they are suspected to be carcinogenic. Natural antioxidants, therefore have gained importance. In the present study carbon tetra chloride was used to induce oxidative stress and inturn evaluate the antioxidant potential of *Chlorophyll.*[94] Supar chlorophyll is water soluble content that is Chlorophyllin, the form of Chlorophyll used in Unicity Super Chlorophyll. That contains is Mg and copper, and science has confirmed the potential health benefits of chlorophyll as supplemementations.

In vitro antioxidant activity:

DPPH Assay: DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by decrease in its absorbance at 517 nm, which is induced by antioxidants[.[95] The significant decrease in concentration of the DPPH radical is due to scavenging ability of Chlorophyll.

Hydrogen peroxide scavenging: Hydrogen peroxide is generated in vivo by several oxidase enzymes. In this method, when an antioxidant is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide is measured spectrophotometrically.[96] Hydrogen peroxide is a weak oxidizing agent which inactivates enzymes by oxidation of the essential thiol (SH-) groups. It rapidly transverses cell membranes and once inside the cell interior, interacts with $Fe^{2+}$and Cu^{2+} to form hydroxyl radicals, which is harmful to the cell.[97]

Chlorophyll showed good scavenging effects of hydrogen peroxide.

Nitric oxide scavenging: Nitric acid was generated from sodium Nitropruside and measured by the greiss reduction. Sodium Nitropruside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrate ions that can be estimated by the use of greiss reagent. Scavengers of Nitric oxide compete with the oxygen, leading to reduced production of nitric oxide.[98] Significant scavenging activity of Nitric oxide was observed for chlorophyll.

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