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Nutritional Profiling, Antioxidant Potential and Collagen Building Properties of Lacto-Fermented, Microencapsulated Guava Juice Powder

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Abstract

Fermented products consist beneficial probiotics, which makes these products render improved nutritional properties and health effects. There is a need for research to explore and establish the use of commonly existing fruits in fermented forms for application in skincare and as anti aging solutions.This study has been conducted to establish collagen building and antioxidant potential of fermented guava fruit juice powder.The human dermal fibroblast cells were used to determine the collagen building potential while chemical enzymatic assays were used to evaluate antioxidant properties of fermented guava juice powder (FGJP) . The antioxidant activity evaluated by the ability to scavenge DPPH radicals showed very high anti-oxidative capacity (IC50 = 0.015 mg/ml) comparable to ascorbic acid (IC50 = 0.018 mg/ml). This antioxidant potential can be attributed to the presence of various phytonutrients, primarily flavonoid (quercetin equivalent) at a concentration of 38 mcg/mg of the powder. The Collagen building potential was evaluated on the normal human fibroblast cell line . The levels of Collagen IV involved in skin cell migration, regeneration, and wound healing were estimated. FGJP imparted proliferation ability in NHDF cells at the concentrations of 0.025, 0.05, and 0.1mg/ml,which is 31% collagen synthesis against untreated cell control. This explains that fermented guava juice powder has a collagen building potential along with antioxidant properties. Such product would be helpful in providing a plant based anti aging solutions especially for vegan consumers.The optimized fermented guava juice powder has a shelf life of 45 days when stored at refrigerated conditions with a potential for combating oxidative stress and supporting in alleviating the aging symptoms like fine-line, wrinkle through collagen building.

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Introduction

With the emergence of an aging population and their consciousness, more attention is given to skin health.[1] Cosmetics are used for artificial beauty but the real essence lies in one's own healthy skin. Human skin is constantly exposed to ultraviolet light, sunlight, and pollution, here the skin barrier serves as the first line of defense.[2] But in addition, the reactive oxygen species, artificial light, pollution, smoking, age, and dietary habits, severely affect the biology of the skin and this leads to the activation of matrix metalloproteinases causing fine lines and wrinkles among others.[2,3] Numerous approaches are introduced to mitigate the skin defects such as the use of actives that have anti-MMPase activity (anti-collagenase, or anti-elastase),[4] or actives that can modulate the signaling cascade that can increase the elastin and collagen production,[5] or actives that act as a carrier to stabilize trace elements that are involved in wound healing.[6] Cosmetic nutraceutical agents are now extensively in demand as they promote the synthesis of existing peptides and collagen reserves to maintain the skin's integrity.[7]

Accordingly, herbal medicines and cosmetics are now gaining importance for their antioxidative properties, cost-effectiveness, high biodegradability, and their abilities to impart minimum side effects.[8] The origin of ayurvedic cosmetics and the utilization of vegetables, herbs, and fruits to innovate skin-rejuvenating cosmetic products dates back to the Indus valley civilization in India.[9]

Phytochemicals are known for multiple pharmacological and cosmeceutical applications. The fermentation process improves the quality of the active ingredients by facilitating the easy absorption of them via human biological system. Recently, many scientists are working on the cosmeceutical importance of Fermented Plant Extracts (FPE), particularly on anti-aging, anti-wrinkle, and whitening properties of FPE.[10]

Guava is cultivated in all tropical and subtropical countries, thus also known as 'Poor man's apple of tropic'. Guava fruit has commercial applications for its taste, flavour, and perfume. Numerous pharmacological applications have been recorded for it, including the antibacterial action of its leaves and the use of fresh fruit and tea made from its leaves to treat diarrhoea, dysentery, diabetes mellitus, and other conditions.[11] However, due to its rich content, guava is also a potential source of antioxidants and it is been explored for its cosmeceutical potential and has been proven to have photo-protective properties.[18] Fruits are rich in antioxidants and vitamins that help in improving the texture of skin. Loaded with vitamin C, the guava extracts are great for the skin. The extract of guava leaf helps in curing acne and other skin issues. So both eating and applying helps in the foundation of a naturally glowing skin.[19] This study aims at evaluating Spray-Dried Fermented Guava Juice (FGJP) for its collagen building potential and antioxidant capacity, in-vitro. The FGJP was tested for determining the presence of phytochemicals which might be responsible for its antioxidative, skin cell proliferating, and collagen-building ability on human dermal fibro-blast cells.

Materials and Methods

Chemical and Materials

Fresh guava were purchased from local fruit vendors. L.Plantarum was provided by CHR Hansen, as a sample for research purposes.Sodium Hydroxide, Maltodextrin and Inulin and other chemicals of commercial grade were used.

Preparation of Spray Dried Fermented Guava Juice

Fermented guava juice preparation was carried out by fermentation with L. plantarum as per the procedure demonstrated. The process included adjustment of guava juice to 6.4 by NaOH. This was followed by inoculation of commercial freeze-dried DVS culture of L. plantarum at 1% w/v concentration. Temperature was maintained at 30ºC for 72 hr to achieve viable cell count of 10 log cfu/mL.[20] Further,to obtain lacto-fermented guava juice powder, spray drying with optimized process parameters using 5% maltodextrin and 5% inulin (w/v) (MD: INU) as drying aid was performed. The fermented guava juice powder was sealed in air-tight bags and stored protected from light in refrigerated conditions for further study.[12]

Nutritional Profiling

The nutritional composition of microencapsulated lacto-fermented guava juice powder was analyzed using the standard methods published by AOAC.[13] To determine the ash content,the samples were heated at 500 ◦C in a muffle furnace for about 5–6 h, while the total moisture content was determined

by drying the fresh sample in the oven at 100–110 ◦C until constant weight. The crude fat content of spray dried lacto-fermented guava juice powder was estimated by using a Soxhlet apparatus and petroleum ether (60–80 ◦C) as an extraction solvent. The crude protein content was analyzed by the micro-Kjeldahl method as nitrogen equivalent. The determination of crude fiber content, total soluble sugars, reducing sugars, non-reducing sugars and total carbohydrates was done by methods reported.[14] Crude fat refers to the crude mixture of fat-soluble material present in the sample and is commonly extracted in anhydrous ether while using the dry Soxhlet extraction method.

Acsorbic Acid Content in FGJP

Ascorbic acid content of FGJP was deterimined by 2, 6-dichlorophenol indophenol (DCPIP) titration method 5 mL of the ascorbic acid working standard (500µg/5 mL) and 10 mL of 4% oxalic acid were pipetted out into a 100 mL conical flask. The contents in the flask were titrated against the dye solution (V1) until the appearance of a pale pink colour that persisted for a few min. 5 mL of the test sample was similarly titrated against the dye solution (V2). Ascorbic acid content present in the test samples were determined using the formula: Amount of ascorbic content (mg/100g) =(500 x V2 x 25 x 100)/(V1 x 5 x 5) Where; 500 = µg of standard ascorbic acid taken for titration V1 = Volume of dye consumed by 500µg of standard ascorbic acid V2 = Volume of dye consumed by 5 mL of test sample 25= Corresponds to total volume of the extract[35]

Phytochemical Assays

Estimation of Antioxidant Activity

The Antioxidant Activity of the FGJP was analyzed by checking its ability to scavenge 2,2-diphenyl picryl hydrazyl radical scavenging (DPPH) radicals in methanol.[15] The stock solution of DPPH (Himedia) was prepared at 1 mg/mL in 50% methanol in water. Equal volumes of DPPH stock and sample at concentration 0.0625, 0.03125, 0.015, and 0.007 mg/mL were added to the tube. Negative control was set by adding equal volumes of DPPH and methanol. The tubes were kept for incubation in the dark for 30 mins. The absorbance was recorded at 515nm using a spectrophotometer.[21]

The percent radical scavenging activity of the sample was calculated using the formula:

(Absorbance of negative control – Absorbance of the sample)*100/Absorbance of the negative control.

Estimation of Total Flavonoid Content

A volume of 500 µL of different concentrations of Quercitin (10mg/mL in DMSO – dilutions prepared in distilled water to give a final concentration of 0.05, 0.025, 0.0125, 0.006, and 0.003 mg/mL in the experiment) was added to tubes. Sample was prepared at 0.1mg/mL and final concentration in the experiment was 0.05 mg/mL. Sample was also added at 500 µL in the tube. Appropriate blanks were maintained. To all the tubes except sample and standard blank, 30µL of 5% $NaNO_2$ was added. After 5 mins of incubation at room temperature, 30µL of 10% $AlCl_3$ was added and the tubes were incubated for 5 mins. After the incubation period, 200µL of 1M NaOH was added to all the tubes, and the volume of the tubes was made upto 1 mL using distilled water. After an incubation period of 30 mins, all the tubes were read at 510nm. A graph was plotted for the standard quercitin to obtain a standard curve with absorbance at y- axis and concentration of the sample at X-axis. The absorbance of the sample was used to determine mg of quericitin equivalent flavonoid content present in 0.05mg of the sample.[16]

Assessment of Collagen Building Potential on Human Dermal Fibroblast (HDF) Cell Lines

The primary culture of Human Dermal Fibro-blast Cells, Dulbecco's modified Eagle's medium, 100X Penicillin-streptomycin antibiotic solution, and MTT: 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole was procured from Himedia Laboratories Pvt Ltd. Fetal Bovine Serum (FBS) and 0.25 X Trypsin was procured from GibcoTM, Thermo Fisher Scientific, Human Collagen IV Elisa Kit was procured from Krishgen Biosystems. The ELISA plate reader from Thermo Labsystems had a built-in software of MRX revelation for capturing data. All the other chemicals used were of analytical grade. The HDF cells were maintained on DMEM media with 10% FBS and 1% Pen-Strep (100X) with a frequency of media change of every two days. The cells were passaged every third days with 2mL of 0.25X trypsin-EDTA solution for 2 mins, the action was terminated using FBS and the cells were centrifuged at 1500rpm for 4 mins. The supernatant was discarded and the cells were split at 1: 2 in T-25 flasks (Nunc, Nalgene, USA).The cytotoxic effect of the FGJP was assessed on the primary culture of

Human Dermal Fibroblasts (HDF) derived from the juvenile foreskin as mentioned.(17) The cells were trypsinized at 70% confluency and approximately 1 x 104 cells per well of HDF cells in a 96-well plate in DMEM media with 10% Fetal Bovine Serum (FBS) and 1% Pen-Strep 100X. The plate was incubated for 24 hours at 37ºC and 5% CO_2. After 24 hours of incubation CB1 and CB2 at different concentrations were added to the well and the plate was further incubated for 24 hours at 37ºC and 5% CO_2. After incubation, the plate was analyzed for percent cell viability in the presence and absence of the FGJPs using 10µl of 5mg/mL MTT dye. The plate was incubated in dark at 37ºC for 4 hours. The dye was discarded carefully and the formazan crystals were eluted in 100µl of DMSO reagent. The plate was again incubated at room temperature for 30 mins, shaken for 30 mins, and was read at 570nm on the ELISA Plate reader (Thermo Labsystems, MRX revelation software).

Calculation

Percent Viability = t/c x 100 where,
t - optical density of test substance
c - Optical density of the zero control

Table 1: Evaluation of Nutritional Parameters for Lacto-fermented Spray Dried Guava Juice Powder

Nutritional Parameters	Results /100g FGJP
Energy	339.98 kcal/100 g
Protein	2.80 g/100 g
Total Fat	<0.1 g/100 g
Total carbohydrates	81.97 g/100 g
Total Sugar	9.31 g/100 g
Added sugar	0.86 g/100 g
Cholesterol	<1 mg/100 g

The FGJP is a low fat low sugar product which makes it a choice for skin care regime of health conscious population.

Results

Nutritional Parameters

Guava is an excellent source of protein, carbohydrates,vitamins, minerals other major and micronutrients so is known as health boosters.Guava fruit is considered the richest source of vitamin C and other nutrients such as vitamin A, phosphorous, iron and calcium. It also contained polyphenols, flavonoids, saponin, etc. (22) Minerals present in guava fruit were reported as as iron (0.26mg), magnesium (22mg), manganese (0.15mg), phosphorous (40mg), potassium (417mg), sodium (2mg), zinc (0.23mg), lycopene (5204µg).(23) The nutritional contents were analyzed on the micro-encapsulated lacto fermented guava juice powder. The results are shown in Table No. 1.

Acsorbic Acid Content in FGJP

Ascorbic acid is essential for the synthesis of a protein collagen, it is a protein that is important in wound healing process and also in formation of connective tissue. It is an antioxidant compound, and protecting against the harms by reactive molecules i.e. called as free radicals. The Federal Food and Drug Administration have accepted the recommended dietary allowance (RDA) as 60mg/day. Daily minimum intake of vitamin C should be 10-15mg/day for an adult, to prevent scurvy disease as well as to avoid the deficiency of vitamin C.[36]

Ascorbic acid was estimated in FGJP and the contents were found to be 9.8mg/100g. which is almost 50% of fresh fruits.

Phytochemical Parameters

Antioxidant Activity

Antioxidants are molecules which slow down the oxidation process. The oxidation reactions may produce free radicals which damage the cells by starting various chain reactions. Free radicals which damage the cells cause skin aging. Antioxidants terminate the free radicals and stop the chain reactions. DPPH method shows that the guava has remarkable antioxidant contents and these antioxidants dose not damage the human neutrophils.[24]

The FGJP was tested for its antioxidative capacity invitro on DPPH radical. The sample showed very high DPPH radical scavenging activity as the IC50 value obtained was 0.015mg/mL which was comparable to ascorbic acid (0.018mg/mL). The antioxidative potential of guava extracts has rendered a new therapeutic path against the oxidative stress and also a promising candidate

to prevent oxidation induced damage to the skin aging. Hence supporting to inhibit further damage due to various external and internal factors causing oxidative stress.

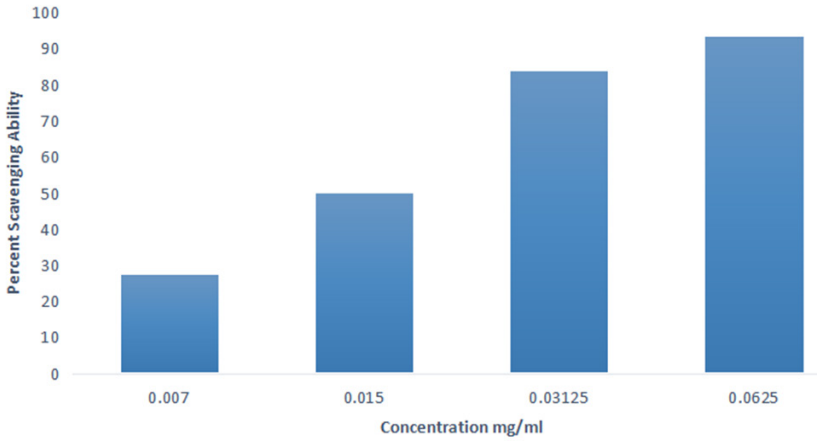

Fig. 1: Percent scavenging activity vs concentration

Total Flavonoid Content

The flavonoids in guava are mainly present in glycoside and ester forms.[25] The free elagic acid and glycosides of apigenin and myricetin and are found to be present in guava.[26] Flavanoid estimation was done in FGJP using the graph plotted for quercitin standard, the linear line equation obtained for the standard curve was y=2.068x + 0.040. After substituting the absorbance for the sample as 0.119 in the curve, the result suggests that 0.05mg/mL of the sample has a flavonoid content equal to 0.038 mg/mL of quercitin, that is 76 mg/100g of FGJP.

Flavonoids may retard and prevent aging-related deterioration of skin aging by targeting cellular pathways important in regulation of cellular senescence.[28]

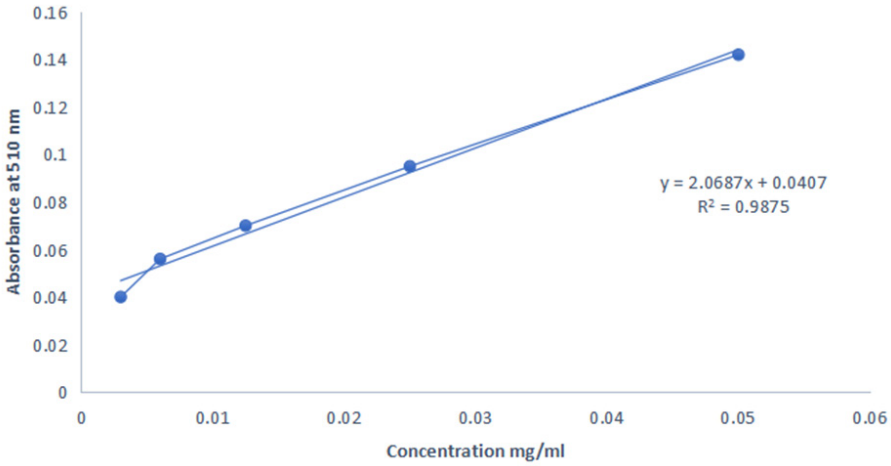

Fig. 2: Absorbance vs Concentration

Effect of FGJP on Cell Viability and proliferation of Human Dermal Fibroblasts

The proliferation of dermal fibroblasts is important in tissue repair as fibroblasts are involved in migration, proliferation, contraction and collagen production, leading to the deposition of the ECM and the formation of granulation tissue.[29] Thus, we investigated the effects of FGJP on the proliferation

of NHDFs at concentrations up to 200 µg/ml using colorimetric MTT assay as represented in Fig. 3.The FGJP was tested for its non-toxic, proliferative activity on human dermal fibroblasts. The cells were treated with different concentrations of FGJP viz., 0.0125, 0.025, 0.05, 0.1, and 0.2 mg/mL. It was found that there was a statistically significant effect on the proliferation capacity of the FGJP on the HDF cells ($p<0.01$ using One-Way ANOVA). All the concentrations of the blend were found to be safe except 0.2 mg/mL where the cell viability decreased as compared to the control of 100% and hence was not considered for measuring the effect of the blend on collagen content of HDF at that concentration. Further the collagen synthesis potential of the FGJP was assessed at these concentrations.

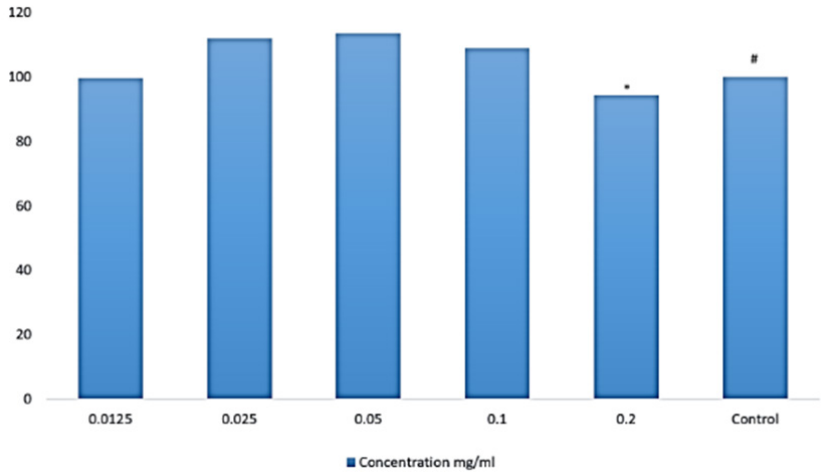

Fig. 3: Proliferation Capacity of FGJP on Human Dermal Fibroblasts (HDF)

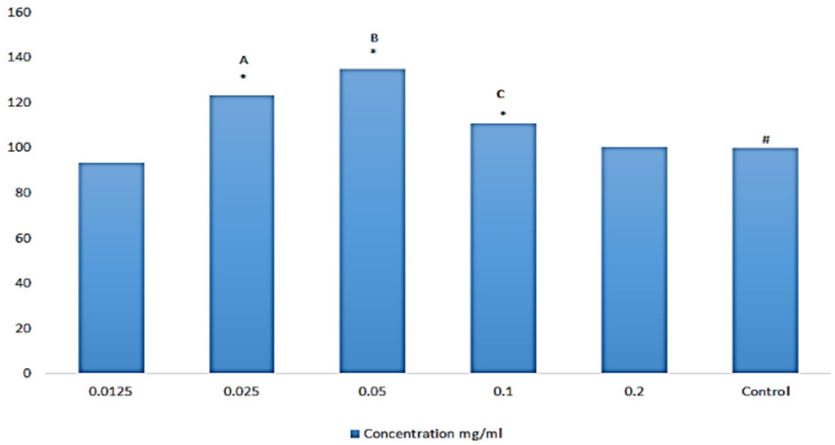

Fig.4: Collagen Type IV Synthesis by SDGJP on Human Dermal Fibroblasts (HDF)

FGJP Increases the Collagen Content in HDF Cells

We further investigated the effect of FGJP treatment on collagen release in HDFs for treatment concentrations ranging from 0.01 to 0.2 mg/ml. Cell culture media samples were collected and total collagen in the media was estimated. Total collagen release into the media showed maximum increase at 0.05 mg/ml.

There was a statistically significant difference that was observed between the treated cells and untreated cells ($p<0.01$) when tested using One-Way ANOVA. At the concentration of 0.0125 mg/mL,

there was no increase observed. At 0.05 mg/mL, an increase in the collagen content was observed in comparison to the untreated cells and it was found to be 31.04%.

Conclusion and Discussion

Skin,being the outermost layer of the human body, is prone to be affected by the external factors such as pollutants, UV irradiation, injury and so on. Skin aging and premature aging is most extensively effected by UV-B exposure in sunlight. While the external causes of aging may be various the effects of aging on skin like wrinkles,fine lines, sagging etc is primarily due to collagen degradation. The collagen damage is triggered due to the oxidative damage within the body which is induced by external factors. Hence, to explore the application of a product in preventing skin aging it is essential to understand how it is helping to prevent collagen damage and build collagen within the body. In this work, we have explored both the aspects of lacto-fermented spray dried guava juice powder.The ability to prevent collagen damage is established via the anti-oxidant potential while the ability to help synthesis collagen is established via cell-line study on human dermal fibroblast cells.

Type IV collagen forms the unique component of the basement membrane of the cells of animal phylum,[30] and once secreted into the extracellular space of the cells,they re-associate to establish interaction between other cells building structures.[31] The bioactivities of Collagen-IV include the adhesion of cells, migration,immobilization of growth factors and regeneration of cells, and subsequent wound healing.[32] Considering, collagen IV has been an integral part of the basal membrane of the cells and is involved in activities like cell proliferation and wound healing, this marker was chosen to study the collagen-building ability of the collagen blend.

Vitamin C (ascorbic acid) is abundant in guava. Ascorbic acid can influence the expression of the procollagen gene, causing collagen organisation and altering fibroblast separation via its effects on the extracellular matrix. A mouthwash made from the root bark is recommended for sore gums, and a gargle made from leaves is recommended for swollen, bleeding gums[32]

Although there is encouraging records that a variety of bioactive compounds derived from vegetables and fruits helps delay skin aging, the possibility of fermented guava juice powder in delaying the skin aging is not being thoroughly established till date. The promising results were seen in both the dimensions studied. 31% collagen synthesis was reported along with significant antioxidant potential, when compared with ascorbic acid.

The analysis of antioxidant content show that lactic fermentation could enrich the contents of antioxidants in guava juice. The significant antioxidant potential of the fermented guava juice powder,ensures that this could be potentially helping to fight against the oxidative stress induced damage of skin.

From the in-vitro assay on the collagen synthesis which was followed by the cyctotoxicity assay on Human Dermal Fibroblast cells,the fermented guava juice powders showed an increase in cell numbers at all concentration, which indicates towards promising collagen building potential with no practical cyctotoxicity.At all the tested concentrations, i.e., 0.0125-0.2 mg/mL, the cell viability was found to be above 80%. At 0.4 mg/mL, an increase in the collagen content was observed in comparison to the untreated cells and it was found to be 31.04%.

This shows that fermented guava juice powder may support in collagen synthesis and hence can act as an anti-aging agent. Based on this research and the results obtained via the cellular models and enzymatic studies it can be interpreted that the lacto fermented guava juice powder could substantially improve skin conditions due to the combined synergistic effect of probiotics and bio-actives from guava.

Human clinical trials need to be done in future to validate these findings along with detailed cellular study to understand the mechanism of action.

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Conflict of Interest

The authors do not have any conflict of interest.

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