ORIGINAL ARTICLE

In vitro and *In vivo* Assessments on Nutraceutical and Safety of Herb-Fruit Based Drink on Female Wistar Rats

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ABSTRACT

INTRODUCTION: The study aimed to assess the safety and nutraceutical properties of ALLURA™ related to women's health and skin beautification. **MATERIALS AND METHODS**: Determinations of total phenolic (TPC) and flavonoid contents (TFC) were done using the colorimetric method, followed by the identification of gallic acid via high-performance liquid chromatography (HPLC). Antioxidant activity was analyzed using 2,2 -diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radicals while its anti-inflammatory activity was measured using enzyme-linked immunosorbent assay (ELISA). Anti-aging and whitening effects were determined by porcine elastase and mushroom tyrosinase activities, respectively. Skin cell growth promotion and rejuvenation were evaluated using in vitro scratch assay. Cytotoxicity assay was done using HSF1184 and 3T3 BALB/c cell lines. While, acute toxicity test was done on two groups (control and treatment) of six Wistar rats each. The nutraceutical properties were evaluated based on proximate analysis. **RESULTS:** ALLURA™ exhibited DPPH-IC^{50} values of 180.40 μg/mL and ABTS-IC^{50} value of 174.40 μg/mL. TPC and TFC were 67.31 mg GAE/g extract and 43.21 mg CE/g extract, respectively while 10.98 mg/g of gallic acid were quantified. ALLURA™ reduced pro-inflammatory cytokines of TNF-α and IL-6 and showed anti-aging (IC^{50}-162.40 μg/mL) and whitening effects (IC^{50}-167.70 μg/mL). ALLURA™ also increased the proliferation of HSF1184 (≤ 1000 μg/mL), producing significant secretion of epidermal growth factor (EGF) and shown to be non-cytotoxic. No mortality was observed at the highest dose of 2000 mg/kg b.w.t. nor the behavioral and morphological changes in rats. The proximate analysis resulted in high content of moisture and low calories. **CONCLUSION**: These findings provided preliminary reports for the first time on the functionality of ALLURA™ for its anti-inflammatory, antioxidant, and nutraceutical properties.

Keywords
ALLURA™, antioxidant, nutraceutical, women's health, herb-fruit drink

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INTRODUCTION

Today, health-conscious communities insist on healthy products, which are natural, safe, effective, and supported by scientific studies. Antioxidants act as free radical scavengers in the body and eliminate the progression of the potentially cancerous cell and help in the body's regular repair and maintenance.[1] Constant inflammation in the body is the root cause of many diseases, especially metabolic diseases. The anti-inflammatory agents act to regulate the inflammation pathways and keep the body in an optimum health state.[2] Therefore, the health supplement with nutraceutical characteristics should have at least such properties as antioxidant and anti-inflammatory.

ALLURA™ is a traditional 'jamu' drink formulated to boost women's general health, improve skin complexion, increase mental alertness, relieve premenstrual symptoms, treat vaginal dryness, and maintain a healthy reproductive

system. It is made using traditional herbs and fruit juices, which is a mixture of Eastern woman's beauty herbs; 'kayu serapat' (*Parameria Leavigata),* 'buah pinang' *(Arecha Catechu)* and 'manjakani' *(Quercus Infectoria)*; a mixture of fruit juices of guava (*Psidium Guajava*), pineapple (*Ananas Comosu*) and date palm (*Phoenix Dactylifera*).

Various scientific studies reported that those ingredients in ALLURA™ possess antioxidant and anti-inflammatory activities.[3-8] Although the individual ingredient used in making ALLURA™ have been proven to possess therapeutic properties, the present study need to be conducted to validate the traditional claims, to justify its possible effectiveness thus supporting the scientifically validated knowledge of ALLURA™ ingredients.

MATERIALS AND METHODS

Sample preparation

ALLURA™ concoction was supplied by Naturemedics Laboratories Sdn. Bhd. Terengganu, Malaysia. One liter of the liquid concoction of ALLURA™ was transferred into several Petri dishes and put in the oven at 50°C for drying. The dried paste was then scrapped and weighted. Both dried paste and liquid of ALLURA™ were kept in tightly closed containers and stored in the fridge at < 15° C until further use.

DPPH scavenging activity

Antioxidant activity was measured using DPPH assay.[9] About 100 µL of the samples at different concentrations were mixed with the DPPH solution (Sigma-Aldrich, USA). The sample solutions were kept at room temperature for 30 mins. Ascorbic acid (Qrec, New Zealand) was used as a standard. The absorbances were measured in triplicates at 515 nm using ELx800 Absorbance Microplate Reader (BioTek Instrument, USA).

ABTS radicals assay

About 7 mM of ABTS stock solution (Sigma-Aldrich, USA) was mixed with 2.45 mM ammonium persulphate (Sigma-Aldrich, USA) and incubated in the dark for 16 hours at room temperature.[9] Prior to assay, the mixture absorbance was adjusted to give 0.70±0.05 at 750 nm. ALLURA™ extract (30 µL) was added to 300 µL of ABTS working solution and let to stand in the dark for 6 min. The absorbance was measured in triplicates at 750nm. Trolox was used a positive control. Radical scavenging activity was calculated based on the following formula:

Radical scavenging activity (%)=[1-(sample absorbance/ control absorbance) x 100

Then, the IC_{50} value was determined from the plotted graph of radical scavenging activity against the concentration of the sample.

Total phenolic content (TPC) and Total flavonoid content (TFC)

TPC was measured according to Folin-Ciocalteu method.[10] The sample (20 µL) was mixed with 100 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, USA). Then, 80 µL of 7.5% w/v sodium carbonate (Na_2CO_3) solution (Sigma-Aldrich, USA) was added to the mixture. Next, the mixture was kept at room temperature for 2 hours. The absorbance readings were measured in triplicates at 760 nm by UV/VIS-spectrophotometer (Schott UVLine 9400, USA) and expressed as milligrams of gallic acid equivalents (GAE)/g dry weight of the extract.

Then, TFC was determined based on colometric method.[11] Initially, 12 µL of 5%, w/v sodium nitrite ($NaNO_3$)(Sigma-Aldrich, USA) was added to the 200 µL of sample and followed by 12 µL of 10%, w/v aluminum chloride ($AlCl_3$) (Sigma-Aldrich, USA) after 5 min. Next, 80 µL of 1M sodium hydroxide (NaOH) (Sigma-Aldrich, USA) was added. The mixture was shaken and the absorbance was measured at 510 nm in triplicates. The result was expressed as mg of catechin equivalence per hundred gram of extract (mg CEQ/ g).

Identification of gallic acid

The presence of gallic acid was identified using high-performance liquid chromatography (HPLC). The sample was dissolved in deionized water at 1000 µg/mL. The

gallic acid standard was prepared in several concentrations to construct a calibration curve. The HPLC separation was conducted through Agilent 1260 series HPLC system (Agilent Technologies Inc., USA). A flow rate at 1.0 mL/min was used with a gradient elution program: 0.0 min, 80% eluent A and 20% eluent B; 10.0 min, 55% eluent A and 45% eluent B. Eluent A consisted of formic acid and water. Eluent B consisted of formic acid and acetonitrile. The column was sustained at 22°C with UV detection of 280 nm.[12]

Anti-inflammatory activity

The assay was carried out by measuring the concentration of pro-inflammatory cytokines of tumor necrosis factor (TNF)-α, interleukin (IL)-6) using lipopolysaccharide (LPS) induced human skin fibroblast (HSF1184) (ATCC CL-193, USA) treated with 10^2 and 10^3 µL of ALLURA™ concentrations.[13] The levels of cytokines from cell supernatants were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol (Invitrogen™, USA). The intensity of the color was measured at 450 nm using ELx800 Absorbance Microplate Reader (BioTek Instrument, USA).

Anti-aging assay

The assay was measured using porcine elastase assay.[14] The enzyme solution was prepared by dissolving porcine pancreatic elastase in Tris–Cl buffer. While the substrate solution was prepared by dissolving N-succinyl-ala-ala-ala-p-nitroanilide in Tris-Cl buffer. Next, 10 µL of the sample was incubated with 130 µL of the enzyme for 5 mins at 25 °C before adding 15 µL of substrate. The positive control was epigallactocatechin gallate (EGCG). The absorbance was measured in triplicates at 410 nm using ELx800 Absorbance Microplate Reader (BioTek Instrument, USA).

Whitening assay

Whitening effect was measured using mushroom tyrosinase assay.[15] About 140 µL of the sample was mixed with 40 µL of 10 µg/mL of mushroom tyrosinase solution and incubated for 10 mins at room temperature. The absorbance was measured in triplicates at 405 nm using ELx800 Absorbance Microplate Reader (BioTek Instrument, USA). Ascorbic acid was used as a positive control. The enzyme inhibition activity was calculated based on the following formula:

Enzyme inhibition activity (%): [1- (sample absorbance/ control absorbance) x 100]

Skin cell growth promotion and rejuvenation

Briefly, human skin fibroblast (HSF1184) (ATCC CL-193, USA) HSF1184 was seeded into a 12-well microplate and incubated for 24 hours.[16] After incubation, the confluent cells monolayer was scratched linearly and exposed to the sample at various concentrations. Platelet-derived growth factor (PDGF) was used as a positive control. The width of the scratch was measured and the image was taken at 0, 12, and 24 hours. Next, the production of epidermal growth factor (EGF) was analyzed via enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Invitrogen™, USA). The cell lysate from the scratch assay was collected for EGF bioassay.[17]

Cytotoxicity test

HSF1184 cell line was grown in the growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, and 1% Penicilin-Streptomycin.[18] Meanwhile, 3T3 BALB/c (ECACC 90011883,UK) cell line was grown in DMEM, 5% fetal calf serum and 1% Penicilin-Streptomycin. All the chemicals were purchased from Gibco, Scotland.

Next, the cytotoxicity effect was determined using neutral red uptake analysis. The cells were seeded in 96-well tissue culture plates and treated for 2 hours with a medium containing neutral red. The cells were subsequently washed with phosphate-buffered saline (PBS), the dye was extracted in each well and the absorbance was measured at 540 nm using ELx800 Absorbance Microplate Reader (BioTek Instrument, USA). The lC_{50} was determined from a plotted graph using Graphpad Prism Version 6 software. The LD_{50} value was estimated based on the following formula:

$$LogLD_{50} = 0.372 \text{ X } logIC_{50} (\mu g/mL) + 2.024$$

Meanwhile, relative cell viability was calculated based on the following formula:

Relative cell viability (%): (absorbance of sample/ absorbance of control) x 100

Acute toxicity study

The study was approved by the Ethics Committee and Research Management and Innovation Centre (Ref. No.:UMT/JKEPT/2018/20) of Universiti Malaysia Terengganu (UMT).[19] The handling and fixed-dose procedures of the animals were according to the established public health guidelines in The Organization for Economic Cooperation and Development (OECD) guidelines 420 for acute toxicity study (OECD, 2002).[20] The animals were individually housed in cages under a 12-h light and 12-h dark cycle in a temperature-controlled room (25-27°C). Before the experiments, the rats were acclimated for a week and allowed free access to a standard laboratory animal diet and water ad libitum. The female rats were divided into two groups with 6 rats each as shown below:

Group control = Normal rats treated with water ad libitum.

Group 1 = Normal rats treated with 2000mg/kg.

ALLURA™ extract was administered using oral gavage. Close observation was made during the first four hours to examine any adverse effect symptoms such as abnormal behavior, abnormal posture, diarrhea, blood in urine, and an increase in heartbeat caused by ALLURA™ consumption.

Physicochemical and proximate analyses

Lotus Laboratory Services (M) Sdn. Bhd. Malaysia conducted the analyses according to Pearson's Chemical Analysis of Food and Association of Analytical Chemists (AOAC) method.

Statistical analysis

One-way analysis of variance (ANOVA) was performed using GraphPad Prism V 6.0 (GraphPad Software Inc., San Diego, CA, USA); Dunnett comparison test was chosen as *a post hoc* analysis method and was considered to be statistically significant. The results were expressed as mean (SD).

RESULTS

Phytochemical compounds and antioxidant activities

Based of Table I, ALLURA™ yielded 67.31 ± 0.90 mg GAE/g of TPC and 51.00 ± 2.20 mg CE/g of TFC value. It also showed an antioxidant potential through DPPH and ABTS assays in a concentration-dependent manner. In the HPLC analysis, as can be seen in Figure 1, gallic acid was identified according to the retention time of its peak against the standard as well as the spiked sample with the standard. The quantification of gallic acid also detected 10.98 ± 0.02 mg/g in the sample.

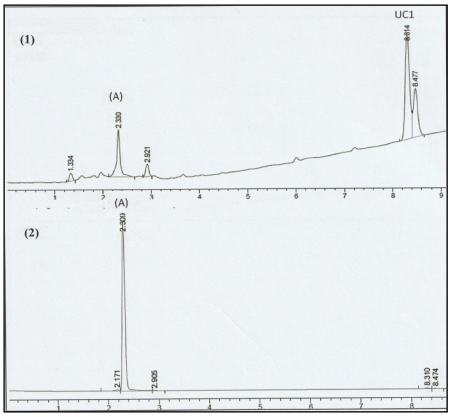

Figure 1: HPLC analysis of gallic acid (peak A) of ALLURA™ (1) and spiked ALLURA™ (2) with the gallic acid standard.

Table I: Antioxidant properties of ALLURA™

Assays	Values
TPC (mg GAE/g extract)	67.31 ± 0.90
TFC (mg CE/g extract)	51.00 ± 2.20
DPPH (IC_{50} µg/mL)	149.90 ± 2.92
ABTS (IC_{50} µg/mL)	174.40 ± 2.70
Gallic acid content (mg/g)	10.981 ± 0.02

Anti-inflammatory activity

Figure 2 indicates the reduction in pro-inflammatory cytokines of TNF-α and IL-6 in LPS induced HSF1184 cell lines in a dose-dependent manner. This result confirmed that ALLURA™ inhibits the pro-inflammatory cytokines in LPS-stimulated HSF1184 cell lines.

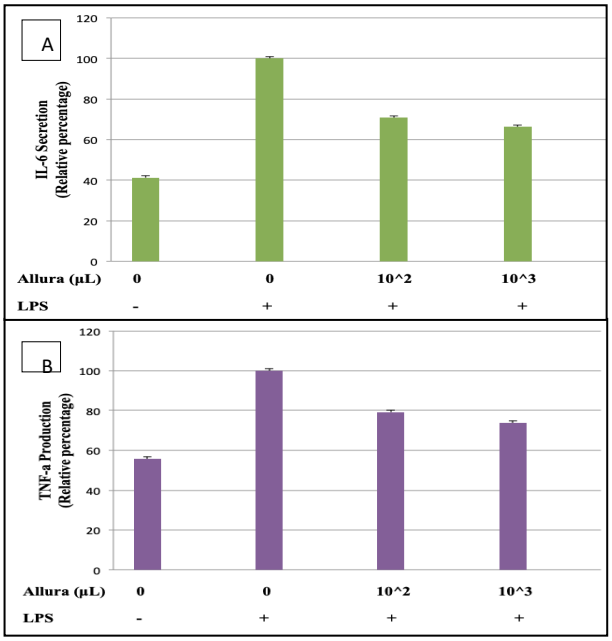

Figure 2: Percentage of pro-inflammatory cytokines secretion when exposed to different concentrations of ALLURA™. Indicated results are expressed as mean (SD) (n=3). A= IL-6; B= TNF-α. Symbol – indicated no induction by LPS. Symbol + indicated an induction by LPS.

Anti-aging and whitening assays

The anti-aging assay showed that ALLURA™ significantly inhibited elastase enzyme ($p<0.05$) with an IC_{50} value of 162.40 µg/mL. ALLURA™ also showed a significant inhibition against tyrosinase ($p<0.05$) with IC_{50} value of 167.70 ± 2.34 µg/mL (ascorbic acid=15.86 ± 0.18).

Skin cell growth promotion and rejuvenation

As shown in Figure 3, ALLURA™ effectively increased the proliferation and migration of HSF1184 cells at concentrations of 0.001-1000 µg/mL within 24 hours. This indicates that ALLURA™ could stimulate the growth of fibroblasts and repair the tissue. Additionally, a significant secretion of EGF from 110 to 180% (relative cytokine viability) was measured in ALLURA™ -treated HSF1184.

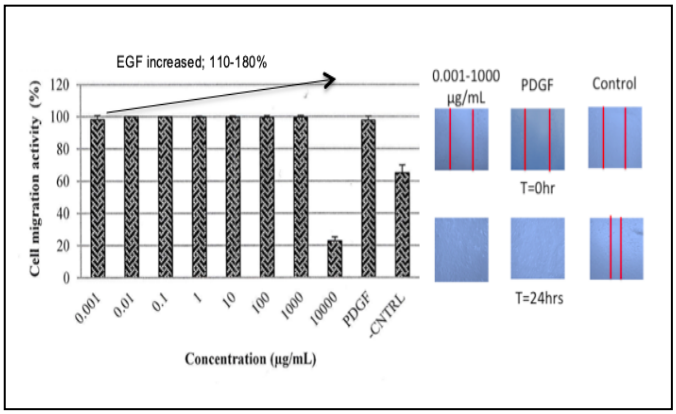

Figure 3: Relative percentage of HSF1184 cell migration within 24 hours with the increment of EGF. Indicated results are expressed as mean (SD), n=3.

Safety analysis

The cytotoxity test showed that ALLURA™ was non-cytotoxic at concentrations below 1000 µg/mL when exposed to two cell lines of HSF1184 and 3T3 BALB/c (Figure 4). The calculated IC_{50} values of ALLURA™ were 3145 µg/mL (HSF1184) and 3151 µg/mL (3T3BALB/c). Meanwhile, the calculated LD_{50} of ALLURA™ were 2114.05 µg/mL and 2115.54 µg/mL for HSF1184 and 3T3 BALB/c, respectively. The estimated starting dose for *in vivo* study based on OECD 425 protocol was around 1057 mg/kg (using factor 0.5).

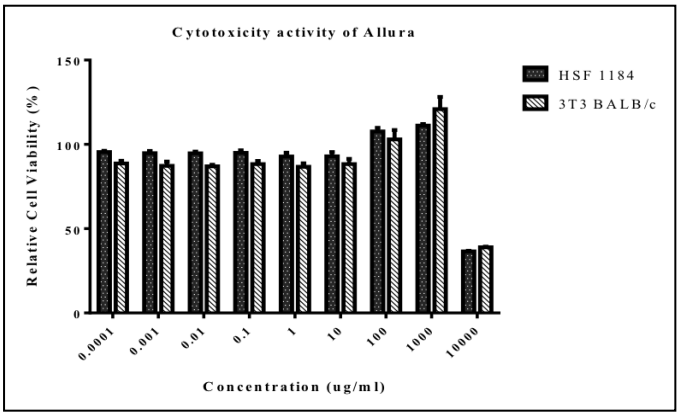

Figure 4: Cytotoxicity effect of ALLURA™ exposed to HSF1184 and 3T3 BALB/c cell lines. Indicated results are expressed as mean (SD), n=6.

The safety of the concoction was also evaluated *in vivo* using an acute toxicity study. An oral administration of ALLURA™ with a maximum dose level of 2000mg/kg did not produce any mortality or alter the behavior, breathing, and nervous responses. The fur, skin, eyes, and nose of all animals are also normal. No convulsion, tremors, salivation, or diarrhea were observed.

Physicochemical and proximate analysis

The proximate composition, pH, color, and taste of the ALLURA™ concoction are shown in Table II. Based on the proximate analysis, low calories (59 kcal/100g) and carbohydrate (14.7%) content were observed.

Table II: Physicochemical and proximate composition of ALLURA™

Parameters	Values
pH	4.45
Colour	Chocolate
Taste	Bittersweet
Protein, % (w/w)	Not detected
Saturated fat, % (w/w)	Not detected
Carbohydrate, % (w/w)	14.7
Calories, kcal/100 g	59.0
Ash, % (w/w)	0.3
Moisture, % (w/w)	85.0

DISCUSSION

Based on the TPC result in this study, ALLURA™ is considered rich in phenolic content. According to Lai and Lim,[21] the sample that has TPC value >20 mg GAE/g sample is categorized as having a high phenolic content. Meanwhile, TFC value of the present study was high as compared to the TFC value of herbal tea from a commercial brand, 17.92 mg QE/mL.[22]

DPPH is usually expressed as IC_{50}, the amount of antioxidant necessary for decreasing the initial concentration of DPPH by 50%. When the IC_{50} value of the sample was lower, the antioxidant activity was higher.[23] The IC_{50} value of the present study showed that ALLURA™ has a high antioxidant capacity as compared to the previous data from Dian-Nashiela *et al.*[22] who reported that *C. caudatus* herbal tea extracted from the leaves had an lC_{50} value of 1055 μg/mL.

In the ABTS assay, the ABTS radical forms a blue-green solution. As antioxidants within the sample reduce the ABTS+ to ABTS−, the blue-green color fades. This change is monitored spectrophotometrically.[24] The ingredients of ALLURA™ contained 'manjakani' and date palm, both of which have high antioxidant activities.[25,26] Based on the HPLC analysis, the presence of gallic acid was detected in ALLURA™. This explained the capability of ALLURA™ in scavenging the radicals in DPPH and ABTS assays. According to Daglia *et al.*[27], gallic acid is regarded as a strong antioxidant by scavenging the reactive oxygen species (ROS).

The production of IL-6 and TNF-α in the LPS-induced HSF1184 were suppressed by the extract suggesting a potential anti-inflammatory effect. The anti-inflammatory effect observed in this study might be related to the antioxidant property of ALLURA™. According to Ranaweera *et al.*[28], the phenolic and flavonoid compounds exert an effective role as anti-inflammatory factors. According to one study, the anti-inflammatory of the herbal supplement was reported in R38 water extract as it decreased the production of IL-6 and TNF-α in the LPS-induced HSF1184.[29]

The elastase enzyme is responsible for elastin degradation in the extracellular matrix of the dermis.[30] Since elastase causes wrinkles and aging in the skin, an assessment of the anti-elastase activity of a plant extract can be a useful indicator of its potential application in cosmetic agents. ALLURA™ exhibited potent anti-elastase activity and this anti-elastase activity could be attributed to the presence of phytochemical compounds as reported in previous studies wherein phytochemical compounds exhibited significant elastase inhibitory properties.[31]

Meanwhile, for the whitening effect, the inhibitory activity of ALLURA™ was determined against tyrosinase, an enzyme that is responsible for melanin synthesis. It is well known that tyrosinase plays a critical role in catalyzing the melanogenic pathway since it promotes the production of reactive metabolites in the process of melanin formation.[32] The capacity of ALLURA™ as a whitening agent is close to the previously reported in the extract of Tunisian medicinal halophytes, which had an IC_{50} value of 125 μg/mL.[14]

Scratch assay is a widely applied *in vitro* technique to understand the wound healing capability of medicinal plant extract.[16] Based on the result observed in this study, ALLURA™ was proved to enhance wound healing.

Significant secretion of EGF was also observed in this study, indicating that it could promote cell proliferation and thus enhance skin rejuvenation.[33] The safety profile of the concoction in terms of production and potential toxicity to the body were also evaluated. *In vitro* cytotoxicity was done to predict potential toxicity using the cultured cells.[34] In the current work, the relative cell viability increased up to 1000 μg/mL of concentration, indicating that ALLURA™ was non-cytotoxic. In the previous study, a herbal supplement namely R-38 showed a similar result as it also showed a non-cytotoxic effect against the HSF1184 cell line. This herbal product contains traditional herbs such as galangal, temulawak, and cat's whisker.[29] Meanwhile, no mortality was reported and no changes in behavior were observed in the animal model indicating that ALLURA™ is safe to be consumed.

The physicochemical and proximate analyses were measured to obtain the nutritional values of ALLURA™. This information is important to ensure that the product meets the appropriate requirement as well as the safety of the end product. High moisture (85%) content was detected, which might be due to the high mixture of fruits in the concoction. There was also no detection of saturated fats and protein. This observation is ideal for a low-calorie drink with no fat content.

CONCLUSIONS

Scientifically, ALLURA™ possesses antioxidant and anti-inflammatory activities; low cytotoxicity and promotes cell proliferation through EGF induction. ALLURA™ also has the potential as an anti-aging and whitening agent. ALLURA™ is in the liquid form and could be innovatively processed into powder form. It is also can be used as one of the active ingredients in the future development of botanical health drinks to amplify its benefits.

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