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RESEARCH ARTICLE

Antioxidant analysis of protein from fresh and dry leaf of *Orthosiphon aristatus* (Blume) Miq.

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Abstract

Orthosiphon aristatus (Blume) Miq. is a folklore plant consumed as brewed tea for various health benefits. The white variety of the plants leaf and stem are predominantly sold in the market in dried form, blooming as a natural herbal product. To date, no proteomics and antioxidant studies are available on the fresh and dried leaf protein extract of *O. aristatus* since most studies take an interest in the crude extracts of plants. Thereupon, this study focuses on the One-dimensional (1D) electrophoretic pattern of the fresh and dry leaf determined via sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Additionally, antioxidant proteins are currently being focused on for their link to controlling disease through their potential to destroy free radicals that are present in excess. Hence, antioxidant assays were conducted for the fresh and dry leaf protein extract using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and ferric reducing antioxidant power (FRAP) assay. The electrophoretic pattern obtained resulted in approximately 16 and 10 bands for the fresh and dry leaves respectively. As for the antioxidant activity, the dry leaf had higher radical scavenging activity and higher reducing power compared to the fresh leaf. The findings obtained provides insight in to the protein content and antioxidant activity found in the protein extract of fresh and dry leaves of *O. aristatus*. Hence, further detailed study of the protein extracts could further unveil their therapeutic potential.

Keywords

Orthosiphon aristatus ; electrophoresis; protein; antioxidant; DPPH; FRAP

Introduction

Despite living in a modern society, traditional medicinal plants are still being prioritized by consumers as an alternative to modern medicines because of their role in treating and preventing various human diseases due to their bioactive compounds and therapeutic effects (1). In Malaysia, traditional plants such as *Aloe vera* (Lidah buaya), *Piper sarmentosum* Roxb. (Daun sirih), *Eurycoma longifolia* Jack (Tongkat ali) and *Centella asiatica* (L.) Urb (Pegaga) (2) can be easily obtained since the climate and natural surroundings make plants easy to grow and cultivate. Many parts of the plant depending on their nutritive values are also being commercialised in various forms such as capsules, tablets, tonics and herbal teas (3). However, in order to promote and commercialize natural products for their efficiency,

detailed studies such as pharmacological activities, bioactive compounds, chemical structures, the interaction of specific target molecules and mechanisms of action will be helpful in expanding the knowledge of the products, which can be further investigated for specific drug developments (4).

Similarly, *O. aristatus* leaves have been traditionally used in Malaysia for the treatment of chronic and infectious diseases, including edema, hepatitis, diabetes, jaundice, influenza and hypertension. *O. aristatus* is among the traditional plants in the limelight due to research claiming their potential in different disease treatment and prevention, such as cancer, gout and detoxification (5). Orthosiphon tea has been very well known for various properties such as antioxidant, cytotoxic and anti-inflammatory while functioning as a diuretic for menstrual disorders, kidney stones and abdominal pains (6). Till date, there has been more than hundreds of chemical compounds found in the plant and they have been categorized into their respective type of bioactive groups. The rich content of bioactive compound groups, such as terpenoids, polypropanoids, polymethoxylated flavonoids and phenolic acids, further instigates the medicinal and pharmacological properties of *O. aristatus* (7). It was also reported that *O. aristatus* exhibited good antioxidant ability when extracted using different solvent and extraction methods (8). Compounds such as rosmarinic acid was found to be the highest phenolic compound in the leaf and root of *O. aristatus* while the presence of ursolic acid at a high level was detected relatively higher in the roots and stems (9). Ursolic acid is known as a good anticancer agent while rosmarinic acid is often related to its cellular protective effects. Scientific studies have shown that *O. aristatus* extracted using various solvents such as 50 % methanol, pure methanol, water and chloroform to exhibit free radical scavenging activity when tested using 1,1-diphenyl-2-picrylhydrazyl (DPPH) *in vitro* model system (10). Nevertheless, it can be noted that most of the studies have been limited to crude extracts of the plant regardless whether it is fresh or dry. The possible potential that can be obtained from the protein extracts of the plant itself remains clueless.

With current advancement in proteomics, proteins and peptides discovered from medicinal plants have been proven to be active therapeutically, becoming an emerging subject of interest in research. For instance, a study reported that a protein in *O. aristatus* named transketolase (TKT) acts as an anti-diabetic protein by impeding hyperglycaemia damage-inducing pathway stimulations (11). Nonetheless, though identification of plant protein has been conducted in vast numbers, only a small amount has been found in databases. Inaccessible and uncharacterised plant proteins may play a crucial role in the development and discovery of protein drugs (12). *Orthosiphon aristatus* has been majorly consumed in the dry form, which can be easily obtained as it is commercialised as dry leaves, dry stems and in tea bags, though some consumers may utilize them in a fresh state. This study may provide preliminary insights on the protein content of the commercialised product being produced in the market. Current studies are still focusing on obtaining the best antioxidant ability of *O. aristatus* in different solvents and by targeting different factors such as genotype, drying methods, drying temperature and environmental factors (13). Hence, with little to no studies on the protein content and antioxidant capacity of the protein extracts to be specific, the present study was outlined to differentiate the protein content in the fresh and dry leaf of the white variety *O. aristatus,* followed by comparisons on the antioxidant potential of the protein extracts.

Materials and Methods

Plant material and sample collection

The white variety *O. aristatus* was grown in the green house at T02, Faculty of Science, Universiti Teknologi Malaysia (UTM), Skudai, Johor, Malaysia. The plant was verified by the Natural Product Division of the Forest Research Institute Malaysia (FRIM) with SBID number (001/19). Young green leaves as shown in Fig. 1 (a) were harvested at an approximate age of 2-3 weeks for further analysis. Commercialised dry leaves as shown in Fig. 1 (b) were purchased online from a company in Selangor.

Fig. 1 (**a**). *O. aristatus* fresh leaf. (**b**). *O. aristatus* commercialised dry leaf.

The harvested fresh and dry leaves were ground into a fine powder using liquid nitrogen. The fine powder obtained was wrapped and sealed securely in aluminium foil prior to being stored in -80 °C refrigerator until protein extraction was conducted.

Protein extraction

Protein extraction was done using Phenol/SDS buffer with three preliminary washes (14). The initial protocol with suggestion of 330 mg, starting material was modified accordingly based on the protein band quality obtained when qualified via SDS-PAGE. Starting material of 330 mg was doubled up to 660 mg for the fresh leaves while 330 mg of dry leaves were used for the protein extraction. Ground samples were transferred into microcentrifuge tube followed by addition of 1 mL 10 % TCA/Acetone. The sample was centrifuged at 4 °C by using an Eppendorf MiniSpin plus® microcentrifuge for 15 min at 13000 rpm. Next, the supernatant was discarded and added with 0.5 mL 80 % (v/v) methanol and 0.5 mL 0.1 M ammonium acetate, followed by vortexing and centrifugation for 15 min at 13000 rpm, 4 °C. Similarly, the supernatant was discarded, added with 80 % (v/v) acetone, followed by vortexing and lastly centrifuged for 15 min at 13000 rpm at 4 °C. Then, samples were let to air-dried at room temperature on ice to eliminate any acetone residue. After that, 0.6 mL of phenol and 0.6 mL of SDS sample buffer in 5 % (v/v) of β-mercaptoethanol was added to the pellet. The mixture was thoroughly mixed and placed on ice for 5 min before centrifuging at 13000 rpm for 15 min at 4 °C. Finally, the upper phenol phase was transferred to a new microcentrifuge tube and added with 1.2 mL of 0.1 M ammonium acetate in 80 % (v/v) methanol and kept overnight at -20 °C. The following day, the mixture was proceeded with similar centrifugation step. Afterwards, the supernatant was removed and 100 % (v/v) methanol was added, followed by the centrifugation step. The final washing step was done using 1 mL of 80 % (v/v) acetone and centrifuged at 13000 rpm for 15 min at 4 °C. The supernatant was discarded and the protein was air dried. Lastly, the pellet was suspended in 0.15 mL of SDS sample buffer added with 5 % (v/v) β-mercaptoethanol and stored at -80 °C prior to use.

Protein quantification and qualification

The Bradford assay (15) was used to quantify the total protein extracted from *O. aristatus* leaf to obtain concentrations, yield and amount. Bovine Serum Albumin (BSA) was used as a standard to plot the standard curve. Series of BSA concentration of standard concentration were prepared from 100 µg/mL stock solution and topped up with ultra-pure water up to 0.5 mL final volume. Meanwhile, protein samples were diluted to 100x by adding 10 µL of protein samples and 0.49 mL of ultra-pure water. 0.5 mL of Bradford reagent was added to both BSA standard and protein samples and vortexed briefly. Next, samples were incubated at room temperature for 30 min and absorbance readings were measured at 595 nm using a spectrophotometer.

The SDS-PAGE was used to determine the one-dimensional electrophoretic protein pattern of *O. aristatus* leaf. The extracted protein's quality was determined via SDS-PAGE by running it on 12 % (w/v) of SDS-PAGE gel and viewed it under the gel documentation system. Approximately 5 ug of extracted protein samples were mixed with SDS sample buffer with 5 % (v/v) β-mercaptoethanol in a ratio of 1:1. The samples were centrifuged at 15000 rpm for 1 min and heated at 95 °C for 4 min prior to being loaded in gel wells. Separation was done using SDS-PAGE on a Bio-rad Protean II system.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

The DPPH assay (16) with slight modifications was carried out by diluting 20 µL of sample with ultra-pure water (10x) prior to the addition of 1.48 mL of 0.1 mM DPPH reagent (3.94 mg in 100 mL 100 % Methanol). The sample was incubated at 37 °C for 30 min for the reaction to take place, followed by absorbance reading measured at 517 nm. The radical scavenging activity (RSA) of the crude extracts was calculated using formula;

$$\% \text{ RSA} = [(\text{Abs control} - \text{Abs sample}) / \text{Abs control}] \times 100.$$

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay (17) with slight modification was carried out using 30 µL sample diluted 10x using ultra-pure water topped up with 90 µL ultra-pure water followed by 0.9 mL of FRAP reagent. The samples were vortexed vigorously and incubated at 37 °C for 30 min to allow the reactions to take place. The solution absorbance was measured at 593 nm. A standard curve of absorbance (A593) against $FeSO_4.H_2O$ standard concentration was constructed using 4 mM $FeSO_4.H_2O$ stock solution. The FRAP values (Fe^{2+}/g) of samples were determined using the equation from the standard curve.

Statistical analysis

The experiments were carried out in triplicate and expressed as mean ± standard deviation (S. D). The antioxidant activity of *O. aristatus* protein was analyzed using t-test and p-value <0.05 was considered significant.

Results and Discussion

The protein quantity of *O. aristatus* fresh and dry extracts is presented in Table 1 and the quality of the electrophoretic patterns is shown in Fig. 2. According to Table 1, the fresh leaf has a higher protein concentration and amount, however with a lower yield amount compared to the dry leaf. This could be due to the difference in weight of

Table 1. Protein quantification of the fresh and commercialised dry leaf via bradford assay.

Sample	Protein concentration ± SEM (µg/mL)	Protein amount ± SEM (µg)	Protein yield ± SEM (µg/mg)
Fresh leaf	869.6 ± 19.9	130.44 ± 3.0	197.64 ± 4.5
Commercialised dry leaf	656.8 ± 8.9	98.52 ± 1.3	298.55 ± 4.0

starting material used. By referring to the protein marker based on Fig. 2, high band count can be observed in the fresh leaf protein with an approximate total of 16 bands at 9, 14, 17, 19, 23, 25, 32, 35, 40, 46, 48, 55, 63, 70 and 180 kDa. Majorly, intense protein bands can be observed around 63, 55, 46, 35, 25, 25 and 23 kDa, similar to a study done on irradiated and non-irradiated *O. aristatus* leaf samples (18). This indicates that the dominant proteins are present in the fresh leaves of this study as well.

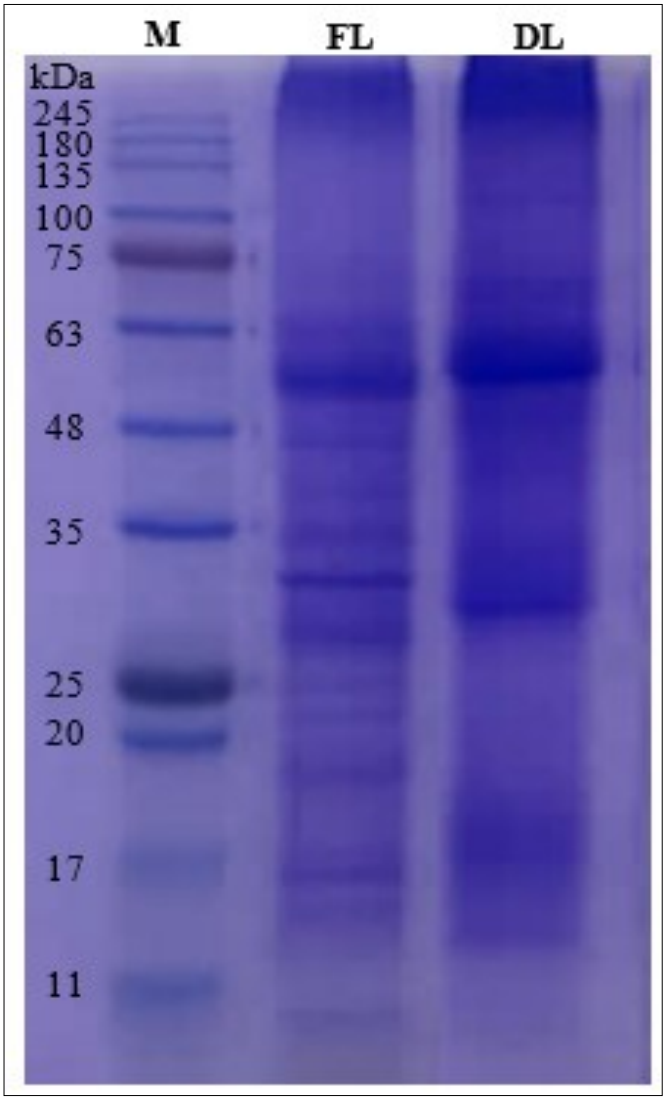

Fig. 2. Electrophoretic pattern of fresh and commercialised dry leaf. Proteins were run on 12 % (w/v) SDS-PAGE gel and each lane was loaded with 5 µg of protein. Lane **FL** represents fresh leaf; Lane **DL** represents dry leaf and **M** represents Marker used for reference. Protein bands identified was labelled according to molecular weight (kDa).

Meanwhile, the dry leaf protein has approximately 10 band counts at 14, 17, 28, 33, 44, 50, 60, 69, 100 and 240 kDa. Compared to the fresh leaf protein bands, the dry leaf had a reduction of 37.5 % of protein bands. The majority of proteins were seen to be lost in the range of 20 to 60 kDa for the dry leaf which are at 23, 25, 32, 35, 40, 46, 48 and 55 kDa. The finding indicates that the process of drying causes protein loss in the leaf. The reduction of protein band counts observed could be due to denaturation of proteins and hampering in protein synthesis. Proteomic analysis done on drought stress induced spring wheat of Bahar and Kafir cultivar observed reduction in protein spots viewed via 2D-SDS PAGE (19). Disruption in protein synthesis and elevated protein degradation may explain the decrease in protein content in the leaves especially, when drought stress is applied (20). A study observed almost similar reduction in the protein of 31.6 % in dry *Murraya koenigii* leaves compared to fresh leaves (21). Nevertheless, some intense bands could also be observed in the dry protein extract approximately at 28, 33, 44, 50 and 60 kDa which may possibly indicate the presence of heat shock proteins as a defence mechanism towards heat stress (22). Additionally, plants may also adapt and alter their metabolism for osmolytes productions and secondary metabolites that facilitates tolerance to stress for survival during heat or stress conditions. Reduction in protein content was observed in *Mentha piperita* and *Catharanthus roseus* when exerted with heat and drought stress which may have caused the protein biosynthesis to be decreased or inhibited and supressed heat shock proteins (20).

Table 2 explains the antioxidant activity of *O. aristatus* protein extracts. The DPPH assay resulted in fresh and dry leaves protein extracts having a very slight difference in the radical scavenging activity with no significant difference at 95 % confidence interval. The fresh leaf had a radical scavenging activity of 44.51 ± 4.96 % while the dry leaves had a radical scavenging activity of 45.68 ± 3.63 %. Next, the ferric reducing capability of both plant extract done using FRAP assay, showed that the dry leaf had a higher capability as compared to the fresh leaf and was significantly different ($p<0.05$) with value of 5172.42 ± 168.0 and 2603.35 ± 120.97 respectively. Both assays mechanism differs when measuring the antioxidant ability in which the DPPH assay detects the scavenging ability of free radical while the FRAP assay test the ability of the sample to reduce Fe^{3+} to Fe^{2+}. Regardless, both plant extracts still has the presence of antioxidant activity though the values could be the influence of tolerance by the plant to different environmental and abiotic stress.

Table 2. Antioxidant activity of fresh and dry leaf protein extract. Values are mean ± SEM in triplicates. * Indicates statistical difference with p-value < 0.05.

Assay	DPPH	FRAP
Samples	**Radical scavenging activity (%)**	**Reducing ability (Fe^{2+}/g)**
Fresh leaf	44.51 ± 4.96	2603.35 ± 120.97*
Dry leaf	45.68 ± 3.63	5172.42 ± 168.00*

Possible production of more antioxidant proteins in the plants due to heat stress may imply that antioxidant capability, though affected by heat and stress, it is still enhanced. Hence, possibly indicating more benefit of the leaves in a dry state as compared to fresh (23). *O. aristatus* leaves showed a high radical scavenging activity ranging from 78 % to 84 % despite being applied with different drying method (24). Almost similar findings were obtained on fresh and dry leaves of *Ocimum gratissimum* and *Petivera alliacea* in which the dry leaf had a higher antioxidant activity. The values obtained was possible to be affected by moisture content, hence making dry leaves extract to be more concentrated due to their dry state (25).

Additionally, *O. aristatus* is well known for its high phenolic and flavonoid content, while having antioxidant compounds such as rosmarinic acid, eupatorin and sinensitin (26). The presence of such antioxidant compound may be enhanced or reduced at different environmental temperature contributing the antioxidant activity of the plant towards oxidative damage. For instance, fresh *M. piperita* and *C. roseus* had better scavenging activity and high reducing power compared to the plants subjected to heat and drought stress (20). Environmental stress in plants can cause the plants biochemical and physiological response to be altered in order to overcome, abstain or counteract the stress effect (27). Many changes may occur in plants such as depletion or accumulation of metabolites, modification in terms of enzyme behaviour and interestingly, induces the production of novel proteins (28).

Many research such as (29) and (30) have been conducted to study the effect of different drying method on *O. aristatus* leaf by evaluating their bioactive compounds, protein content, pharmacological activities and henceforth. *O. aristatus* extracted using water solvent resulted in discovery of sinensitin in oven dried samples and discovery of rosmarinic acid under shade the drying method (23). Meanwhile, comparing to spray method and freeze-dried method using methanol and water in ratio 1:1, high protein content and glycosaponins was detected in the freeze-dried method, which contributed to the potent antiangiogenic activity due to presence of antioxidant compounds (31). Apart from that, shade dried samples were found to have the highest secondary metabolites and highest phenolic compound (32). This suggests that despite applying heat to the plant species, though the biochemical profiling and compounds are altered, they are still present and contributes to their pharmacological activities such as antioxidant activity, ani-inflammatory activity and anti-diabetic activity in the dry form. Reports are on *O. aristatus* using different drying method and temperature namely solar drying, shade drying and cabinet drying (40 °C, 50 °C and 60 °C) resulted in the leaves retaining their total flavonoid, total phenol and antioxidant content though in different value (29). Additionally, shade drying was concluded to be the best drying method as it retained the best and most biochemical component tested. Meanwhile (30), concluded that drying the *O. aristatus* leaves will be optimum at 40 °C due to results projecting the highest total flavonoid content, rosmarinic acid, caffeic acid and total phenolic content . *O. aristatus* was also claimed to possess anti-inflammatory activity as leaves dried under 40 °C infused in ethanol was able to inhibit inflammation by more than 50 % when tested in male white rats injected orally (33). Hence, based on the outcome of this study, it can be said that the leaves do possess a certain amount of pharmacological activity, although they are used in dry form. However, the temperature or drying method applied to the leaves plays a significant role, as too much heat may have the tendency to destroy all the chemical components that contribute to the therapeutic property of *O. aristatus*.

Crude extracts were also mostly utilised in most studies because protein extracts are generally much harder to be isolated due to their amino acid profiling and variation in the sequences, which creates their own chemical and physical characterization (34). Additionally, the purification process following the extraction process may give better concentrated extract and better biological activity with no contamination, though it is achieved through a series of steps such as the elimination of disruptive compounds through TCA/acetone or phenol precipitation, rinsing procedure and fractionization (35). Often, protein extraction is time consuming as many steps and solvents are required to breakdown the protein tissues and cells, which have to be broken down or solubilized by using appropriate buffers, correct temperature, pH and time to ensure no loss or degradation of protein occurs and minimal protein activity. However, due to their own amino acid constituents, protein extracts possess their own antioxidant capability. 63 % of DPPH radical scavenging activity and 78 % hydroxyl radical scavenging was detected in protein extract of *Leucas linifolia* (36). Similarly, protein extract of fresh leaf and commercialised leaf of *M. oleifera* was found to have 46.26 % and 88.32 % DPPH radical scavenging activity respectively (37). The protein extract of the *O. aristatus* fresh and dry leaf also exhibited a certain level of antioxidant potential that may indicate the presence of antioxidant proteins. This can be supported by an earlier work where antioxidant proteins such as peroxidase, glutaredoxin, myrosinase and glutathione S-transferase were detected from protein identified from fresh and dry leaf of *O. aristatus* (11). Many plant proteins and peptides have been isolated and discovered to possess therapeutic potential such as antioxidant, antimicrobial and anticancer activities. Approximately 13 and 16 proteins related to antibacterial were found in the white and purple variety of *O. aristatus* with heat shock protein 90–1 being one of them, indicating the potential of the plant as an antibacterial agent (38).

With this said, *O. aristatus* may emerge as a new drug in the near future with further in-depth studies in terms of its protein profiling and identification, especially since the plant is already very well-known till date to have abundant pharmacological activities. For instance, sinensitin in *O. aristatus* leaves has been found to induce apoptosis by exhibiting an anti-proliferative effect, making the plant as a strong therapeutic candidate for preventing hepatocellular carcinoma cells in humans (39). Ethanolic extract of *O. aristatus* was also able to enhance non-alcoholic fatty liver disease through the reduction of hepatic steatosis, improvement in terms of abnormalities in liver enzymes and preventing the complications caused by atherosclerosis, additionally increasing the antioxidant capability of the liver leading to the improvement of the liver metabolism (40). A review concluded that *O. aristatus* possess diuretic potential and has the ability of controlling the elimination of sodium and potassium in urine (41). With this said, narrowing the studies by going further in the protein profiling of the plant may give us a better understanding of how the proteins may work and act in pathways that leads to disease, becoming a prominent figure in medicinal field. Most of the *O. aristatus* leaf products are blooming in the market in dry form.

Conclusion

The results of this study show that the fresh leaves of *Orthosiphon aristatus* (Blume) Miq. has higher protein bands compared to the dry leaves indicating that the fresh leaves possess better pharmacological properties that could be useful in medicinal discoveries. Nevertheless, both extracts were found to have antioxidant activity manifested by the DPPH and FRAP assays indicating heat processing did not jeopardise the antioxidant proteins in the plant. Hence, this shows that the plant protein extract can be further investigated by looking into the best temperature that can preserve the pharmacological properties of the leaves if drying or heating is to be applied. Thus, further detailed studies on the pharmacological properties of the plant protein in fresh or dry form will be beneficial. Additionally, a detailed investigation of the differences in the proteins identified in fresh and dry state will be valuable for further understanding of their potential in medicinal discoveries.

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Authors' contributions

HR conceived and conducted the research and data analysis. ZR supervised the research project. HR and ZR reviewed the results and discussion. Both authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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