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Isolation of Acidic Mucilage from the Outer Seed Coat of Shaddock (*Citrus grandis* Osbeck) and Evaluation of Its Functional Properties

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Abstract

After water imbibition, the outer layer seed coat of shaddock (*Citrus grandis* Osbeck) produces transparent gel-like mucilage (MSS), but its characteristics have never been studied before. This study aimed to assess the physico-chemical and functional properties of MSS. Extractions of MSS with deionized water at room temperature yielded about 3.5% based on the dry weight of seed. The major components were neutral sugars and uronic acids in the amounts of 33.5% and 49.6%, respectively. The acidic nature of MSS was confirmed by ruthenium red staining. Its water holding capacity and viscosity were 44.53 $g \cdot g^{-1}$ DW and 1660 cP at 10 g/L, respectively. MSS showed a weak quenching activity against DPPH radical, and moderate ferrous ion-chelating and superoxide anion radical scavenging activities, with IC_{50} value of 1.5 g/L and 1.1 g/L, respectively. A methyl thiazolyl tetrazolium (MTT) assay demonstrated that MSS significantly stimulated the viability of mouse skin fibroblasts (NIH/3T3) at 5 - 300 mg/L. These results impart the potential usefulness of the MSS to food, cosmetics and other applications.

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Keywords

Antioxidation, Cell Viability, Shaddock Seed, Viscosity, Water Holding Capacity

1. Introduction

Many angiosperms, including Brassicaceae, Solanaceae, Linaceae, and Plantaginaceae, among others, produce a pectinaceous mucilage layer in their outer seed coat, known as myxospermy [1] [2]. This seed mucilage is thought to play many important roles, including to decrease the rate of water loss and increase the moisture supply to the seed [3] [4], maintain seed viability under harsh desert conditions [5], initiate or ensure seed germination [6], and prevention of seed dispersal by adherence to soil, and promotion of seed spreading by attachment to animals [7], among others. Evidently, the seed mucilage presents some special physical and chemical properties to achieve the aforementioned ecological and physiological roles.

Mucilage is a complex heterogeneous polysaccharide. Polysaccharides have emerged as an important class of bioactive natural products [8] and widely used in food, cosmetics, textiles, and pharmaceutical systems for various purposes, such as thickener, stabilizer, emulsifier, excipient, and gelling agent. In addition, many studies elucidate that polysaccharides isolated from plants have antioxidative activities [9]-[12].

Shaddock (*Citrus grandis* Osbeck) is an important economic fruit in Taiwan during autumn. Our preliminary study indicated that the outer seed coat of shaddock contained a high amount of transparent gel-like mucilage (MSS) and had never been studied before. In general, the seeds are removed and discarded while the pulp is eaten. The objective of this paper was to characterize MSS with respect to its physicochemical properties such as the viscosity, water holding capacity, antioxidative potency *in vitro* and cytotoxicity against the mouse embryo fibroblast (NIH 3T3) cells. Such information contributes toward the sustainable reuse of agricultural wastes and imparts the potential usefulness of MSS to various applications.

2. Materials and Methods

2.1. Shaddock Seeds

The seeds were removed from ripe shaddock fruits (*Citrus grandis* Osbeck) that were purchased from a local market in the northern Taiwan. After being dried at room temperature, they were stored in a desiccator box until use.

2.2. Ruthenium Staining

The dried seeds were imbibed in water for about 15 min to form a gel around wetted seed. Then, they were incubated with the cationic dye ruthenium (2 g/L).

2.3. Preparation of the Mucilage

The dried seeds were extracted three times with water (water to seed ratio of 5:1) on a reciprocal shaker (BT-150, Yihdern, Taiwan) under the room temperature for 20 min each time. The extracts were mixed and vacuum filtrated through a glass microfibre filter (Whatman GF/A, GE Healthcare, USA). The filtrate was lyophilized in a freeze dryer (DM-25ES, VirTis, NY, USA). The dry MSS yield was estimated.

2.4. Quantitative Analysis

Ash content was determined using the AOAC oven method [13]. Total neutral sugar was determined by the phenol-sulphuric acid method [14] using glucose (10 - 80 μg) as the standard. Uronic acid analysis followed the method of Blumenkrantz and Asboe-Hason [15], using phenolic m-hydroxydiphenyl reagent and calibrated against a standard glucouronic acid (25 - 150 μg). Total protein was determined using the Bio-Rad protein micro-assay, with bovine serum albumin (2 - 20 μg) as the standard. Total phenolic content was determined by Folin Ciocalteu reagent [16]. Briefly, 0.1 ml solution of MSS in methanol (80%) was mixed with 0.5 ml of Folin Ciocalteu reagent (diluted 10-fold with water) and 0.4 ml of aqueous Na_2CO_3 (75 g/L). The mixture was kept for

30 min in the dark and the total phenol content was determined at 765 nm with gallic acid (25 - 300 μg) as the standard.

2.5. Viscosity Analysis

The viscosities of MSS solutions (1 - 10 g/L) were measured using Dial Reading viscometer (LVT, Brookfield, Massachusetts, USA) with spindle No. 18 at 26˚C. The viscosities of xanthan (1 - 5 g/L) and gum arabic (1 - 10 g/L) were compared.

2.6. Water Holding Capacity (WHC) Analysis

The WHC of MSS was determined by an adaptation of the filtration method of Robertson and Eastwood [17]. Briefly, a dry sample of 25 mg was soaked in 10 mL water and shaken with an orbital shaker (200 rev/min) at room temperature for 24 h and filtered through filter paper (ADVANTEC No. 1, Tokyo, Japan). After 10 min, the sample with the paper was weighed (wet weight) before drying at 60˚C until a constant weight (dry weight) was obtained. The difference of the two weights gave WHC.

2.7. Determination of Antioxidant Activity

The antioxidant activity of the MSS was evaluated by using the DPPH free radical scavenging method described by Yamaguchi *et al*. [18], chelating ferrous methods according to Dinis *et al*. [19], and superoxide anion radical scavenging using the method of Li *et al*. [20], with slight modifications.

Briefly, 20 μL of the MSS or butylated hydroxytoluene (BHT) at various concentrations were mixed with 100 mM Tris-HCl buffer (pH 7.4, 80 μL), and added to 100 μL DPPH radical in ethanol (0.25 mM). The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance was measured at 517 nm. The percentage of DPPH radical scavenging activity was calculated as $[(A_0 - A_1)]/A_0 \times 100$, in which A_0 is the absorbance of the control, and A_1 is the absorbance of the MSS and BHT.

For chelating effect determination, briefly, 100 μL of the MSS or EDTA at various concentrations were mixed with 2 mM ammonium ferrous sulphate (10 μL). The reaction was initiated by the addition of 5 mM ferrozine (20 μL) and the mixture was shaken vigorously and left standing at room temperature for 10 min, and the absorbance of the mixture was determined at 562 nm. The percentage of inhibition of ferrozine-ferrous complex formation was calculated by $[(A_0 - A_1)]/A_0 \times 100$, in which the notations of A_0 is the absorbance of the control, and A1 is the absorbance of the MSS and EDTA.

For superoxide anion radical scavenging determination, 100 μL of the MSS or ascorbic acid at various concentrations were mixed with 100 μL NBT solution (300 μM), 100 μL PMS solution (120 μM), and 100 μL NADH solution (936 μM). The mixture was shaken vigorously and left to stand for 5 min at room temperature in the dark, and the absorbance of the mixture was determined at 560 nm. The percentage of superoxide anion radical scavenging activity was calculated as $[(A_0 - A_1)/A_0] \times 100$, in which A_0 is the absorbance of the control, and A_1 is the absorbance of the MSS and ascorbic acid.

2.8. Cell Viability Assay

Mouse embryonic fibroblasts (cell line NIH 3T3, BCRC 60071) were purchased from the Bioresource Collection and Research Centre in Hsinchu (Taiwan).

NIH-3T3 cells (1×10^4/well) in their exponential growth phase were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 5% CO_2 at 37˚C. Following a 12 h incubation period, the cells were exposed to various concentrations (5 - 300 mg/L) of the MSS solution that was filtrated through a filter (PTF205030, BIOFIL, Guangzhou, China) for another 12 h. The viability of cells was assessed using the MTT assay as described elsewhere. Briefly, the medium was aspirated and MTT was added to cells at a concentration of 0.25 g/L. Cells were incubated at 37˚C for 3 h and the formazan product was solubilized with dimethylsulfoxide. The absorbance was detected in the microplate reader at 570 nm. Concanavalin A (ConA) (5 μg/mL) was the positive control.

2.9. Statistical Analysis

The results are presented as means of at least three replicates ± standard error (SE). The data were subjected to

one-way ANOVA and the differences between means were measured at the 5% probability level using Fisher's protected least significant difference (LSD) test (CoHort Software, Monterey, CA, USA).

3. Results and Discussion

3.1. Preliminary Characterizations

After imbibition at room temperature for 15 min, the transparent gel-like mucilage appeared at the surrounding of seed coat, and its thickness was approximately 30% of the seed width at the middle of its length (**Figure 1(A)**). It was significantly stained by ruthenium red (**Figure 1(B)**), revealing that MSS was an acidic polysaccharide.

A simple aqueous extraction process could obtain MSS. The yield of MSS was approximately 3.5% of the dry mass of the whole seed (**Table 1**), and its level was higher than that of flax seeds (about 2%) [21], but lower than that of yellow mustard seeds (about 5%) [22]. The major constituents were neutral sugar and uronic acid, 33.5% and 49.6%, respectively (**Table 1**). These results are consistent with the ruthenium red staining (**Figure 1(B)**). The ash content of 4.9% was lower than those of sage seed gum (9.2%) [23] and flaxseed gum (7.4% - 8.4%) [24], but higher than those reported for locust bean gum (0.7% - 1.5%) [25] and gum arabic (2.7% - 3.2%)

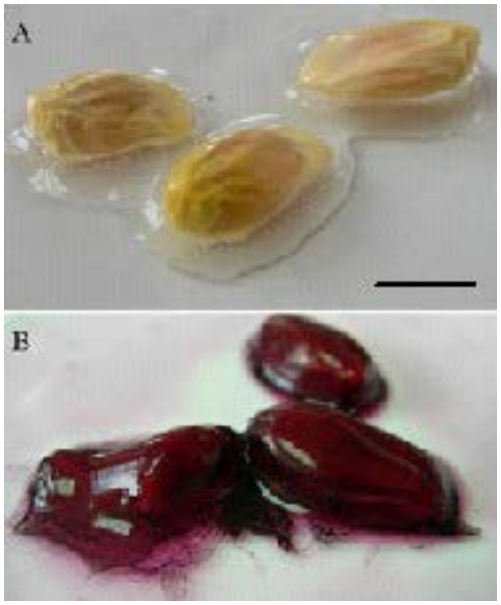

Figure 1. The appearance of shaddock seeds after been submerged in water (A) and stained with 0.2% (w/v) ruthenium red (B). Bar is 1 cm.

Table 1. The characteristics of MSS. The data were presented as mean ± SE (n = 3). *Expressed as weight percent of dry seed.

Parameter	Quantity
Yield*	3.5% ± 0.4%
Ash	5.00% ± 0.06%
Neutral sugar	33.5% ± 0.6%
Uronic acid	49.6% ± 2.8%
Protein	0.7% ± 0.1%
Total phenol	0.2% ± 0.0%

[26]. By contrast, the protein and the total phenol contents were both minor, only 0.7% and 0.2%, respectively (**Table 1**).

3.2. The Physical Characteristics

MSS was effective at increasing the viscosity of solutions at levels as low as 5 - 10 g/L, reached to 1660 cP at 10 g/L (**Figure 2**). Xanthan and gum arabic had high and low viscosity [27], showing 1455 cP at 5 g/L and only 1.6 cP at 10 g/L, respectively (**Figure 2**). Wannerbergera *et al*. [28] reported that the viscosity range of 10 g/L solutions of linseed mucilage from 23 varieties was 20 - 280 cP at room temperature. Hence, this moderate consistency offers the potential applications of MSS.

Similar to the trend of viscosity, the WHC analysis reveals that MSS could hold 44 times their weight of water (**Table 2**). This capacity was significantly lower than that of xanthan (274 times) but higher than that of gum arabic (7.9 times), even though the levels between MSS and gum arabic were not statistically significant ($P > 0.05$) (**Table 2**). In addition, the WHC levels in the polysaccharides isolated from acorn fruit at 4.3 [29], and in some fibre-rich feedstuffs, such as rice bran, sweet potato vine, and Tofu residue at 2.4, 6.3, and 8.3, respectively [30], were all lower than that of MSS.

These WHC and viscosity features are consistent with MSS ecological and physiological roles, such as facilitation of seed hydration [3] [4], affect further dispersal of the seed [7].

3.3. Antioxidant Activity *in Vitro*

The stable DPPH radical is a widely adopted target for evaluating the free radical scavenging ability of various samples [31]. MSS showed a weak quenching activity against DPPH radical, by only inhibiting 15% at 400 g/L, significantly lower than BHT with IC_{50} value reaching 8.2 mg/L (**Figure 3(A)**). Guendez *et al*. [32] addressed that there was a positive relation between the activity of scavenging DPPH radical and the content of polyphenol

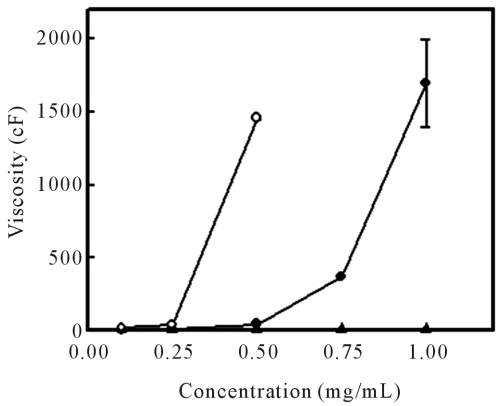

Figure 2. The viscosity of MSS (closed circles), xanthan (opened circles), and gum arabic (closed triangles) at different concentrations. The data were mean cP ± SE (n = 3).

Table 2. The water holding capacity of MSS, xanthan and gum arabic. The data were presented as mean ± SE (n = 3). Different lowercase letters show significant difference ($P < 0.05$).

	Water holding capacity g of water/g DW
Xanthan	274.0 ± 45.8[a]
MSS	44.5 ± 3.7[b]
Gum arabic	7.9 ± 1.6[b]

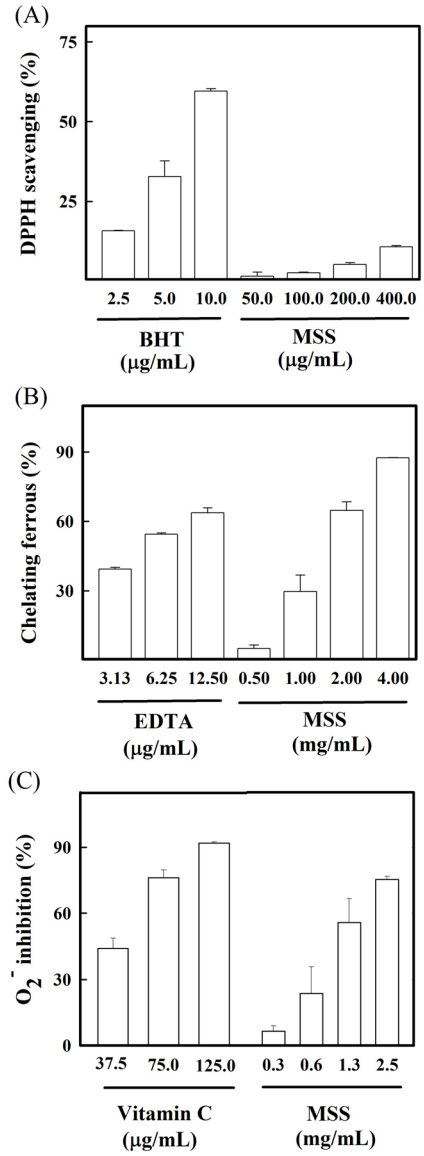

Figure 3. The DPPH free radical scavenging (A), ferrous chelating (B), and superoxide anion radical scavenging (C) activities of MSS and the relative standards, butylated hydroxytoluene (BHT), EDTA and vitamin C, respectively. The data were presented as mean ± SE (n = 3).

in the seeds of different grape species. Hence, a weak free radical scavenging ability may be attributed to MSS to contain low levels of phenol (**Table 1**).

Iron is essential for oxygen transport, respiration and activity of many enzymes, which are required for sustaining life; however, ferrous, not ferric, is the most powerful pro-oxidant among the various species of metal ions and is able to generate free radicals from peroxides by the Fenton reaction to catalyze oxidative changes in lipids, proteins, and other cellular components [20] [33] [34]. There is a good correlation between chelating ferrous ability and uronic acid content in the polysaccharides from Zizyphus jujube [35]. MSS contained uronic acids (**Table 1**) and exhibited a moderate ferrous ion-chelating activity that increased on increasing concentration from 0.5 to 4.0 g/L, even though its IC_{50} value was 1.5 g/L, not comparable with EDTA (IC_{50} = 6.2 mg/L) (**Figure 3(B)**). The results indicated that MSS could act moderately as a ferrous chelator to minimize moderately the harmful oxidation induced by ferrous ion.

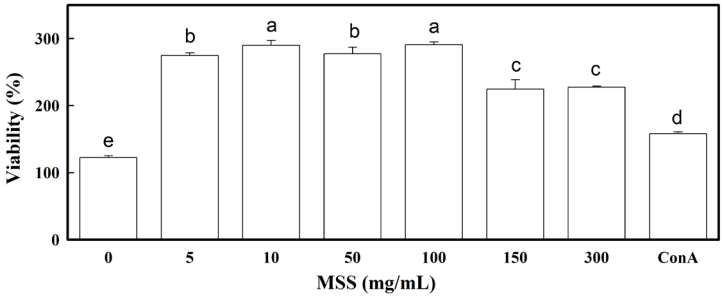

Figure 4. NIH 3T3 cells were exposed to various concentrations of MSS. The data were presented as mean ± SE (n = 3). Different lowercase letters show significant difference ($P < 0.05$).

Of the reactive oxygen species, superoxide anion radical is generated first [20]. Superoxide anion radical decomposes to form others reactive oxygen species, such as singlet oxygen, hydroxyl radicals and hydrogen peroxide [36]. Therefore, its scavenging is very important to evaluate the antioxidant efficiency of various samples. **Figure 3(C)** shows the dose-dependent curve for the superoxide anion radical scavenging activity of MSS and its IC_{50} value was 1.1 g/L, even though that was markedly lower than Vitamin C (IC_{50} = 49.0 mg/L).

3.4. Cell Viability

MTT assay showed that MSS significantly stimulated the viability of mouse skin fibroblast NIH3T3 at 5 - 300 mg/L, reached to 130% increase at 100 mg/L, and the increasing efficiency was profoundly higher than ConA, which had only 20% increase, a value much lower than that of MSS (116%) at the same concentration (5 mg/L) (**Figure 4**). Fibroblasts are important in supporting normal wound healing, and increasing the number of fibroblasts in an artificial dermal substitute is conducive to improved healing in experimental wounds [37]. Therefore, it is worthwhile to study whether MSS has the potential to be used on healing skin wounds.

4. Conclusion

The present work clearly demonstrated that MSS could be easily obtained via a simple extraction process from the outer layer seed of shaddock, and its yield was about 3.5% of the dry seed. MSS exhibited a moderate viscosity, water holding capacity, and ferrous ion-chelating as well as superoxide anion radical scavenging activities. MSS not only showed nontoxicity, but also stimulated the proliferation of NIH3T3 cells. These results impart the potential usefulness of MSS to food, cosmetics and other value-added applications. Several kinds of seed coat mucilage present in different plants, hence, another important subject will understand the composition and linkage of the purified MSS and further identify its polysaccharide type.

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