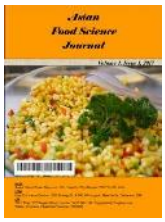

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Antioxidant Activities and Functional Properties of Complex Polysaccharide (Mucilage) in Vegetable Fern (*Diplazium esculentum*)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AS and NFB designed the study, performed the statistical analysis, wrote the protocol and managed the analyses of the study. Author INH wrote the first draft of the manuscript. Authors INH and AS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Diplazium esculentum is a type of edible fern which has long been used in traditional dishes and is also known to contain mucilaginous material in a form of complex polysaccharide called mucilage. This study aimed to determine antioxidant activities and functional properties of mucilage extracted from *D. esculentum*. Extraction of mucilage from young fronds of *D. esculentum* was done using three different extraction methods i.e. hot water, alkaline and acidic. All subsequent analyses were done according to the established procedures. The results showed that hot water and alkaline extractions gave a significant ($P < 0.05$) higher yield (2.6 and 2.5%, respectively) of mucilage as compared to acidic extraction (1.6%). It was also found that mucilage extracted using hot water exhibited a significant higher ($P < 0.05$) ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals (scavenging activity = 51.3%), as compared to other mucilage including gum Arabic. Hot water and acidic mucilage were found to be significantly different ($P < 0.05$) from alkaline mucilage and gum Arabic in terms of antioxidant activity measured via ferric thiocyanate (FTC)

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analysis with higher percentages of peroxide inhibition (22.7% and 27.4%, respectively). Hot water mucilage was further studied for functional properties. This mucilage exhibited a clear thixotropic flow behaviour with higher viscosity profile and also better emulsion stability (68.0%) at 0.5% concentration, as compared to gum Arabic. These findings suggest that *D. esculentum* mucilage could be an interesting functional food ingredient with significant antioxidant properties though further study should be done in order to fully understand its potential as one of alternative food hydrocolloids.

Keywords: Vegetable fern; Diplazium esculentum; extraction; mucilage; antioxidant activities; functional properties.

1. INTRODUCTION

Ferns are typically found in hot wet climate areas and thus they are very common in Asia region including Malaysia. One of them is *Diplazium esculentum* (local name: Paku Tanjung), popularly known as a vegetable fern since young fronds of this plant are commonly taken as food by locals [1] which contributed to their dietary intake. The fronds were also reported to have a relatively high antioxidant activity as compared to other edible plants such as *Eryngium foetidum* and *Cosmos caudatus* [2]. Moreover, in some species of plants including *D. esculentum*, the epidermal cells of leaves and stems contain a large quantity of complex polysaccharides called mucilage. Being regarded as a hydrocolloid, one of the key functional roles of mucilage is to thicken and stabilize emulsion-based food systems. Some commercial hydrocolloids like gum Arabic and galactomannans exhibit emulsifying activities at oil-water interface of the emulsion to stabilize the system. Several applications of these polysaccharides for this particular functional property are including beverage emulsions, sauces and dressings. In addition, gum Arabic exhibits a free-radical scavenging capacity and thus it is able to inhibit oxidation of oil droplets in the emulsion system. However, gum Arabic has also been reported to show an adverse biological effect that causes hypersensitivity to human [3] and thus searching for alternative hydrocolloids is of current interest.

Mucilage or gum can be extracted using various extraction methods such as alkaline, acidic, hot water and enzymatic. Previously, alkaline method has been reported to give higher yield (20.0%) of malva nut gum as compared to dilute acid (6.0%) and hot water (1.0%) extraction methods [4]. Differently, hot water method was more efficient in giving higher yield (5.4%) of barley mucilage as compared to other methods i.e. enzymatic, acidic and alkaline extraction methods, with 5.2%, 4.7% and 3.9% yield, respectively [5]. Some works have also been done to study physicochemical and functional properties of new plant mucilage from leafy vegetables including edible fern. Singthong et al. [6], reported on functional properties of mucilage from *Tiliacora triandra*, a vegetable that is used in many cuisines of the northeast Thailand. More recent study demonstrated that the hot water extraction at 90.8°C for 4.8 hours could optimally provide mucilage from *Paris polyphylla* leaves with a strong antioxidant activity [7]. Moreover, a comparative study on chemical and functional properties of mucilage from young fronds of *Asplenium australasicum* (a wild edible fern in Taiwan) obtained using water and alkali extraction methods has been documented [8]. Research on this fern has been extended by using acidic extraction condition providing the mucilage with better emulsifying properties [9]. In short, these studies found great potential application of the mucilage as a natural functional food ingredient. Thus, mucilage from *D. esculentum* may offer another alternative for the food industry. As reported for the plant itself, mucilage from *D. esculentum* may also serve an additional functionality in terms of antioxidant activities yet no related study is available. Therefore, this study was initiated to gain new knowledge on antioxidant activities of mucilage extracted from young fronds of *D. esculentum* as affected by different extraction methods. Subsequently, functional properties of the mucilage obtained from the best extraction method were also determined.

2. MATERIALS AND METHODS

2.1 Materials

Young fronds (young leaves and stems) of *D. esculentum* were collected from wet areas in Kuala Terengganu, Terengganu, Malaysia. Gum Arabic was purchased from Sigma Company (M) Sdn. Bhd. All chemicals and reagent used were of analytical grade.

2.2 Extraction of Complex Polysaccharide

Three types of extraction methods were employed namely hot water, alkaline and acidic extraction methods as described by El-Mahdy and El-Sebaiy [10] as well as Ahmad et al. [5]. For all methods, young fronds of *D. esculentum* were firstly homogenized with deionized water at 1.5:1 ratio. After refluxing with 80% ethanol for 4.5 hour, the viscous homogenate was mixed with either 1 M NaOH (pH 10) (alkaline method) or 1 M citric acid (pH 5) (acidic method) at 1:8.5 ratio and heated at 55°C for 90 min to inactivate enzymes. After centrifugation at 4700 g for 20 min, the supernatant was mixed with absolute ethanol at 1:0.8 ratio and allowed to stand for 1 hour. Differently in the hot water extraction, the viscous homogenate was heated at 70°C for 30 minutes to inactivate enzymes. For all methods, the crude mucilage was precipitated using three volumes of acetone followed by washing with absolute ethanol to remove impurities. Then, the mucilage was dried overnight in an oven at 40°C followed by crushing using a blender. All extractions were carried out in three different batches and the respective yields of mucilage were calculated based of weight of fresh sample.

2.3 Determination of Antioxidant Activities

Antioxidant activities of each mucilage were examined using 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric thiocyanate (FTC) and thiobarbituric acid (TBA) analyses [11] in comparison to commercial gum Arabic. In the DPPH analysis, 4 ml of the mucilage solution (1%) was mixed with 1 ml of DPPH ethanolic solution (0.2 mM) and allowed for reaction (30 min in dark place). The absorbance (A) was read at 517 nm using a UV/Vis spectrophotometer (Varian Cary 50 Probe, Malaysia). A control sample was a mixture without mucilage. The scavenging activity was calculated as follows;

$$\text{Scavenging activity (\%)} = [(1 - A_{sample}) / A_{control}] \times 100\%$$

For the FTC analysis, a mixture of 4 mg mucilage, 4 ml of absolute ethanol, 4.1 ml of 2.52 % linoleic acid in absolute ethanol, 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of deionized water, was placed in a vial with a screw cap and then the mixture was held in a dark oven at 40°C. 0.1 ml of this mixture was added with 9.7 ml of 75 % ethanol and 0.1 ml of 30% ammonium thiocyanate. Precisely 3 min after an addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance (A) of the resulting red color was measured at 500 nm for every 24 hours until the absorbance of the control reached the maximum. The same sample prepared in FTC was later subjected to TBA analysis. 2 ml of 20% trichloroacetic acid and 0.1 ml of 0.67% TBA aqueous solutions were added into 1 ml of sample solution from the previous FTC method. The mixture was placed in a boiling water bath, 100°C for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min and the absorbance of the supernatant was measured at 532 nm. The percentages of inhibition for both FTC and TBA analyses were calculated on the seventh day of experiment as follows;

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

2.4 Determination of Functional Properties

The following analyses involved only the mucilage obtained from the best extraction method (hot water). Solubility of the mucilage was determined according to the previous study [12]. Mucilage solutions (30 ml, 1.0% w/v each) were placed in different water baths at 30, 60, 70 and 90°C for 30 min and stirred continuously. The solutions were then centrifuged at 800 g for 15 min. For each solution, 10 g of supernatant was collected and dried in a convection oven at 125°C for overnight until a constant weight was reached and the percentage of solubility was calculated by the following formula:

$$\text{Solubility (\%)} = (\text{Weight}_{final} - \text{Weight}_{initial}) / 10 \times 30 \times 100$$

Viscosity and emulsion stability were determined according to the modified procedure of Scariani et al. [12]. The viscosity was measured using a viscometer (Digital viscometer, Brookfield, USA) with CP21 spindle. Aliquots (8 ml) of 2.0% w/v mucilage suspensions were used for the analysis. The assay was carried out at different temperatures (25, 40, 50ºC). To determine the emulsion stability, mucilage solutions (60 ml) with different concentrations (0.5%, 0.25% and 0.1%) were each mixed with a commercial corn oil (6 ml) and the mixtures were homogenized for 1 min to form the emulsions. The emulsions were then heated in a water bath at 80°C for 30 min and subsequently were centrifuged at 800 g for

10 min. The emulsion stability was calculated by using the following formula:

$$\text{Emulsion stability (\%)} = (\text{Height}_{\text{emulsion layer}} - \text{Height}_{\text{whole layer}}) \times 100$$

2.5 Statistical Analysis

This study applied a one-level treatment arrangement structure involving three different extraction methods namely hot water, alkaline and acidic, in order to determine their effect on antioxidant activities (responses) of the resultant mucilage. A completely randomized treatment assignment was used in this study with triplicate (n = 3) independent observations for each response. Additionally, gum Arabic was used as a material for comparison. A one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison was used to find mean differences among samples with a significant level of $P < 0.05$. Statistical analysis was carried out using a Minitab (Release 14) statistical package.

3. RESULTS AND DISCUSSION

3.1 Yield and Antioxidant Activities

In this study, three different extraction methods were applied in order to compare their efficiency in giving relatively high mucilage yield with preserved antioxidant activities. The mucilage precipitation however was similarly done by using acetone. The results showed that hot water extraction gave the highest yield (2.6%) of mucilage but not significantly different from alkaline extraction (2.5%) while acidic extraction gave the lowest yield (1.6%) (Table 1). Previously, Ahmad et al. [5] used different types of extraction method for mucilage extraction from barley and similarly found that hot water extraction gave the highest yield. Koocheki et al. [13], applied temperatures ranging from 25ºC to 85ºC, for extraction of *Eruca sativa* seed mucilage. It has been found that the highest temperature could produce the highest yield. As employed for hot water extraction in the present case, an increase in polysaccharide yield is suggested to be due to effect of temperature on mass transfer rate of water soluble polysaccharides in the cell wall [14]. However it is important to mention that the alkaline condition could increase the mucilage yield by hydrolyzing insoluble constituents into their soluble counterparts [15]. In addition, Somboonpanyakul et al. [4] stated that most of polysaccharides were either covalently linked to other components or physically trapped within the cell wall matrix, which could be effectively released under the alkaline condition. Thus, these previous findings could explain the close yield values for both hot water and alkaline extractions.

In terms of colour, extraction methods may affect the colour of mucilage being extracted [16,5]. It was observed in this study that each mucilage obtained from both hot water and alkaline methods was dark brown in colour. On the other hand, the mucilage was green in colour for acidic method. An alkaline medium has been reported to give more colour to mucilage extracted from sweet mesquite seeds than a neutral medium [16]. A lighter mucilage colour has a technologically advantage when such ingredient was applied in beverages with transparent appearance [5]. Further purification process may lead to lighter colour of mucilage which is more suitable for industrial applications.

Total free radical scavenging activity was determined by their efficiency to scavenge DPPH free radicals. The efficiency was based on the reduction of DPPH stable free radicals and any molecules that can donate an electron or hydrogen to DPPH radicals can react with them and thereby bleach the DPPH absorption [17]. In addition, during the scavenging of DPPH radicals, the colour of the reaction mixture changed from purple to light yellow with decreasing wavelength at 515 nm. It was found that the mucilage extracted using hot water exhibited a significant higher ($P < 0.05$) scavenging effect on the DPPH free radicals (51.3%), as compared to other mucilage including gum Arabic (Table 1). However, the scavenging effects of all mucilage extracted from *D. esculentum* were found to be slightly lower than the antioxidant activities of mucilage extracted from *Hsian-tsao* leaf with the DPPH scavenging activity of 68.6% [18].

Ferric thiocyanate analysis is used to measure antioxidant activity on hydroperoxide compounds formed at the initial stage of oxidation while TBA test is used to measure antioxidant activity on secondary products of oxidation such as aldehydes and ketones [19]. In the present case, a similar sample preparation was used in both FTC and TBA analyses. During the FTC analysis, hydroperoxides will react with ferrous chloride to form a reddish ferric chloride pigment. Thus higher antioxidant activity of mucilage was indicated by decreased absorbance value due to decreased concentration of hyrdoperoxides [20]. As supported by Farrukh et al. [21], plant extracts that showed lower absorbance values indicated

higher antioxidant activities. The mucilage extracted using acidic method was found to be significantly different ($P < 0.05$) from the one extracted using alkaline method and gum Arabic in terms of antioxidant activity measured *via* TBA and FTC analyses with higher percentages of inhibition (66.4% and 27.4%, respectively). Meanwhile, all samples including gum Arabic showed more efficiency in inhibiting secondary oxidation product (malonaldehydes in TBA) rather than primary ones (hydroperoxides in FTC). Conversely, an aqueous extract of *D. esculentum* has been reported to exhibit a higher antioxidant activity in FTC rather than in TBA analysis as indicated by a stronger antioxidant activity at an initial stage of lipid peroxidation [22]. Alkaline mucilage somehow showed the lowest antioxidant activities amongst all samples including gum Arabic in most of the antioxidant analyses. It is believed that both acidic and hot water extractions could efficiently retain most antioxidant compounds (e.g. phenolic) originally existed in *D. esculentum* plant [22].

3.2 Functional Properties

The mucilage obtained from hot water extraction method (or *D. esculentum* mucilage) was further characterized in terms of functional properties. It was observed that, *D. esculentum* mucilage exhibited a better solubility compared to gum Arabic which was more pronounced at higher temperatures (Fig. 1). Solubility of polysaccharide was resulted from breaking of the H bonds among polysaccharides chains, which exposed the OH-groups to water [12]. In the previous study done by Mirhosseini and Amid [23], it has been shown that the water solubility values of durian seed gum determined at 80ºC (which was close to 70°C, in the present case) were ranging from 21.4% to 53.2% which were greater than the present study. Moreover, according to the previous study done by Sciarini et al. [12], the water solubility of *Gleditsia triacanthos* gum extracted by alkaline condition increased (40% - 50%) when temperature increased from 70 to 90ºC. This reflects that a heating process is required to completely dissolve some of the hydrocolloids in order to achieve a maximum solubility.

Fig. 2 depicts that *D. esculentum* mucilage exhibited a thixotropic behaviour, a typical behaviour of commercial gums used in the food industry. The viscosity of mucilage solution decreased as a function of time especially at 25ºC at which viscosity was very much reduced in the earlier time compared to temperatures at 40 and 50ºC. This behaviour may be due to diminished interaction among molecules with an increase in kinetic energy of the molecules at higher temperature [12]. Interestingly, a higher viscosity profile was observed for the mucilage at 25°C compared to gum Arabic at all measurements.

Functional property of *D. esculentum* mucilage was also assessed in terms of its capability to stabilize the emulsion. This is related to capacity of the mucilage as a functional ingredient to maintain the emulsion structure and its resistance to rupture [24]. Considering its functional property, *D. esculentum* mucilage provided the highest emulsion stability (68.0%) at 0.5% concentration amongst the concentration tested. There was no layer of oil that appeared in the emulsion at this concentration, but a thin layer of oil occurred in the emulsion with lower concentrations since there were less emulsified oils. Similarly, Tsai et al. [25] reported that the emulsion stability provided by green algae mucilage was 69.0% with no layer of oil appeared in the emulsion at concentration over 0.5%. These may suggest that at mucilage concentration of 0. 5% and above, the mucilage may completely surround the oil droplets and increase the viscosity of the continuous phase to prevent oil droplet coalescence [26]. This is due to the fact that increase in mucilage concentration will increase emulsifying capacity of the mucilage by decreasing the surface tension and hence will increase the stability of emulsion droplets.

Table 1. Yield and antioxidant activities of *D. esculentum* Mucilage as affected by different extraction methods

Extraction Method	Yield (%)	DPPH scavenging activity (%)	% inhibition	
			FTC	TBA
Hot water	2.6^{a} ± 0.3	51.3^{a} ± 1.4	22.7^{ab} ± 0.4	46.1^{b} ± 4.3
Alkaline	2.5^{a} ± 0.2	2.7^{c} ± 0.1	18.4^{b} ± 0.0	43.3^{b} ± 1.3
Acidic	1.6^{b} ± 0.1	32.2^{b} ± 1.9	27.4^{a} ± 2.6	66.4^{a} ± 0.5
Gum Arabic	-	35.7^{b} ± 2.0	11.2^{c} ± 1.3	29.6^{c} ± 3.0

$^{a-c}$ Means in a column with different letter are significantly different ($P < 0.05$) ($n = 3$)

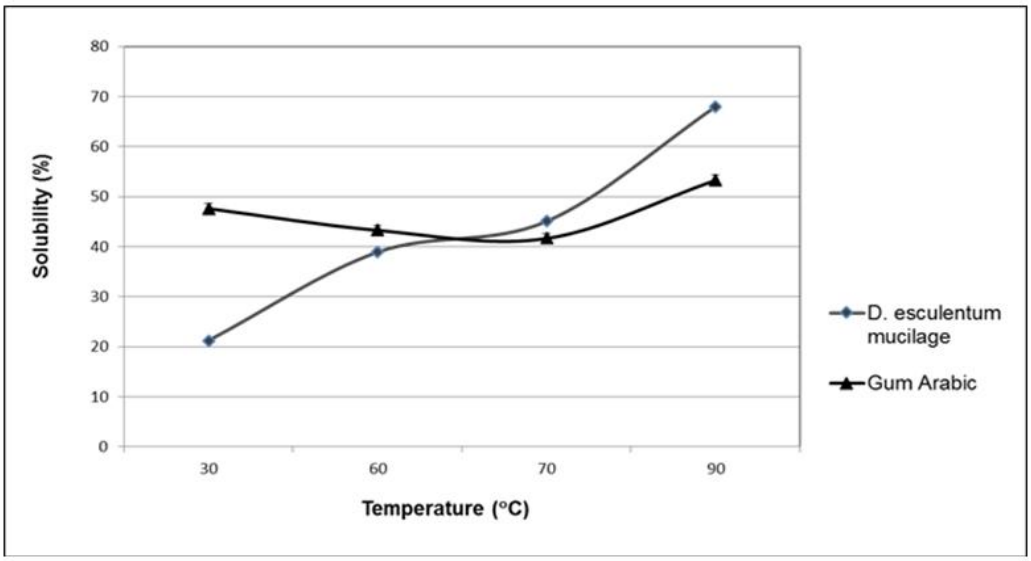

Fig. 1. Solubility of *D. esculentum* mucilage and gum Arabic (1% w/v solution)
Data are presented in mean from 3 replications with maximum standard deviation of 2.6%

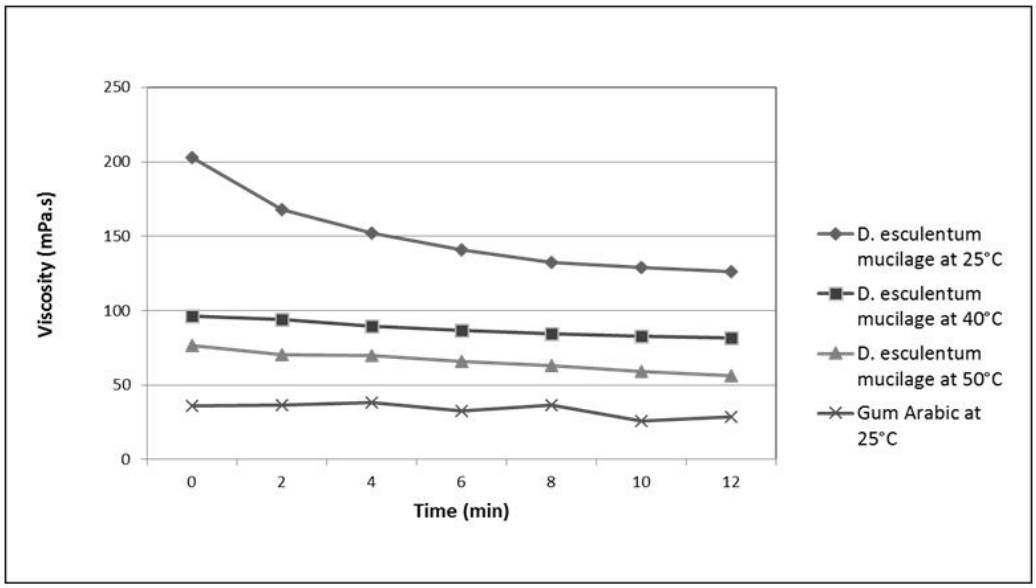

Fig. 2. Viscosity of *D. esculentum* mucilage and gum Arabic (0.5% w/v solution)
Data for gum Arabic at other temperatures were not available. Data are presented in mean from 3 replications with maximum standard deviation of 0.9 mPa.s.

It is also worth noticing that the *D. esculentum* mucilage was able to provide a much better emulsion stability (Fig. 3) compared to gum Arabic, reflecting that this mucilage has an acceptable ability to stabilize food emulsion systems. It is mainly due to higher viscosity of the emulsion continuous phase. This will implicate a fairly stronger emulsion structure which will cause lower droplet mobility and subsequently lower degree of synerisis (or creaming). As a result, the emulsion will be more stable with the presence of this mucilage in contrast to gum Arabic. Nor Hayati et al. [26] have previously shown that destabilization of emulsion with 0.5% gum Arabic could happen due a very week emulsion structure and it is likely that this drawback could be overcome with *D. esculentum* mucilage.

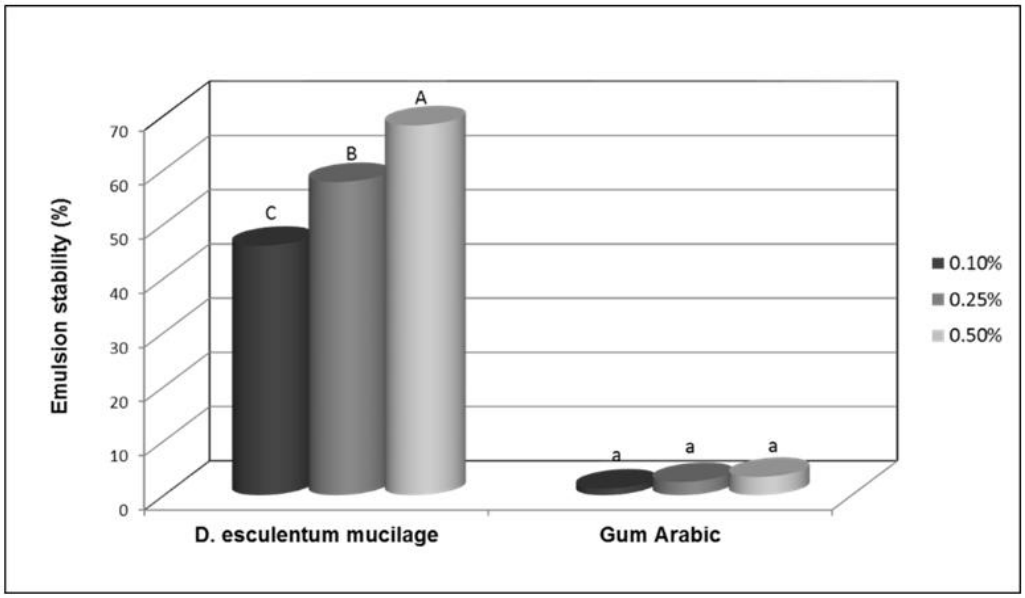

Fig. 3. Emulsion stability as affected by *D. esculentum* mucilage and gum Arabic at 25°C.
Data are presented in mean from 3 replications. Means with the same letter within the same sample group, are not significantly different. Maximum standard deviation = 0.9%.

4. CONCLUSION

This study revealed that mucilage obtained via acidic and hot water extraction methods showed significantly higher antioxidant activities compared to alkaline mucilage and even commercial gum Arabic. Instead of acidic extraction method, hot water extraction could somehow be a method of choice in order to produce higher mucilage yield with good antioxidant activities. Hot water mucilage was further studied in terms of functional properties and it was found that this mucilage exhibited better viscosity profile and emulsion stability than gum Arabic, reflecting its potential to be used as an alternative hydrocolloid for food industry especially in emulsion-based food products.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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