



## Original article

A biological active artificial saliva formulation containing flower mucilage from Ceylon Spinach (*Basella alba* Linn.)Aranya Manosroi<sup>a,b</sup>, Kassara Pattamapun<sup>c</sup>, Charinya Chankhampan<sup>a,b</sup>, Bang-on Kietthanakorn<sup>d</sup>, Worapong Kitdamrongtham<sup>a,b</sup>, Jie Zhang<sup>e</sup>, Jiradej Manosroi<sup>a,b,\*</sup><sup>a</sup> Manose Health and Beauty Research Center, Mueang, Chiang Mai 50200, Thailand<sup>b</sup> Faculty of Engineering and Technology, North-Chiang Mai University, Chiang Mai 50230, Thailand<sup>c</sup> Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200, Thailand<sup>d</sup> Thai - China Flavours and Fragrances Industry Co., Ltd (TCFF), Lad Bua Luang, Ayutthaya 13230, Thailand<sup>e</sup> Department of Natural Medicinal Chemistry, China Pharmaceutical University, Nanjing 21198, PR China

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## ABSTRACT

Ceylon Spinach (*Basella alba*) is an edible perennial vine found in tropical Asia and Africa, known as vegetables containing mucilage. Its mucilage from flowers was extracted by microwaving and precipitated with 95% ethanol. Five artificial saliva formulations composing of mucilage from Ceylon Spinach, calcium chloride (CaCl<sub>2</sub>), potassium chloride (KCl) and sodium fluoride (NF) were developed. The best formulation No.5 containing 0.61% of the mucilage with the non-Newtonian pseudoplastic flow (8.9 ± 0.2 cP) and the wetting time (12.50 ± 2.24 min) similar to the normal human saliva was selected. This artificial saliva formulation exhibited biological activities including an antioxidative activity by DPPH free radical scavenging with the SC<sub>50</sub> of 14.26 ± 2.00 mg/ml (0.05 folds of ascorbic acid), and the adhesion inhibition of *S. mutans* on hydroxyapatite beads at 17.01 ± 7.75%, while the natural human saliva exhibited an increase bacterial adhesion of 33.10 ± 9.70%. The safety of this formulation which gave no cytotoxicity on normal human gingival fibroblasts at 99.20 ± 21.09% cell viability was also demonstrated. The results from this study have indicated high biological activity and safety of the developed formulation containing mucilage from Ceylon Spinach which is potential to be used as artificial saliva for xerostomia patients.

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## 1. Introduction

Human saliva is the complex fluid secreted by major and minor salivary glands. Saliva secretion is under the control of the autonomic nervous system. The three major salivary glands are parotid, sublingual and submandibular. Human saliva consists of water, glycoproteins, enzymes, antimicrobial substances and electrolytes. Glycoproteins in saliva are responsible for the viscoelastic characteristics giving a lubricative film, which enables the free movement of oral tissues. The mucin and electrolytes in saliva maintain the

oral mucosa in its hydrated state and thus providing mucosal integrity (Preetha and Banerjee, 2005). The major functions of human saliva are lubrication, antimicrobial and cleansing activity, remineralisation of enamel with calcium and phosphate and facilitating eating and speech. Salivary gland dysfunction such as xerostomia (subjective sensation of dry mouth) and hyposalivation (diminished salivary flow) (Hahnel et al., 2009) are relatively common problems that can give difficulties in speech, problems with eating, mucosal infections, denture intolerance, increased dental caries, periodontal disease and loss of life quality. The usual therapies for xerostomia and hyposalivation are drinking large quantities of water, using chewing gum, candies and artificial saliva. The aims of using artificial saliva are to ensure lubrication of oral tissues, relieve the sensation of dry mouth, and protect the tooth tissues from decay. There are several approaches to produce artificial saliva, including the imitation of natural saliva which is quite complex. Usually, the commercially available artificial saliva formulation composes of carboxymethylcellulose (CMC), sodium carboxymethylcellulose (SCMC) and hydroxyethylcellulose as thickening and lubricating agents. In fact, mucilage can be found

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in various parts of many plants. These mucilages can be used as thickening, moisturizing and lubricating agents in artificial saliva formulations. Beside mucilages in these plants, there are also several bioactive compounds which are advantageous for the development as artificial saliva, e.g. antioxidant and anti-adherent activity. Antioxidants can enhance the immune system and prevent cancer in oral cavity, while the anti-adherent activity is one of the important properties to prevent the adhesion of microorganisms on the teeth.

Mucilage is water soluble polysaccharide found in a widespread number of plants and also in some microorganisms. There are many plants which contain mucilage such as *Basella alba* Linn., *Hibiscus esculentus* Linn., *Litsea glutinosa* (Lour.) C.B. Robinson, *Ocimum canum* Sims., *Plantago ovate* Forssk., *Scaphium scaphigerum* G. Don. and *Trigonella foenum-graecum* Linn. (Palanuvej et al., 2009). *Basella alba* Linn. (called in common name as Ceylon Spinach or Phak Plung in Thai), family Basellaceae is a wildy cultivated, cool season vegetable (plants that have adapted to cool climates. They prefer temperatures between 55° and 75° F, which are late winter, early spring, late summer, autumn and early winter) with climbing growth habit (Fig. 1). Ceylon Spinach is an edible vegetable and has long been used as thickening agents in soups and stews. It is rich in vitamins A and C, as well as iron, calcium and soluble fiber. In addition, mucilage from this plant has also been used as topical Thai traditional medicines for the treatment of irritant, bruise, ringworm and laboring. Its stem and leaves are used as mild laxative, diuretic and anti-pyretic. The Ayurvedic treatment in India has used its leaves and stems for anticancer such as melanoma, leukemia and oral cancer (Adhikari et al., 2012). Its mucilage is composed of mainly polysaccharides with the pH ranging between 5.3 and 5.4, containing D-galactose as a major monosaccharide and exhibiting slow swelling capacity (Chatchawal et al., 2010). Our previous study has demonstrated that the mucilages of *B. alba* Linn. extracted by distilled water at pH 11 using microwave for 3 min gave the highest DPPH radical scavenging and metal chelating activity of 1.01 and 11.14 folds of vitamin C and EDTA, respectively (Manosroi et al., 2015). Therefore, the objective of this present study was to develop a biological active artificial saliva formulation containing mucilage from Ceylon Spinach by evaluating the physicochemical properties (viscosity, rheology wetting time) and biological activities including anti-oxidant activity and anti-adherent activity.

## 2. Materials and methods

### 2.1. Materials

Ceylon Spinach was purchased from the local fresh market in Chiang Mai province, Thailand during April-May 2012 and identi-



Fig. 1. The whole plant of Ceylon Spinach (*Basella alba* Linn.) showing its flowers, Family Basellaceae.

fied by a botanist of the botanical garden at Faculty of Pharmacy, Chiang Mai University in Thailand. A voucher specimen (BAL-258) was deposited in the herbarium of Chiang Mai University (CMU) herbarium and flora database, Department of Biology, Faculty of Science, Chiang Mai University, Thailand. Vitamin C (L-(+)-ascorbic acid), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), sulforhodamine B (SRB) and resazurin sodium were from Sigma Chemical Co. (St. Louis, MO, USA). Calcium chloride was purchased from Merck, Germany. Potassium chloride and sodium fluoride from Ajax Finechem Pty Ltd., Australia were used. Tris (hydroxymethyl) methylamine was purchased from Fisher Scientific UK Limited, UK. All other chemicals and reagents were of analytical grade.

### 2.2. Mucilage extraction

The fresh flowers of Ceylon Spinach (100 g) were cut into small pieces, macerated with 700 ml distilled water for 24 h and microwaved at 600 W intensity for 5 min. The mixture was then pressed through a muslin cloth. The filtrate containing the mucilage was centrifuged at 4,660g (centrifuge machine, Fisher Scientific Inc., New York, U.S.A) 25 °C for 30 min. The supernatant was collected, mixed with 95% ethanol (3 folds in volume) to precipitate the mucilage and re-centrifuged at 4,660g for 15 min. The precipitate was collected and the remaining ethanol in the precipitate was removed by a rotary evaporator (Buchi, Flawil, Switzerland) (50 ± 2 °C) until all ethanol was evaporated and lyophilized by a lyophilizer (Christ FOC-1 Model K-40 equipment, Balzers-Pfeiffer GmbH, Asslar, Germany) at -50 ± 2 °C. The dried lyophilized powder of the mucilage was kept at room temperature (25 ± 2 °C) until use.

### 2.3. Phytochemical assays

The mucilage was analyzed for phytochemical constituents (anthraquinones, glycosides, tannin, carotenoids, flavonoids and alkaloids) using the standard methods (Manosroi et al., 2010). For anthraquinone, 0.05 g of the mucilage was put into a dry test tube, added with 2 ml of chloroform and shaken for 5 min. The mucilage was filtered. The filtrate was mixed with an equal volume of 10% ammonia solution and shaken. A pink violet or red color in the ammoniacal layer (lower layer) indicated the presence of anthraquinone. The qualitative assay of reducing sugars was performed by TLC method. The mucilage dissolved in water was spotted on the silica gel plate in comparing to the standard reducing sugars (glucose, fructose and sucrose). The filtrate was resolved on the TLC plate coated with silica gel 60. The mobile phase was butanol/acetic acid/diethyl ether/water (9:6:1:3). The spot on the plate was sprayed with 10% H<sub>2</sub>SO<sub>4</sub> and heated. Sucrose, glucose and fructose were used as the standards. For tannins, 0.05 g of the mucilage was mixed with 2 ml of 15% FeCl<sub>3</sub> solution. The blue-black precipitate indicated the presence of tannins. For carotenoid, each mucilage sample was extracted with chloroform in a test tube with vigorous shaking. The resulting mixture was filtered and 0.1 ml of H<sub>2</sub>SO<sub>4</sub> was added. The blue color at the interface showed the presence of carotenoids. For the presence of flavonoid, 2 ml of the mucilage solution mixed with 1 ml of the concentrated HCl and magnesium ribbon gave the pink tomato-red color. For alkaloids, an amount of 0.05 g of the mucilage in 2 ml of 1.5%v/v HCl was boiled on a water bath and 6 drops of the Dragendorff's reagent were added. The orange precipitate indicated the presence of alkaloids.

### 2.4. The development of artificial saliva formulations containing mucilage from Ceylon Spinach

#### 2.4.1. Preparation of the artificial saliva formulations

Five developed artificial saliva formulations were prepared (Table 1). The compositions of the formulations were mucilage

**Table 1**

Compositions of the 5 developed artificial saliva formulations.

Compositions (g)	No.1	No.2	No.3	No.4	No.5
Ceylon Spinach mucilage	0.5500	0.7300	0.8300	0.6600	0.6100
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.0242	0.0226	0.0216	0.0232	0.0237
KCl	0.1428	0.1334	0.1282	0.1371	0.1397
Na <sub>2</sub> HPO <sub>4</sub>	0.1265	0.1265	0.1265	0.1265	0.1265
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	0.1530	0.1530	0.1530	0.1530	0.1530
NaF	0.0010	0.0010	0.0010	0.0010	0.0010
20% concentrated paraben	1	1	1	1	1

Note : 20% concentrated paraben solution contained 18% methyl paraben and 2% propyl paraben.

from Ceylon Spinach, calcium chloride (CaCl<sub>2</sub>), potassium chloride (KCl) and sodium fluoride (NF) as electrolytes and minerals, and 20% concentrated paraben (18% methyl paraben and 2% propyl paraben) as preservative. Briefly, the mucilage from Ceylon Spinach together with the electrolytes and minerals were dispersed in 100 ml of 20 mM phosphate buffer (pH 7.4). The mixture was then homogenized by a homogenizer (Brinkmann, Kinematica GmbH, Switzerland) at room temperature (25 ± 2 °C) for 15 min and left for 30 min. Then, 1% of the concentrated paraben was added. The formulations were filtered through a Whatman® No. 1 filter paper and kept in a tight bottle for the further experiment.

#### 2.4.2. Physico-chemical properties of the developed artificial saliva formulation containing mucilage from Ceylon Spinach

**2.4.2.1. Natural human saliva collection.** The natural human saliva was collected from healthy volunteers (N = 5, 25–35 years old). The volunteers gargled their mouths with drinking water prior to saliva collection.

**2.4.2.2. Viscosity and rheology determination.** Viscosity and rheology of the developed artificial saliva formulations were determined by a viscometer (Myr VR 3000 model, Tarragona, Spain) with the range of the shear rate between 5 and 200 rpm. All measurements were carried out at 25 ± 2 °C and 50 ml volume of the formulation were used in each test. The shear stress of the artificial saliva formulations was calculated by the following equation:

$$\text{Shear stress} = \frac{\text{shear rate}}{\text{Viscosity}}$$

**2.4.2.3. Wetting time determination.** Wetting time of the developed artificial saliva formulations was measured by the modified method previously described (Krishnamoorthy et al., 2011). Briefly, a filter paper (Whatman® No. 1) was placed in a Petri dish containing 6 ml of the sample (distilled water, natural saliva from human volunteer and the developed artificial saliva formulation). A tablet with methylene blue dye powder on the upper surface was carefully placed on the filter paper in the Petri dish. The time (mins) required for the sample to reach the upper surface of the tablet by observing from the completely wetting with methylene blue was noted as the wetting time.

**2.4.2.4. Selection of the best artificial saliva formulation.** The formulation with the best physical properties (clarity, precipitation, viscosity/rheology and wetting time) was selected for the further study.

#### 2.5. Biological activities of the selected artificial saliva formulation containing mucilage from Ceylon Spinach

##### 2.5.1. DPPH radical scavenging activity

Free radical scavenging activity of the selected artificial saliva formulations was determined by the modified DPPH assay

(Manosroi et al., 2013). Briefly, 50 µl of five serial concentrations (0.001–10 mg/ml) of the sample and 50 µl of ethanolic solution of DPPH were added into each well of a 96-well microplate (Nalge Nunc International, NY, USA). The reaction mixtures were allowed to stand for 30 min at 25 ± 2 °C and the absorbance was then measured at 515 nm by a well reader (Bio-Rad, Model 680 Microplate Reader, USA) against the negative control (distilled water). Ascorbic acid (0.001–10 mg/ml) was used as a positive control. The experiment was done in triplicate. The percentages of free radical scavenging activity were calculated as the following: Scavenging (%) = [(A – B)/A] × 100, where A was the absorbance of the negative control and B was the absorbance of the sample. The sample concentration providing 50% of scavenging (SC<sub>50</sub>) activity was calculated from the graph plotted between the percentages of the scavenging activity and the sample concentrations.

##### 2.5.2. Cytotoxicity on normal human gingival fibroblasts

**2.5.2.1. Cell culture.** Normal human gingival fibroblasts were obtained from the gingival tissue by the explant technique at Faculty of Dentistry, Chiang Mai University in Chiang Mai, Thailand. They were cultured in the 30-mm diameter tissue culture dishes in the complete culture medium containing α-Modified Eagles culture medium (MEM-Alpha, Hyclone, Utah, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, Utah, USA), penicillin (100 U/ml; Hyclone, Utah, USA) and streptomycin (100 mg/ml; Hyclone, Utah, USA). Cells were incubated in a temperature-controlled, humidified incubator (Shel Lab, model 2123TC, USA) with 5% CO<sub>2</sub> at 37 °C and subcultured every 5–7 days. The cells at the 5th to 8th passage were used in this study.

**2.5.2.2. Sample preparation.** The extract at 6.1 mg/ml and the developed artificial saliva formulations containing 6.1 mg/ml of mucilage from Ceylon Spinach were used for cytotoxicity test compared to natural human saliva at 1 mg/ml. All samples were filtered through a membrane filter (0.2 µm) before use.

**2.5.2.3. Cytotoxicity by SRB assay.** The cells were seeded in 96-well plates at an amount of 10,000 cells/well and allowed to attach overnight at 37 °C in 5% CO<sub>2</sub> incubator. Then, the cells were exposed to the sample for 24 h. After incubation, the adherent cells were fixed by adding cold 50%w/v trichloroacetic acid and further incubated for 1 h at 4 °C. Then, the cells were rinsed with distilled water, air-dried and stained with 0.4% SRB in 1% glacial acetic acid for 30 min at room temperature (27 ± 2 °C). The unbound SRB was removed by washing with 1% glacial acetic acid solution for four times. After air-drying, 100 µl per well of 10 mM Tris base were added to dissolve the bound stain. After mixing, the absorbance was measured at 540 nm with a microplate reader (Biorad, Milan, Italy). The untreated cells were used as a negative control. Cell viability (%) was calculated by the following equation:

$$\text{Cell viability (\%)} = \left( \frac{\text{Absorbance}_{\text{treated cells}}}{\text{Absorbance}_{\text{untreated cells}}} \right) \times 100$$

### 2.5.3. Anti-adherent activity determination

**2.5.3.1. Bacterial culture.** *Streptococcus mutans* (obtained from Faculty of Dentistry, Chiang Mai University, Thailand) was inoculated into tryptic soy broth and incubated at 37 °C for 24 h. The absorbance at 550 nm (OD<sub>550</sub>) was measured and the cell concentration was adjusted to obtain the OD<sub>550</sub> of 0.5. The bacterial concentration was diluted one fold to obtain the bacterial concentration of 1 × 10<sup>6</sup> CFU/ml.

**2.5.3.2. Sample preparation.** The selected artificial saliva formulation containing the Ceylon Spinach mucilage and the natural human saliva from the human volunteers were used. Briefly, an amount of 20 ml of the natural saliva was collected from the human volunteers, centrifuged at 10,000g, 4 °C for 15 min and the clear solution was collected. Distilled water was used as a negative control. The developed artificial saliva formulation, the natural saliva and the distilled water were filtered through a membrane filter (0.2 μm) before use.

**2.5.3.3. Anti-adherent activity.** The anti-adherent activity was performed as previously described (Manosroi et al., 2015). Briefly, hydroxyapatite (HA) was dispersed in phosphate buffer (pH 6.8) at the concentration of 5 mg/ml. An amount of 200 μl of the HA suspension was added into each well of the 96-well plate, centrifuged at 1,000g for 15 min and the supernatant was discarded. The natural saliva (200 μl) was added into each well, incubated at 37 °C with shaking at 80 rpm for 120 min and centrifuged at 1,000g for 15 min. The HA was washed by phosphate buffer. The 100 μl of *S. mutans* with 100 μl each of either the selected artificial saliva formulation, natural saliva or distilled water were added, incubated at 37 °C with shaking at 80 rpm for 120 min and then incubated without shaking at 37 °C for 60 min. The plate was centrifuged at 1,000g for 15 min and washed with phosphate buffer. The resazurin solution (100 μl) was added into each well and incubated at 37 °C with shaking at 80 rpm for 60 min. The fluorescence intensity was measured by a spectrofluorometer (Jusco, Hachioji, Japan) with the excitation/emission at the λ of 562/595. The % adhesion was calculated in comparing to the control (distilled water) as the followings:

$$\% \text{ Decrease of adherence} = \frac{F_{\text{control}} - F_{\text{sample}}}{F_{\text{control}}} \times 100$$

$$\% \text{ Increase of adherence} = \frac{F_{\text{sample}} - F_{\text{control}}}{F_{\text{control}}} \times 100$$

where  $F_{\text{control}}$  was the fluorescence intensity of the control and  $F_{\text{sample}}$  was the fluorescence intensity of the sample.

### 2.6. Statistical analysis

The results were presented as mean ± SD of three independent experiments. ANOVA was used for the analysis of the test results at the significance level of  $p$ -value < 0.05.

## 3. Results and discussion

### 3.1. Physical properties of the Ceylon Spinach mucilage and the developed artificial saliva formulations containing mucilage from Ceylon Spinach

#### 3.1.1. Physical appearances

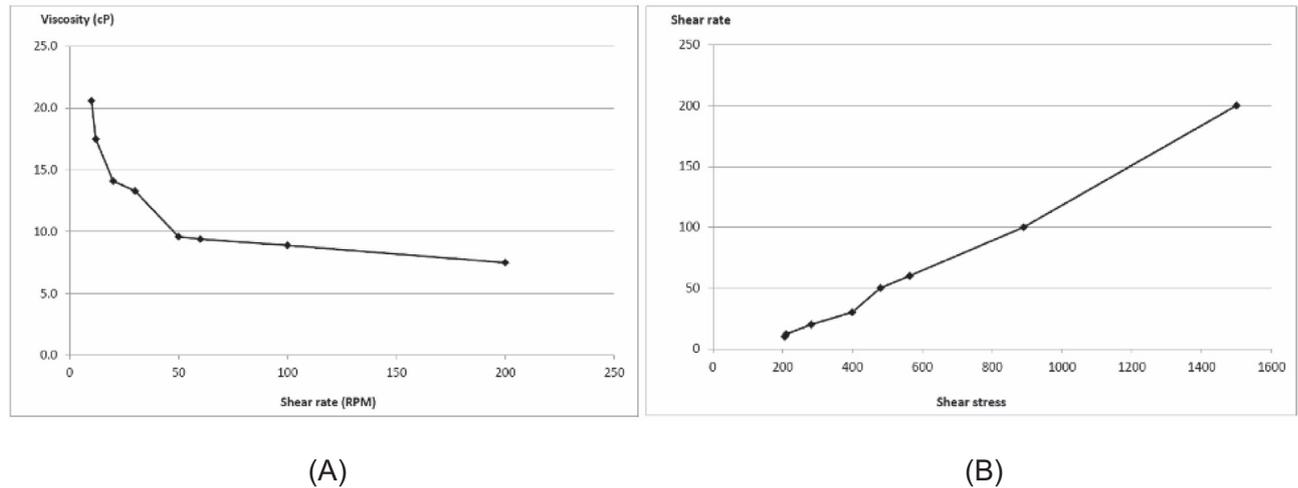
The lyophilized powder of the mucilage from Ceylon Spinach was in green-brown appearance with the characteristic greenish odor. The percentage yield of the mucilage was 0.44% w/w of the fresh plant. The Ceylon Spinach mucilage gave the positive results of phytochemical test of flavonoids and fructose. The 5 developed artificial saliva formulations gave different physical appearance (Fig. 2). Formula No.5 containing 0.61 g of the mucilage from Ceylon Spinach, 0.0237 g of CaCl<sub>2</sub>, 0.1397 g of KCl and 0.1530 g of NaF gave the best physical appearance which was translucent light green solution without precipitation.

#### 3.1.2. Viscosity and rheology of the developed artificial saliva formulations

The formulations which showed good physical appearance with no precipitation having the viscosity value and rheology behavior similar to natural human saliva which is a non-Newtonian pseudoplastic flow that is the most important physical characteristic of human saliva and gave no microbial contamination were selected. All of the 5 developed artificial saliva formulations gave good physical appearance with no precipitation with formulation nos. 1, 2, 3, 4 and 5 showing the non-Newtonian pseudoplastic flow with difference viscosity values of 6.8 ± 0.1, 14.4 ± 0.5, 17.3 ± 0.3, 10.1 ± 0.5 and 8.9 ± 0.2 cP, respectively. Thus, only the developed artificial saliva formulation No. 5 gave the viscosity of 8.9 ± 0.2 cP which was very close to the natural human saliva (9.0 ± 0.1 cP) was selected for the further experiment. The viscosity of the artificial saliva formulation No.5 decreased with increased shear rate, which was an essential property of saliva as shown in Fig. 3. This formulation also indicated the non-Newtonian pseudoplastic flow the same as the natural saliva (Park et al., 2007). Saliva has important rheological properties that may affect mouth feel and other sensory perceptions. Mucin glycoproteins containing in natural saliva are known to be important factors for the extensional rheological properties (viscosity, elasticity and stickiness). Also, different rheological behaviours of saliva from each gland secretion



Fig. 2. Physical appearances of the 5 developed artificial saliva formulations containing mucilage from flowers of Ceylon Spinach.



**Fig. 3.** Viscosity (A) and rheology behavior (B) of the selected artificial saliva formulation No.5 containing mucilage from Ceylon Spinach showing the non-Newtonian pseudoplastic flow similar to the natural human saliva.

could be due to the influence of mucin concentration, mucin conformation and/or the mucin type within the glandular saliva (Diogo Löfgren et al., 2015). As known, the main constituents of plant mucilages are galactose, arabinose, rhamnose, uronic acids, galacturonic acid, protein, Ca and Mg (Williams and Phillips, 2000). The major composition in the Ceylon Spinach mucilage which is responsible for viscosity is pectin, a polysaccharide with rhamnose and neutral sugar with the  $\alpha$ -(1–4) glycoside linkage. Pectins containing a significant amount of galacturonic residues in which the presence of the counterions in their solution can affect the inter- and intra-molecular interactions. The reduction of these intramolecular forces can let the coils to contract to the more compact conformation, with the consequent of the reduction in intrinsic viscosity (Kontogiorgos et al., 2012). In fact, the viscosity of polysaccharide solution has been reported to increase with increased concentrations, but decrease with increased pH and temperatures (Ahiakpa et al., 2014). It has been previously indicated that the higher concentration of the Ceylon Spinach mucilage, the lower the pH and the higher viscosity were obtained (Chatchawal et al., 2010). At higher concentration, the mucilage can have a very rigid structure which can attribute to the formation of hydrogen bonds between the polysaccharide and water (Rao et al., 2011). The selected artificial saliva formulation No.5 containing mucilage from Ceylon Spinach exhibited similar rheological properties of non-Newtonian pseudoplastic flow to the natural saliva with the viscosity of 8.9 cP.

### 3.1.3. Wetting times of the developed artificial saliva formulations

The wetting time is an important property of saliva, since the shorter wetting time can improve food swallowing in xerostomia and hyposalivation patients. Thus, food can be wet in few minutes to make it easier to chew and be swollen. Out of the 5 developed formulations, the wetting time of the artificial saliva formulation

No.5 containing mucilage from Ceylon Spinach was  $12.50 \pm 2.24$  min which was close to the normal human saliva ( $10.87 \pm 1.79$  min) (Table 2). The mucin glycoprotein in natural saliva has a lubricating, wetting and softening effect from its hydrophilic and hydrophobic segments that acts as a surfactant resulting the surface tension reduction between water and air (Mikos and Peppas, 1989). Negative charge moieties of the mucin (glycosylated region) can interact with water molecules, create a hydration shell and improve hydration and lubrication (Coles et al., 2010). Thus, loss of negatively charged glycan residues is a proposed mechanism for oral dryness through the reduced water retention capacity of mucin, leading to reduced mucosal hydration. Moreover, the contact angle of saliva can reflect the degree of wetting of saliva on surfaces and hence gives an insight into the interaction of saliva with surfaces (Vijay et al., 2015). Thus, the wetting property of formulation No.5 may be from the bound moisture of the mucilage that improves wettability by the mechanism of surface tension reduction. In addition, polymers in the Ceylon Spinach mucilage have been reported to have hydrophilic constituents, with hydroxyl and carboxyl groups (Pareek et al., 2010), that can be swollen in water thereby exposing to the maximum number of the adhesive sites (Shaikh et al., 2011). Hence, the formula No.5 which gave superior physical properties of viscosity and wetting time similar to natural saliva was selected for the further study.

### 3.2. Antioxidative activities of the selected artificial saliva formulation No.5 containing mucilage from Ceylon Spinach

Antioxidative activities of the selected artificial saliva formulation containing mucilage were shown in Table 2. The formulation gave the DPPH scavenging activity with the  $SC_{50}$  value of  $14.26 \pm 2.00$  mg/ml which was 0.05 folds of ascorbic acid, and was similar to mucilage from the Ceylon Spinach (the  $SC_{50}$  value of  $13.84 \pm 0.14$

**Table 2**

Physical properties of the selected artificial saliva formulation No.5 containing mucilage from Ceylon Spinach.

Sample	Physical appearance	pH value	Rheology	Wetting time (min)
Natural human saliva	turbid solution	$7.32 \pm 0.51$	non-Newtonian pseudoplastic	$10.87 \pm 1.79$
Ceylon Spinach mucilage	green-brown solid	$5.58 \pm 1.09$	non-Newtonian pseudoplastic	$10.65 \pm 0.69$
The artificial saliva formulation No.5 containing mucilage from Ceylon Spinach	translucent light green solution	$7.41 \pm 0.35$	non-Newtonian pseudoplastic	$12.50 \pm 2.24$

mg/ml). However, DPPH scavenging activity was not observed in natural human saliva. The  $SC_{50}$  value of the mucilage from the Ceylon Spinach observed in this study was different from the previous study which gave the  $SC_{50}$  value of 514.41  $\mu\text{g/ml}$  (Chatchawal et al., 2010). This difference may be from the different extraction method. The observed antioxidant activity of the developed artificial saliva formulation No.5 may be from the presence of polysaccharide containing in the mucilage of Ceylon Spinach such as galactose, arabinose, glucose and mannose (Dong et al., 2012a,b). Several previous works have reported the antioxidant activity of polysaccharides from plants. Polysaccharides including rhamnose, glucuronic acid, galacturonic acid, glucose, galactose and arabinose from Wampee [*Clausena lansium* (Lour.) Skeels] gave high antioxidant activities of 78% hydroxyl radical-scavenging ability and 48.35% antioxidative activity (Wu et al., 2013). Water-soluble polysaccharides from Wolfberry (*Lycium barbarum* L.), Sweet Cherry (*Prunus avium* L.), Kiwi (*Actinidia chinensis* L.) and Cranberry fruits (*Vaccinium macrocarpon* Aiton) indicated the antioxidant activities assayed by the oxygen radical absorbance capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) (Fan et al., 2010). The mucilage from Ceylon Spinach of this present study contained not only polysaccharides, but also flavonoids the same as the previous study (Olajire and Azeez, 2011). Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and chelating metal ions. They also found ascorbic acid and especially phenolic compounds, which have been reported to have antioxidant activities from their redox properties of acting as a reducing agents hydrogen donors and singlet oxygen quenchers. The radical scavenging activities of Ceylon Spinach may be related not only to the phenolic contents, but also the flavonoid, anthocyanin and proanthocyanin contents (Oloyede et al., 2013). Kaempferol which was a flavonoid found in Ceylon Spinach has been reported to exhibit strong DPPH radical scavenging activity with the  $SC_{50}$  value of 4.349  $\mu\text{g/ml}$  (Yu et al., 2013). Hence, the synergistic effect of all phytochemicals in Ceylon Spinach mucilage may be responsible for this free radical scavenging biological activity.

### 3.3. Cytotoxicity of the selected artificial saliva formulation No.5 containing mucilage from Ceylon Spinach

Several studies have investigated cytotoxicity of mucilaginous polysaccharide content in plants. For examples, Okra (*Abelmoschus esculentus* Linn. moench) polysaccharides showed no cytotoxicity on human cancer cell line with nearly 100% cell viability and no morphological changes (Ilango et al., 2011). Mucilage from *Grewia optiva* at 10, 20 and 30  $\mu\text{g/ml}$  gave non-toxic on normal (NIH3T3) cell lines (Kumar and Kulkarni, 2012). Sushila et al. have also demonstrated that the extract from *Basella alba* whole plant indicated significant dose dependant cytotoxicity on the Jurkat cell lines, but gave no toxicity on lung cancer cell line (A549) with 88.33  $\pm$  1.20% cell viability at 25  $\mu\text{g/ml}$  (Sushila et al., 2010). There was no report of Ceylon Spinach mucilage and its artificial saliva formulation on human gingival fibroblast cytotoxicity. This present study has been the first report to determine the safety for oral administration of the artificial saliva formulation containing mucilage from Ceylon Spinach on human gingival fibroblasts. Cytotoxicity of the selected artificial saliva formulation containing mucilage from Ceylon Spinach determined by SRB assay was shown in Table 3. The formulation with the pH value of 7.41  $\pm$  0.35 exhibited no cytotoxic effect on the treated cells with 99.20  $\pm$  12.09% cell viability. However, the mucilage from Ceylon Spinach showed high cytotoxic effect of only 23.90  $\pm$  10.43% cell viability, owing to the acidity (pH 5.58  $\pm$  1.09) of the mucilage. pH is one of the most important factors of growth promoting properties including contact inhibition, growth rate and cell mobility.

**Table 3**

Antioxidative activity and cytotoxicity on human gingival fibroblasts of Ceylon Spinach mucilage and the artificial saliva formulation No.5 containing mucilage from Ceylon Spinach.

Sample	Antioxidative ( $SC_{50}$ , mg/ml)	Cell viability (%)
Ascorbic acid	0.72 $\pm$ 0.00	87.44 $\pm$ 10.38
Natural human saliva	NA	98.28 $\pm$ 8.92
Ceylon Spinach mucilage	13.84 $\pm$ 0.14*	23.90 $\pm$ 10.43*
The artificial saliva formulation No.5 containing mucilage from Ceylon Spinach	14.26 $\pm$ 2.00*	99.20 $\pm$ 12.09

Note: NA = no activity; Cell viability > 90% = non-toxic, 60–90% = slightly toxic, 30–59% = moderately toxic and <30% = highly toxic, \* significant difference ( $p < 0.05$ ) in comparing with ascorbic acid.

This result was different from the previous study showing that the aqueous extract of *Basella alba* (Ceylon Spinach) indicated the  $LC_{50}$  value at >1000 ppm assayed by the brine shrimp (*Artemia salina*) lethality bioassay, indicating of no cytotoxicity (Balasuriya and Dharmaratne, 2007). As previously reported, the pH range of 6.8–8.2 enhanced human-human hybridoma (HB4C5) cell viability, whereas the outside ranges demonstrated a decrease in cell viability (Zungu et al., 2007). Also, it has been previously demonstrated that Ceylon Spinach mucilage at 2 mg/ml showed relatively mild toxicity on Chang liver cell line with 84.4% cell viability (Chatchawal et al., 2010).

Ascorbic acid (vitamin C) is a common positive control of antioxidant activity test by DPPH free radical scavenging. For cytotoxicity test, several studies have shown that vitamin C increases numbers of collagen bundles in the regenerating periodontal tissue, detoxifies histamine in gingival inflammation and reduces gingival oxidative stress. Vitamin C reduces the cytotoxic and apoptotic effects of *P. gingivalis* on human gingival fibroblasts (HGF) (Staudte et al., 2010). This present study has expected that the effect of the developed artificial saliva formulation on HGF was similar to vitamin C and showed the safety of the developed artificial saliva formulation for oral use.

### 3.4. Anti-adherent activity of *S. mutans* of the selected artificial saliva formulation No.5 containing mucilage from Ceylon Spinach

*S. mutans* has been implicated as a primary causative agent of dental caries. Thus, inhibition of *S. mutans* adhesion to the tooth surface is a major goal for the prevention of dental caries. Anti-adherent activity of the selected artificial saliva formulation containing mucilage from Ceylon Spinach were shown in Table 4. In this study, the natural saliva demonstrated an increase adherence of *S. mutans* on the HA (hydroxyapatite) beads of 33.10  $\pm$  9.70% over the control (distilled water). This may be from the compositions in the natural saliva, such as proteins and polysaccharides. Natural saliva contains a multitude of proteins which are important for oral microbial ecology and biofilm formation. Adsorption of specific salivary proteins, such as acidic proline-rich proteins and agglutinin, promotes the adhesion of *S. mutans* onto the HA surfaces by providing ligands for bacterial attachment (Shimotoyodome et al., 2007). Moreover, high molecular weight salivary glycoprotein promotes adhesion of *S. mutans* (Shimotoyodome et al., 2006). Hydrophobic interactions have been demonstrated to be important in bacterial adhesion. Also, the negative charges containing in polysaccharides might be necessary for adsorption onto the HA surfaces. Ceylon Spinach mucilage increased the adherence of only 4.83  $\pm$  3.47% which was less than the natural saliva of 6.85 times. Thus, Ceylon Spinach mucilage appeared to reduce *S. mutans* adherence on the HA beads in comparing to the natural saliva. The mechanism of the mucilage from Ceylon Spinach on the reduction of *S.*

**Table 4**

Anti-adherent activity of *S. mutans* of the selected artificial saliva formulation No.5 containing mucilage from Ceylon Spinach.

Sample	%Decrease of adherence	%Increase of adherence
Natural human saliva	–	33.10 ± 9.70
Ceylon Spinach mucilage	–	4.83 ± 3.47
Vehicle of the artificial saliva formulation	3.66 ± 0.88	–
The artificial saliva formulation No.5 containing mucilage from Ceylon Spinach	17.01 ± 7.75	–

Note:

$$\% \text{ Decrease of adherence} = \frac{F_{\text{control}} - F_{\text{sample}}}{F_{\text{control}}} \times 100.$$

$$\% \text{ Increase of adherence} = \frac{F_{\text{sample}} - F_{\text{control}}}{F_{\text{control}}} \times 100.$$

*mutans* adhesion might be due to the amino sugars, such as glucosamine containing in the mucilage which can inhibit the binding of *S. mutans* to the saliva-coated HA (Hajishengallis et al., 1992). The low molecular weight proteins reduced the saliva-promoted *S. mutans* adherence by competitively inhibiting *S. mutans*-binding glycoprotein adsorption onto the HA surface has also previously reported (Shimotoyodome et al., 2007). Thus, the amino acid such as arginine, leucine, isoleucine, lysine, threonine and tryptophan containing in the Ceylon Spinach mucilage may inhibit *S. mutans* adhesion to the HA surface (Khare, 2007).

The selected artificial saliva formulation containing mucilage from Ceylon Spinach exhibited a decrease adhesion of *S. mutans* on HA beads of 17.01 ± 7.75%, whereas the vehicle of the formulation decreased the adherence of 3.66 ± 0.88%. Hence, it appeared that the Ceylon Spinach mucilage containing in the developed artificial saliva formulation may enhance the anti-adherent activity of *S. mutans* of the vehicle of about 13%. The reduction of bacterial adhesion of the vehicle may be due to the bacterial growth reduction by its compositions of parabens and fluoride. Parabens are antimicrobial agents which can inhibit irreversibly glycolysis by the cariogenic dental plaque bacterium, *S. mutans* (Ma and Marquis, 1996). Sodium fluoride can reduce the growth of *S. mutans* by the sub-minimum inhibitory concentration (MIC) levels of fluoride (MIC: 282 ppm F<sup>-</sup>) (Dong et al., 2012a,b). Also, the commercial fluoride varnishes showed a strong inhibitory effect on the bacterial adhesion of approximately 67–98% reduction (Chau et al., 2014). The anti-adherent activity of *S. mutans* of the selected artificial saliva formulation No.5 was thus the synergistic effects from the Ceylon Spinach mucilage and other compositions in the vehicle. Hence, the selected artificial saliva formulation No.5 containing mucilage from Ceylon Spinach indicated superior anti-adherent activity of *S. mutans* to the normal human saliva which is one of the important advantages of this developed artificial saliva formulation.

#### 4. Conclusions

The results from this study have suggested the potential application of mucilage from Ceylon Spinach for artificial saliva because of not only its superior physical properties including rheological property and wetting time similar to the natural human saliva, but also having higher antioxidant and anti-adherence activities than the natural human saliva, and with no cytotoxic effect on normal human gingival fibroblasts. Moreover, Ceylon Spinach is an edible vegetable and has been used for foods. Thus, the developed artificial saliva formulation containing mucilage from this plant is safer for consumption than other chemical artificial saliva formulations. This present study has also demonstrated the possibility of using mucilage from edible plants which are safe and cost effective to prepare a biological active artificial formulation for the substitution of natural human saliva and other chemical artificial saliva formulations.

#### Declaration of Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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