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Antibacterial Activity and Cytotoxicity of *Aster sphathulifolius* Maxim. Against Oral-bacteria and Oral-cancer Cell Lines

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ABSTRACT

The aim of this study is to investigate anti-bacterial activity and cytotoxicity of *Aster sphathulifolius* Maxim. (*A. sphathulifolius*) against oral pathogens and oral carcinoma cell lines. For the private hygiene, the health of mouths is very important for maintaining entire body healthy. Some chemical agents, used in products for health of mouths, are reported to have bad side-effects. Therefore there is a growing interest in safe and natural materials with pharmaceutical activity. Anti-bacterial activity of *A. sphathulifolius* extract was performed using agar-diffusion assay against several oral pathogens and anti-carcinogenic activity was carried out by cell viability assay for oral cancer cell lines. Whole plant-extract of *A. sphathulifolius* showed the anti-bacterial activities against all tested oral pathogens except of *Staphylococcus aureus* (*S. aureus*). Anti-bacterial activities against *Streptococcus mutans* (*S. mutans*), *Streptococcus sobrinus* (*S. sobrinus*), *Streptococcus pyogenes* (*S. pyogenes*) and *Staphylococcus epidermidis* (*S. epidermidis*) were observed by most of all parts of *A. sphathulifolius*. However, the growth of *S. aureus* was inhibited by only root-extract of *A. sphathulifolius*., *A. sphathulifolius* showed the outstanding cytotoxicity against human oral gingival carcinoma cells with approximately 75.1 and 93.1% cell death in 250 and 500 $\mu\text{g mL}^{-1}$ of concentrations, respectively. *A. sphathulifolius* extracts had the anti-bacteria activity against oral pathogens and the cytotoxicity of oral carcinoma cell lines together. Taken together, it is anticipated that *A. sphathulifolius* might be used for the oral health care, as mouthwashes and toothpastes form with an alternative therapeutic drug.

Key words: Anti-bacterial activity, *Aster sphathulifolius* Maxim., cytotoxicity, oral cancer cells, oral pathogen

INTRODUCTION

Aster sphathulifolius Maxim (*A. sphathulifolius*), a perennial plant for ornament, is inhabiting in the seashore (Lee, 2006). In addition, *A. sphathulifolius* has been used as traditional medicine for chronic hepatitis, obesity, a cough, a common cold and cystitis (Hangul Dongeuibogam, 2011). It was reported that terpene hydroperoxides and labdane diterpenes isolated from the aerial parts of *A. sphathulifolius* had cytotoxicity against cancer cell lines, including human lung cancer cell, ovarian cancer cells, human colon carcinoma (Lee *et al.*, 2005, 2006). Influenza virus A/PR/8/34 was suppressed by methanol extract of whole *A. sphathulifolius* (Won *et al.*, 2013). Unfortunately, the improvement of oral health using the extract of *A. sphathulifolius* has never been studied and reported so far.

Since, the syndrome of deaths by human influenza A (H1N1) virus-infection in 2009, the risen attentions for personal health care has demanded the safe and effective oral-care products from natural materials and versatile oral-care activities (EBN Industrial News, 2011). In contrast with

the shrink of dental service in hospital, the attention for oral-health care and oral hygiene products has been increased in the aspect of precaution. This trend is being certified by the boost in sales of oral hygiene products (KDAN, 2011).

In a mouth, the oral cavity provides a suitable nutritional and physiological circumstance for the growth of oral-bacteria. Generally, the human oral microbial flora includes more than approximately 30 bacteria and the flora and the species-ratio of oral bacteria change according to the individual age, health condition and status of hygiene. Control of oral pathogens is necessary for the care of oral health.

Dental caries and halitosis are associated with oral pathogens. Dental caries is a destructive disease that can progress to inflammation and to death of vital pulp tissue in dental hard tissues. Dental caries is caused by a few species of cariogenic bacteria and specific dietary patterns, such as sugar intake and eating frequency (Huxley, 1977a, b; Beighton *et al.*, 1996). *Streptococcus mutans* (*S. mutans*), *Streptococcus sobrinus* (*S. sobrinus*) and *Lactobacillus casei* (*L. casei*) are well-known cavity-causing bacteria. And *S. mutans* is the major contributor to acid production in dental caries (Loesche, 1986; Van Houte, 1994). Generally, the erosion of teeth by the acid causes the inflammatory destruction in the soft and hard tissues (Hu *et al.*, 2011). This inflammatory destruction begins by plaque-constituents on the surface of teeth-hard root beside soft tissues supporting periodontium. This symptom is limited in gingiva or worsened to deeper places showing destruction of the periodontal ligament and the alveolar bone.

Oral Squamous Cell Carcinoma (SCC) is the most common malignancy of the oral cavity and has more fatality rate deaths than any other oral diseases. Among the clinical treatments of cancer, the chemotherapy was performed frequently, in combination with surgery or radiotherapy (Forastiere *et al.*, 2003; Psyrri *et al.*, 2004). Novel anti-cancer chemicals contributed to the improvement of survival rate. In other hands, the novel therapeutics has the adverse systemic and cytotoxic effects frequently. Many chemical agents used for the chemotherapy are limited to use due to its bad side-effects. Therefore the natural plant extracts has been considered as alternative medicines and side-effect free chemotherapeutic agents. Many researchers have reported the protective role of fruits and vegetables for the treatments of oral cancer and the role of their high polyphenol (particularly flavonoids) contents for its anti-cancer activity (Yang *et al.*, 2008; Block *et al.*, 1992; Levi *et al.*, 1988). In addition, some researchers have studied the anti-bacterial activity of plant extracts against oral pathogens. Some herbal medicine extracts and its constituents, such as (-)-epigallocatechin, were investigated to have anti-bacterial activities against cariogenic *S. mutans*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Porphyromonas gingivalis* (Lee *et al.*, 2010; Park *et al.*, 2010; Jeon and Han, 2004).

In this study, we focused on the investigation of *A. sphathulifolius* for the activity to kill cariogenic bacteria and to affect the cytotoxicity against oral-carcinoma cells. It is anticipated that novel oral care products or phytotherapeutic medicine using extract of *A. sphathulifolius* can be developed on the basis of this result.

MATERIALS AND METHODS

Chemicals and reagents: Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cetylpridinium chloride (CPC) and streptomycin were purchased from Sigma-Aldrich (St.Louis, MO, USA). Cell culture medium and reagents were obtained from GIBCO (Invitrogen Inc., NY, USA). Ethanol and other reagents were used as first grade reagents.

Plant materials: Methanolic whole-and part-extracts of *Aster sphathulifolius* Maxim. (*A. sphathulifolius*, voucher specimen: KRIB 0020347-0020348) were obtained from Plant Extract Bank (PEB), Korea Research Institute for Bioscience and Biotechnology (KRIBB), Taejeon Korea. *A. sphathulifolius* extracts were redissolved in DMSO to 100 mg mL⁻¹, stored at -20°C and used it as a stock.

Oral-microorganisms and growth condition: Oral bacterial strains used in this study is as in the following; *Streptococcus mutans* (*S. mutans*) ATCC (American Type Culture Collection, Manassas, VA, USA) 25175, *Streptococcus sobrinus* (*S. sobrinus*) ATCC 27607, *Streptococcus pyogenes* (*S. pyogenes*) ATCC 21059, *Streptococcus anginosus* (*S. anginosus*) KCTC (Korean Collection for Type Culture, Daejeon, Korea) 5141, *Staphylococcus aureus* (*S. aureus*) ATCC 25923, *Staphylococcus epidermidis* (*S. epidermidis*) ATCC 12228. *Streptococcus* and *Staphylococcus* strains were cultured at 37°C in Brain Heart Infusion medium (BHI) (Bacto™, BD237500, Sparks, MD USA) and Nutrient Broth (NB) (Difco™, BD234000, USA), respectively. Stock cultures of bacteria were kept in 35% glycerol (in phosphate buffered saline) at -70°C. All bacterial strains were cultured by inoculation of the thawed microbial stock suspensions (100 µL) into 5 mL of BHI and NB medium using a shaking incubator at 37°C for 24 h in aerobic condition.

Antibacterial assay: The antimicrobial activities of *A. sphathulifolius* extracts were evaluated by agar-well diffusion assay with some modification (Owen and Palombo, 2007). After the well was cut by a sterile cork-borer (5 mm diameter) onto the surface of each agar plate (1.5%), 50 µL of each bacterial suspension (OD₆₀₀ = 0.2-0.3, approximately 1×10⁷ CFU mL⁻¹) were spread uniformly. *A. sphathulifolius*, extracts (10 µg well⁻¹) were dispensed into the wells. Agar plates were left at room temperature for 30 min to allow the liquid to diffuse into the agar before overnight incubation at 37°C. All assays were carried out in triplicate. A clear zone of the inhibited microbial growth on the agar plate was considered as an exhibition of antibacterial properties. The solvent used for the preparation of *A. sphathulifolius*, extracts was used for a blank well. After incubation, the inhibition zones around wells were measured in mm using a caliper (Fazeli *et al.*, 2007). CPC and streptomycin (10 µg well⁻¹) were used as positive controls.

Oral-cancer cell lines and cell culture: Human oral gingival carcinoma cell (YD-38), human oral squamous carcinoma cell (KB) and human oral tongue carcinoma cell (YD-15) were obtained from Korean Cell Line Bank (KLCB, Seoul Korea). Cells were cultured using 90% RPMI 1640 with L-glutamine (300 mg L⁻¹), 25 mM HEPES, 25 mM NaHCO₃, 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units mL⁻¹) and streptomycin (100 mg mL⁻¹) in a 5% CO₂ humidified incubator at 37°C.

Determination of cell viability: Cell viability was examined by an MTT assay (Lee *et al.*, 2009). Human oral cancer cells were plated at a density of 1×10⁵ cells well⁻¹ in 96-well tissue culture plate (Corning Inc., USA) and incubated at 37°C for 24 h. Plated cells were treated with indicated concentrations of *A. sphathulifolius*, extracts. After 24 h incubation, MTT was added to all wells at a concentration of 0.5 mg mL⁻¹ and incubated for 4 h at 37°C. After discarding all medium from the plates, 100 µL of DMSO was added to all wells. The plates were placed for 5 min at room temperature and were shaken, so that complete dissolution of formazan was achieved. Absorbance

of the MTT formazan was determined on 540 nm by a UV-spectrophotometric plate reader (Spectra Max M2, Molecular Devices Inc., USA). Viability of cells was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

Statistical analysis: All result was expressed as Mean±Standard Deviation (SD) and were analyzed using one way analysis of variance (ANOVA) and Dunnett's multiple comparison test for individual comparisons. Results were considered statistically significant when p-values were less than 0.05.

RESULTS AND DISCUSSION

Antibacterial activity of *A. sphathulifolius* against oral-bacteria: Antimicrobial activities of *A. sphathulifolius*, extracts were examined against six oral pathogens using agar-well diffusion susceptibility assay (Fig. 1 and Table 1). As shown in Fig. 1, the whole plant-extract of *A. sphathulifolius* showed the anti-bacterial activities against all tested oral pathogens, except of *S. aureus*. Whole plant-extract of *A. sphathulifolius* had the inhibitory activity on the growth of *S. mutans* with approximately 5.5 ± 0.7 mm of clear zone around an agar well. For positive controls of anti-bacterial activity, the antibiotics streptomycin and CPC, which is a chemical anti-bacterial agent in the commercial mouthwashes, showed a clear zone with 2.25 ± 1.2 and 4.75 ± 0.9 mm of

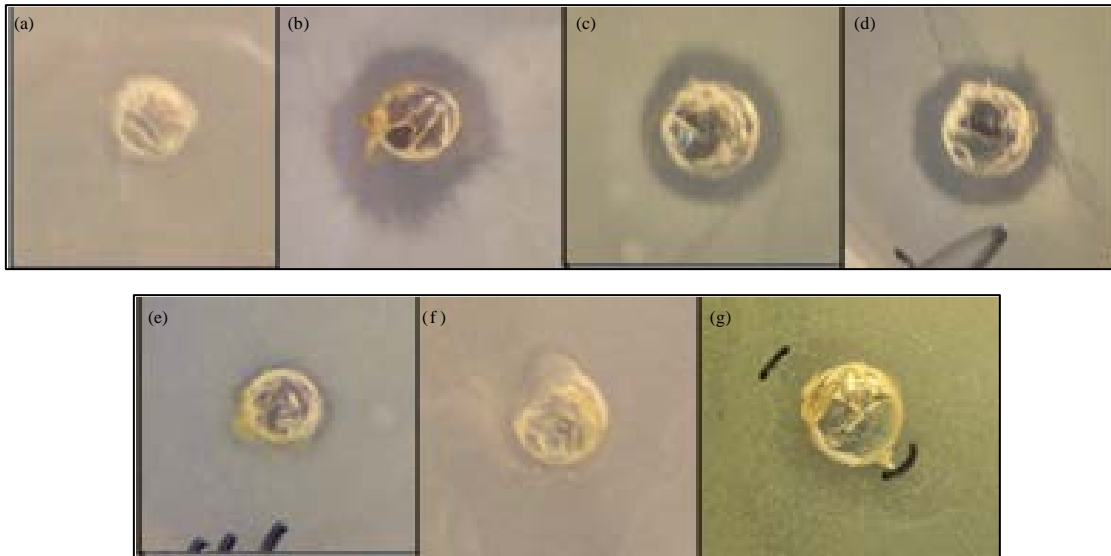


Fig. 1(a-g): Clear zones on oral pathogen spreading agar plate by whole plant-extract of *Aster sphathulifolius* Maxim. Wells were cut by a sterile cork-borer (5 mm diameter) onto the surface of each agar plate (1.5%) and 50 μ L of each bacterial suspension ($OD_{600} = 0.2-0.3$, approximately 1×10^7 CFU mL^{-1}) were spread uniformly. *A. sphathulifolius* extracts (10μ g well $^{-1}$) were dispensed into the wells. Agar plates were left at room temperature for 30 min to allow the extracts to diffuse into the agar before overnight incubation at 37°C. The solvent used for the preparation of *A. sphathulifolius* extracts was used for a blank well. After incubation, the inhibition zones around wells were measured in mm using a caliper. (a) Control, (b) *S. mutans*, (c) *S. sobrinus*, (d) *S. pyogenes*, (e) *S. anginosus*, (f) *S. aureus* and (g) *S. epidermidis*

Table 1: Agar diffusion susceptibility of oral-pathogens to *A. sphathulifolius* extract

<i>Aster sphathulifolius</i>	<i>Streptococcus mutans</i>	<i>Streptococcus sobrinus</i>	<i>Streptococcus pyogenes</i>	<i>Streptococcus anginosus</i>	<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>
<i>Aster sphathulifolius</i> (whole plant)	5.5±0.7	3.0±1.4	3.5±0.7	1.5±0.7	-	2.5±0.7
<i>Aster sphathulifolius</i> (flowers)	2.0±1.4	1.0±0.0	2.5±0.7	1.5±0.7	-	1.0±0.0
<i>Aster sphathulifolius</i> (leaves)	-*	3.5±0.7	2.5±0.7	-	-	1.0±0.0
<i>Aster sphathulifolius</i> (stems)	1.5±0.7	2.5±0.7	1.5±0.7	-	-	1.0±0.0
<i>Aster sphathulifolius</i> (roots)	1.5±0.7	3±1.4.0	-	1.5±0.7	1.5±0.7	-

*not detected

semi-diameter, respectively (data not shown). Anti-bacterial activity of *A. sphathulifolius* whole plant-extract against *S. mutans*, which is a major dental caring bacterium, was superior to that of streptomycin and CPC. Susceptibilities of *A. sphathulifolius* whole plant-extract against *S. sobrinus*, *S. pyogenes*, *S. anginosus* and *S. epidermidis* showed the inhibitory clear zones with 3.0±1.4, 3.5±0.7, 1.5±0.7 and 2.5±0.7 mm, respectively.

In addition, the anti-bacterial activities of part extracts of *A. sphathulifolius* were investigated against oral-pathogens (Table 1). Anti-bacterial activities against *S. mutans* were observed in treatment of all part-extracts of *A. sphathulifolius* but were a little bit weaker than that of whole plant-extract of *A. sphathulifolius*. All parts (flower, leaves, stems, roots), extract of *A. sphathulifolius* showed the susceptibilities against *S. sobrinus*. And cell growth of *S. pyogenes*, *S. anginosus* and *S. epidermidis* was suppressed by two or three parts-extract of *A. sphathulifolius*, as shown in Table 1. Especially only the root-extract of *A. sphathulifolius* inhibited the growth of *S. aureus* slightly.

Recently, as the attention and recognizing the importance on the personal beauty and oral health care increase, many oral care products are developed and sold in commercials (EBN Industrial News, 2011). In many mouthwashes, CPC is contained in mouthwashes in a concentration of 50 mg 100 mL⁻¹ for anti-bacterial activity against oral pathogen (Radford *et al.*, 1997; Feres *et al.*, 2010). However, some opinions about the bad side-effect of CPC are drawing the demand for alternative natural anti-bacterial agent without side-effect (Gilani and eHOW, 2011).

It was reported that several plants, such as soybean, garlic, horseradish, *Aralia continentalis*, *Pinus thunbergii*, *Morus bombycis* and ginger, have anti-bacterial activity against *S. mutans* and its anti-bacterial activity might be caused by high contents of flavonoids (Lee and Kim, 2006; Bakri and Douglas, 2005; Park *et al.*, 2003, 2009; Baek, 2007). There has been no report concerning a biological activity of *A. sphathulifolius* as well as oral health, so far. In this study, the anti-bacterial activities of *A. sphathulifolius*, extracts against *S. mutans* and *S. sobrinus* were superior to antibiotics streptomycin and CPC.

Cytotoxicity of *Aster sphathulifolius* maxim against oral-cancer cell lines: Cytotoxicity of *A. sphathulifolius* against human oral gingival carcinoma cell, human oral squamous carcinoma cell and human oral tongue carcinoma cell was investigated, as shown in Fig. 2. Whole plant-extract and part-extracts of *A. sphathulifolius* were treated to three oral carcinoma cell lines in indicated concentrations. After 24 h of incubation, the potent cytotoxicity against oral carcinoma cell lines was detected in a concentration-dependent manner. Whole plant-extract of *A. sphathulifolius* showed the outstanding cytotoxicity against human oral gingival carcinoma cell with approximately 75.1 and 93.1% cell death at 250 and 500 µg mL⁻¹ of concentration, respectively. Flowers-and leaves-extract showed approximately 92.5 and 92.4% of cytotoxicities

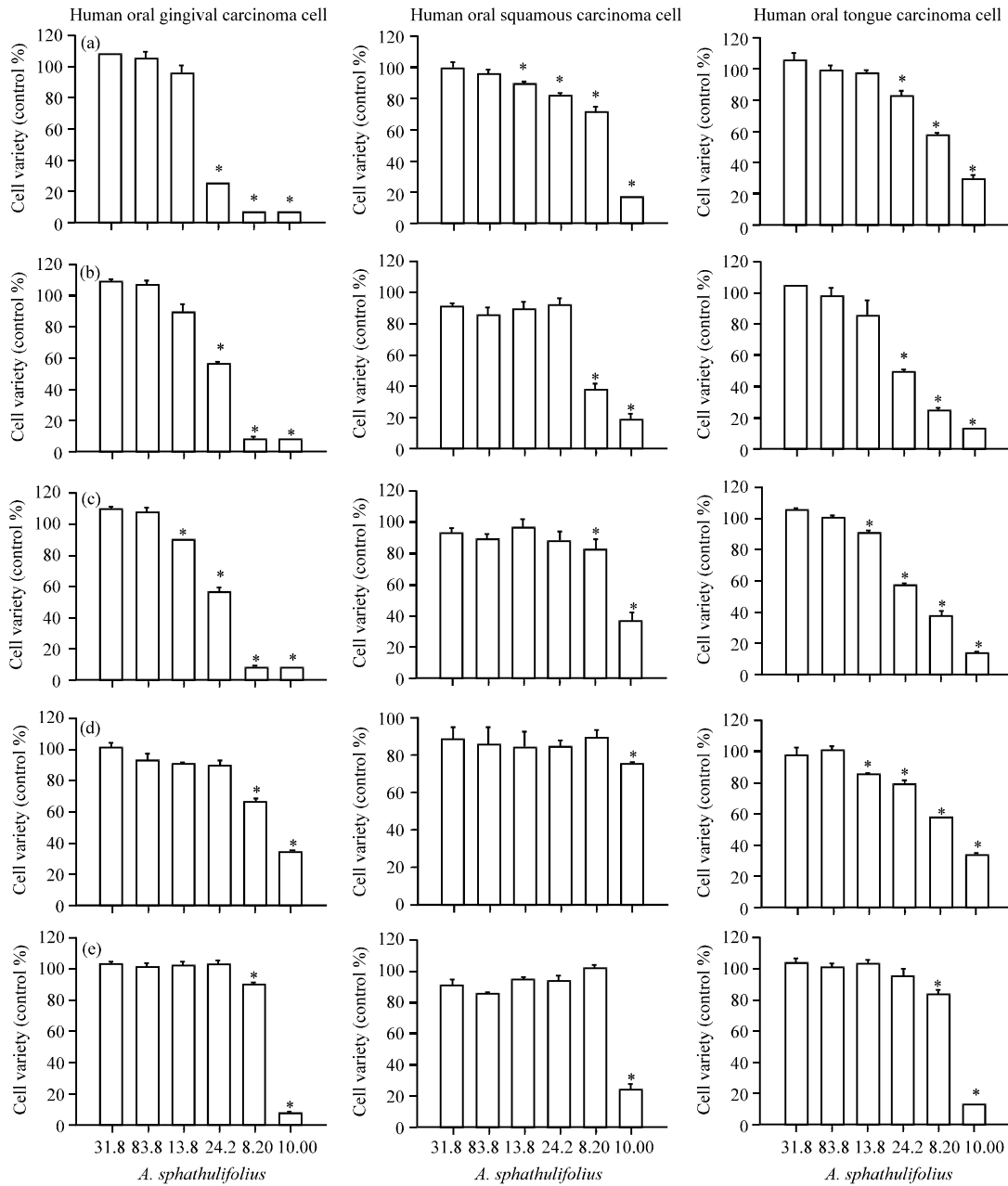


Fig. 2(a-e): Cell viability of human oral carcinoma cell lines by *Aster spathulifolius* extracts. Human oral cancer cells were plated at a density of 1×10^5 cells well⁻¹ in 96-well tissue culture plate (Corning Inc., USA) and incubated at 37°C for 24 h. Plated cells were treated with indicated concentrations of *A. spathulifolius* extracts. After 24 h incubation, MTT was added to all wells at a concentration of 0.5 mg mL⁻¹ and incubated for 4 h at 37°C. After discarding all medium from the plates, 100 μ L of DMSO was added to all wells. Absorbance of the MTT formazan was determined on 540 nm by a UV-spectrophotometric plate reader (Spectra Max M2, Molecular Devices Inc., USA). Viability of cells was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells (a) Whole plant, (b) Flowers, (c) Leaves, (d) Stems and (e) Roots

Table 2: Cytotoxicity (IC₅₀) of *A. sphathulifolius* extracts against human oral-cancer cells

Extract of <i>A. sphathulifolius</i>	IC ₅₀ (µg mL ⁻¹)		
	Oral gingival carcinoma cell	Oral squamous carcinoma cell	Oral tongue carcinoma cell
Whole plant	167.5	>500.0	>500
Flowers	284.9	397.5	289
Leaves	276.5	>500.0	357
Stems	>500.0	>1,000.0	>500
Roots	>500.0	>500.0	>500

against oral gingival carcinoma cell at 500 µg mL⁻¹ of concentrations. However, the cytotoxic activity (IC₅₀ = 167.5 µg mL⁻¹) of whole plant-extract was superior to those of flowers-and leaves-extract, which were IC₅₀ = 284.9 and 276.5 µg mL⁻¹, respectively (Table 2).

Cell viability of human oral squamous carcinoma and tongue carcinoma were significantly suppressed to 13-18% by the treatment of a whole plant-extract *A. sphathulifolius* (1000 µg mL⁻¹). Flowers-extract had the potent cytotoxicity (approximately 81-93%) against human oral gingival carcinoma, human oral squamous carcinoma and human oral tongue carcinoma with 284.9, 397.5 and 289 µg mL⁻¹ of IC₅₀, respectively (Table 2). Otherwise the stems-and roots-extract of *A. sphathulifolius* showed comparatively weak cytotoxicity against oral carcinoma cell lines. Oral cancer takes approximately 3% of all cancers and tongue and gingival carcinoma make up a high proportion of oral cancer cases (Sato *et al.*, 2011). Although the surgery, chemotherapy and radiation therapy for the treatment of oral cancer have been improved, the 5 year survival rate by oral cancer remains at approximately 50% (Shibahara *et al.*, 2002; Cooper *et al.*, 2004).

CONCLUSION

In this study, *A. sphathulifolius* extracts were investigated to have oral health enhancing acidity. Most of all, *A. sphathulifolius* possesses the anti-bacterial activity against oral pathogens and the cytotoxicity of oral carcinoma cell lines together. And it is expected that *A. sphathulifolius* might be used in mouthwashes and toothpastes for oral health care and might be developed as a phytotherapeutic drug for the treatment and prevention of oral caries and oral cancer. In addition, this is the first report for the anti-bacterial activity and cytotoxicity of *A. sphathulifolius* against oral pathogens and oral carcinoma cell lines for the improvement of oral health care.

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