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Antioxidant, Anti-Oral Cancer, and Antimicrobial Activity of Medicinal Plant Extracts: Development of Mouthrinse Formulations

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Abstract

Nowadays, there is growing interest in using medicinal plants as antimicrobial agents to treat microbial infections in the oral cavity. This study was aimed to evaluate phytochemical properties, anti-oral cancer and antimicrobial activity against *C. albicans* and some potential oral lactic acid bacteria (*Lactobacillus casei and Lactobacillus plantarum*). Plant extracts including *Cinnamomum verum* (cinnamon), *Acorus calamus* (sweet flag), *Hibiscus sabdariffa* (roselle), *Zingiber officinale* (ginger) and *Alpinia officinarum* (lesser galangal) were tested and used to formulate mouthrinses for oral health care. Cinnamon extract possessed the highest phenolic, flavonoid and tannin content, and antioxidant activity (1,152.58 mg ascorbic acid equivalent/g by phosphomolybdenum assay and 39.64 % nitric oxide radical scavenging activity), while roselle extract had the highest total alkaloids. Only lesser galangal extract displayed anti-oral cancer activity with 97.83 % cytotoxicity. High performance liquid chromatography (HPLC) analysis revealed the presence of galangin and kaempferide in the lesser galangal rhizome extract. The cinnamon extract showed the strongest inhibitory activity against *Candida albicans* (4 mg/mL MIC). Cinnamon extract (24 mg/mL) and cinnamaldehyde (0.08 mol/L) exhibited the best killing effect and the highest cellular protein leakage in *C. albicans*. In addition, these plant extracts were used to develop mouthrinse formulations. The extracts of cinnamon and ginger at 10 % in mouthrinse formulations could strongly inhibit the growth of *C. albicans*. In comparison, 8 and 10 % roselle extracts showed the best inhibitory effect against *Lactobacillus casei*. In summary, cinnamon and lesser galangal were potential plants with antimicrobial, antioxidant, anti-biofilm and anti-oral cancer activities, and thus can be utilized to formulate oral care products.

Keywords: *Alpinia officinarum*, Cinnamon, Cinnamaldehyde, Phenolics, Flavonoids, Mouthwash, Tannins

Introduction

Oral diseases like dental caries and oral cavity cancer are worldwide health problems. Many biofilm producing microbes colonize in oral cavity. The biofilm developed on teeth surface is associated with the growth of *Lactobacillus* and *Streptococcus*. They accumulate on teeth surface by producing extracellular polysaccharides [1]. Besides, fungal infections in oral mucosa are the most common infection. Fungi of up to 85 species can be found in oral cavity. Among these, *Candida albicans*, one of the potential fungal strains in candidiasis, attacks oral tissue when the balance of oral microorganisms changes. This yeast can exist in multiple states [2]. Its ability to switch from budding to hyphal forms is essential for its biofilm formation and virulence. This yeast can join with lactic acid bacteria to produce biofilm in their role of pathogenesis [3]. Presence of pathogenic microorganisms in oral cavity and their by-products from metabolism can affect immune response, leading to pathogenesis of oral diseases such as oral cancer [4]. Hence, it is essential to protect oral cavity from infection. Controlling of oral pathogens by antibiotics such as ampicillin, chlorohexidine and vancomycin has been reported to cause antibiotic resistance and diarrhea [5]. Researches have shown that plant-based nutraceuticals have potential to use in the management of oral infections and cancer [6,7]. Therefore, an alternative way is to use medicinal plants with both antimicrobial and anti-oral cancer activities.

Several plants such as pomegranate, green tea, clove, garlic, ginger, turmeric, red chilli and etc. have been reported to possess bioactive compounds with anti-oral, antimicrobial and antioxidant activities [7-9]. Antioxidant activity is an important activity of plants rich in polyphenols including flavonoids,

phenolic acids, lignans, stilbenes and etc. These compounds play an important role in cell component protection from oxidative damage by acting as free radical scavengers, thus decreasing risk of chronic diseases related to oxidative stress [8]. Therefore, it is interesting to use these plants in health care products. People in developing countries normally used traditional medicinal plant formulations for oral hygiene aids such as mouthrinses. Considering the need to prevent oral diseases, the aim of the current study is to determine antioxidant, anti-oral cancer, antimicrobial and other phytochemical properties of some selected plants, study anti-biofilm and time-kill effect against *C. albicans*, and finally apply the selected plants in mouthrinse formulations.

Materials and methods

Plant materials

Dried spices of sweet flag (*Acorus calamus*), lesser galangal (*Alpinia officinarum*) and ginger (*Zingiber officinale*) rhizomes, cinnamon (*Cinnamomum verum*) stem barks and roselle (*Hibicus sabdariffa*) flowers were purchased at a local market in Bangkok, Thailand.

Preparation of plant extracts

Dried plant materials (15 g of each) were soaked in 85 % ethanol (150 mL), and shaken at 150 rpm for 48 h, 30 °C. After filtering, the filtrates were evaporated using vacuum rotary evaporator and air dried in vacuum dryer. The extracts were diluted with 10 % dimethyl sulphoxide (DMSO) to prepare 500 mg/mL stock solution.

Microbial strains and inoculum preparation

Candida albicans TISTR 5579, *Lactobacillus plantarum* TISTR 050 and *Staphylococcus aureus* TISTR 118 were obtained from the Microbiological Resources Centre for Southeast Asian Region, Thailand. *L. casei* BCC 4380 and *E. coli* DMST 4212 were obtained from BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, Pathum Thani and the culture collection of the Department of Medical Science, Ministry of Public Health, Thailand, respectively. These yeast and bacteria were cultured at 30 °C for 72 h on Malt Extract broth (MEB) and at 37 °C for 24 h on Tryptic Soy agar (TSA), respectively. Inocula of 10^8 cells/mL were prepared by adjusting the cell turbidity to match the turbidity of no. 2 Mcfarland standard.

Antioxidant activity assay: Total antioxidant capacity by phosphomolydenum method

All plant extracts were tested using the protocol as described by Prieto *et al.* [10]. Briefly, 0.1 mL sample (1 mg/mL) was mixed with 1 mL reagent solution (the mixture of 0.6 M sulfuric acid, 28 mM Na_2HPO_4 and 4 mM ammonium molybdate in the ratio of 1:1:1), incubated at 95 °C for 90 min and left to cool down to room temperature. Then, the absorbance was measured at 695 nm using spectrophotometer (Shimazu, UV-1,800, Japan). Standard curve of ascorbic acid (1 - 2,000 μg/mL) was prepared. Total antioxidant activity was expressed as mg ascorbic acid equivalent (AAE)/g or /mol of sample.

Nitric oxide radical scavenging efficacy

All samples were tested using the method as described by Baipai *et al.* [11]. Briefly, 0.5 mL sample (1 mg/mL) was mixed with 2 mL of 10 mM sodium nitroprusside in phosphate buffer saline (PBS, pH 7.4) and incubated at 37 °C for 60 min in the light. Then, 1.5 mL of this mixture was added with 1.5 mL Griss reagent (1 % sulfanilamide in 5 % phosphoric acid mixed with 0.1 % N-1-naphthyl ethylenediamine dihydrochloride) and incubated at 25 °C for 30 min in the dark. Then, the absorbance was measured at 546 nm using spectrophotometer (Shimazu, UV-1,800, Japan). Percentage of radical scavenging was calculated as followed:

$$\% \text{ Radical scavenging} = (A_{control} - A_{sample}) / A_{control} \times 100$$

where $A_{control}$ was the absorbance of all reagents without the tested sample, and A_{sample} was the absorbance of the reaction mixture with the tested sample. The positive controls, ascorbic acid, butylated hydroxytoluene (BHT), α-tocopherol (Sigma-Aldrich, Germany), galangin (Thermo Fisher Scientific, UK), kaempferide (Tokyo Chemical Industry Co., LTD, Japan), cinnamaldehyde (Sigma-Aldrich, USA) and eugenol (Sigma-Aldrich, Germany) were also analyzed. The assay was performed in triplicate.

Other phytochemical properties

Total phenolic content of all samples was analyzed according to the method of Singleton *et al*. [12]. Total flavonoids and tannins were evaluated using the procedure as stated by Kathirvel and Sujatha [13]. Total alkaloid content was analyzed by the method of Shamsa *et al.* [14].

Anti-oral cancer activity

This assay was performed according to the method of O'Brien [15]. Briefly, KB cell line (ATCC CCL-17) from oral epidermoid carcinoma was grown in a complete medium (minimal essential medium added with 10 % heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 0.01 mg/mL insulin, 1X non-essential amino acid and 1.5 g/L sodium bicarbonate), and incubated at 37 °C in relative humidity controlling chamber with 5 % CO_2. The logarithmic phase cells were harvested and adjusted to 2.2×10^4 cell/mL in the complete medium. Then, each plant extract (5 µL, 50 µg/mL final concentration) and 45 µL cell suspension were added into each well of 384-well plate, and incubated at 37 °C for 3 days at the same condition as above. Rezasurin (0.0625 mg/mL) was added into each well, and incubated at 37 °C, 4 h before fluorescence measurement at 530 nm excitation and at 590 nm emission wavelengths using bottom-reading mode of fluorometer. The signal was subtracted with blank before calculation. Ellipticine and doxorubicin were used as positive controls at final concentration of 5 µg/mL. The percentage of cytotoxicity was calculated using the following equation.

$$\% \text{ Cytotoxicity} = [1-(FU_T/FU_c)]\times100$$

Where FU_T and FU_c values are the fluorescent units from cells treated with the test sample and treated with 0.5 % DMSO (negative control), respectively. The cytotoxicity of less than 50 % was reported as non-cytotoxic, but that of more than or equal to 50 % cytotoxicity was reported as cytotoxic. Cytotoxicity (%) was plotted against the concentration of sample to obtain dose-response-curve and IC_{50} value using SOFTMax Pro software (Molecular Devices, USA).

Analysis of active compounds in a selected plant extract with anti-cancer activity: High performance liquid chromatography (HPLC) analysis

The selected plant extract was dissolved in methanol. The mixture of acetonitrile-deionized water-*O*-phosphoric acid (70:30:0.1, v/v/v) was used as mobile phase. This mixture was filtered through a 0.45-µm nylon membrane syringe filter before injection into YMC-Triart C18 analytical HPLC column (YMC CO., LTD., Japan) at 37 °C using HPLC instrument (Hitachi, Model: Chromaster, Japan). The elution system was isocratic. The detector used was a diode array detector. The flow rate was 0.8 mL/min. Wavelength of detection was set at 254 nm for standard compounds. The chromatographic peak of the sample was confirmed by addition of each standard compound and comparing their retention time with those of the standard compounds. The amount of each compound in the extract was calculated from the standard curves.

Thin layer chromatography (TLC) analysis

Detection of antioxidant compounds in the selected plant extract with anti-oral cancer activity was performed using TLC technique according to the method described by Soonthornchareonnon *et al.* [16]. Briefly, 4 µL of 2 standard compounds (10 mg/mL) and a selected plant extract (400 mg/mL) were spotted onto TLC plate (aluminium plate coated with silicagel 60 F_{254}, Merck, EMD Millipore, Germany). The plate was developed in 25 mL mixed solvents of hexane, dichloromethane and methanol (12:80:12, v/v/v). The spots developed were air-dried and detected by spraying with anisaldehyde/sulfuric acid solution. Then, this TLC plate was dried in hot air oven at 105 °C for 15 min. The TLC detection was undertaken in duplicate for each sample, and based on retardation factor (Rf value). Rf value was calculated as: Rf = distance travelled by sample/ distance travelled by solvent.

Antimicrobial activity

The minimum inhibitory concentrations (MICs) of all samples were determined by an agar dilution method [17]. Plant extracts (5 - 50 mg/mL) or each positive control (0.078 - 5.0 mg/mL α-asarone, 0.02 - 0.2 mg/mL kaempferide and galangin, 0.01 - 0.38 mol/L eugenol and cinnamaldehyde and 0.781 - 50 mg/mL chlorhexidine dihydrochloride) in MEA for *C. albicans* and TSA for all test bacteria were prepared. Then, microbial suspension was inoculated onto dried surface of agar medium. Their growth

was recorded after incubation. The lowest concentration of the sample that completely inhibited visible growth was reported as the MIC.

Anti-biofilm activity against *Candida albicans*

This assay was performed by the method of Tabbene *et al.* [18]. Briefly, *C. albicans* (10^7 CFU/mL) was mixed with Yeast Extract Peptone Dextrose broth [19] containing 1 % yeast extract, 2 % peptone and 2 % dextrose, and the test sample or positive control (cinnamaldehyde, eugenol, galangin and kaempferide) in the microplate well, and incubated at 30 °C for 24 h. Then, biofilm was formed, and inhibited by crystal violet assay. Briefly, the liquid in each well was discarded, and the well was rinsed 3 times with PBS. The cells were then fixed with 99 % methanol (200 µL/well) and left for 15 min. After discarding methanol, 0.05 % crystal violet (200 µL) was filled in each well, left for 15 min and discarded the excess dye. The stained cells attached were rinsed with sterile deionized water. Each well was filled with 33 % acetic acid. The absorbance was measured at 595 nm using microplate reader (FLUOstar Omega, BMG Labtech, Germany). The percentage of inhibition was calculated [18]. The experiment was done in triplicate.

Effect of cinnamon extract and cinnamaldehyde on *Candida albicans* time-kill curve

This assay was performed according to the method of Klepser *et al.* [20]. Briefly, *C. albicans* cell suspension (10^7 cells/mL) was transferred to PBS added with cinnamon extract at 1X - 6X MIC and cinnamaldehyde at 2X - 4X MIC, and incubated at 30 °C in a shaker at 150 rpm. The mixture was taken at 0, 2, 4, 6 and 24 h of incubation to determine total viable count and analyze for cellular protein leakage.

Determination of cellular protein leakage

The sample was taken from the same test tube of time killing assay. Then, the cells and supernatant were separated by centrifugation**.** The supernatant (100 µL) was analyzed for protein content by Bradford method [21]. The experiment was done in triplicate. Statistical analysis of all data was performed using ANOVA and Duncan's multiple range test at 95 % confidence level by the IBM SPSS 22.0 version statistical package, USA.

Formulation of plant extract mouthrinses

The mouthrinse formulations were developed by mixing with the base ingredient mixture [22] with each plant extract at 6 - 10 % concentration. Then, antimicrobial activity was determined by agar diffusion method [23]. The commercial mouthrinses, Listerine original mouthwash (Johnson & Johnson Consumer Co., Ltd.) and C-20 Chlorhexidine antiseptic mouthwash (Osoth Inter Laboratories Co., Ltd.) were used as positive controls. The experiments were done in triplicate. All data were performed statistical analysis as above.

Results and discussion

Antioxidant activity: Phosphomolybdenum method

This assay measures reducing capacity of the sample (an antioxidant compound) which reduces molybdenum (VI) oxide to molybdenum (V) oxide. If more reducing capacity is detected this indicates that antioxidant activity of the sample is stronger [10]. Cinnamon and sweet flag extracts showed strong antioxidant capacity with 1,152.58 and 1,030.07 mg AAE/g extract, respectively. However, galangin and kaempferide possessed stronger antioxidant activity (668.38 - 763.23 mg AAE/g extract) than those of lesser galangal and ginger extracts (**Table 1**).

Nitric oxide radical scavenging method

Nitric oxide is one of free radicals which leads to inflammation and some chronic diseases. This assay tests the sample that can inhibit nitric oxide formation. The more percentage of inhibition indicates the more inhibition of nitric oxide generation. Cinnamon extract had the highest radical scavenging activity (39.64 % radical scavenging, **Table 1**), but lower than those of cinnamaldehyde and eugenol.

Other phytochemical properties

Among all plant extracts tested, cinnamon extract contained highest contents of phenolics, flavonoids and tannins (339.3 mg GAE/g, 255.3 mg CE/g and 681.17 mg TAE/g, respectively), followed by ginger and lesser galangal extracts. Of all, kaempferide had highest total phenolics (1,603.51 mg

GAE/g), tannins (546.3 mg TAE/g) and alkaloids (69.39 mg AE/g), but galangin had highest flavonoids. However, the most abundant alkaloids were found in roselle extract (**Table 1**).

In the current study, strong antioxidant activity found in cinnamon, sweet flag and roselle extracts may be due to the action of their active compounds. Singh *et al.* [24] stated that cinnamon is widely used in food and industrial products. Its essential oil contains cinnamaldehyde, cinnamyl acetate, cinnamic acid, eugenol, caryophyllene and cinnamic acid as its major compounds. These compounds exhibit several activities such as antioxidant, antimicrobial, wound healing, anti-depressant, and etc.

Table 1 Phytochemical properties of plant extracts.

Samples	Antioxidant activity		Total phenolic (mg GAE/g) ± SD	Total flavonoid (mg CE/g) ± SD	Total tannin (mg TAE/g) ± SD	Total alkaloid (mg AE/g) ± SD
	Phosphomolyb-denum (mg AAE/g) ± SD	NO (%) ± SD				
Plant extracts						
Sweet flag	1,030.07 ± 2.32	28.41 ± 1.87	130.88 ± 1.61	22.95 ± 0.32	104.01 ± 2.14	25.51 ± 6.82
Lesser galangal	618.38 ± 1.66	32.69 ± 1.76	150.88 ± 1.22	44.48 ± 0.19	469.14 ± 6.95	7.81 ± 0.31
Cinnamon	1,152.58 ± 3.38	39.64 ± 1.70	339.30 ± 1.61	255.30 ± 0.50	681.17 ± 5.88	10.12 ± 3.56
Roselle	824.05 ± 3.87	30.12 ± 1.76	116.84 ± 1.05	32.13 ± 0.33	111.42 ± 1.07	37.39 ± 0.10
Ginger	647.59 ± 0.79	20.18 ± 2.10	201.75 ± 0.61	94.10 ± 0.33	497.84 ± 5.34	9.69 ± 2.10
Positive control compounds						
Cinnamaldehyde[a]	176.54 ± 0.07	48.37 ± 1.36	128.17 ± 0.78	63.25 ± 0.09	90.13 ± 0.92	11.85 ± 1.48
Eugenol[a]	165.54 ± 0.29	43.04 ± 1.49	654.50 ± 0.00	203.86 ± 0.00	104.64 ± 0.50	15.91 ± 2.74
Galangin	668.38 ± 0.79	44.84 ± 1.42	790.53 ± 1.82	232.35 ± 0.83	473.77 ± 3.74	66.48 ± 10.18
Kaempferide	763.23 ± 1.19	43.06 ± 1.28	1603.51 ± 1.61	190.93 ± 0.50	546.30 ± 3.21	69.39 ± 5.14
BHT	1,276.29 ± 1.36	53.14 ± 1.16	86.32 ± 1.05	13.99 ± 0.19	73.46 ± 6.95	70.91 ± 0.63
α-tocopherol	1,222.51 ± 2.44	51.05 ± 1.11	83.51 ± 1.61	20.33 ± 0.33	70.68 ± 1.07	72.60 ± 0.10
Ascorbic acid	1,006.70 ± 0.00	51.52 ± 1.29	168.07 ± 2.65	13.66 ± 0.50	493.83 ± 4.27	72.24 ± 0.63

[a]mg/mol concentration; NO, nitric oxide radical scavenging; AAE, ascorbic acid equivalent; AE, atropine equivalent; GAE, gallic acid equivalent; CE, catechin equivalent; TAE, tannic acid equivalent.

Chandra and Prasad [25] revealed that sweet flag contained amino acids, terpenoids, carbohydrate, protein, glucoside, asarone, essential oil, alkaloid and eugenol. Barbieri *et al.* [26] reported high amount of alkaloids in roselle extract. The dark red sepals of its flower are rich in antioxidant compounds such as vitamin C and also contain pigments, glycosides, calcium and other minerals and hepiscin chloride [27].

Ginger and lesser galangal extracts displayed moderately strong antioxidant activity. Antioxidant activity of ginger related to their active compounds including, vitamins, carotenoids and phenolic compounds (6-gingerol, 6-shogaol and 6-paradol) [28], alkaloids, saponins, glycosides, terpenoids, anthraquinones, flavonoids and tannins [29]. Lesser galangal rhizome extract has been reported to contain alkaloids, diaryheptanoid with pyridine ring called officinin B and alpinin A, and other flavonoids [30]. Abundant tannin content was also found in lesser galangal extract [31].

Antimicrobial activity of plant extracts

Cinnamon extract exhibited the strongest antifungal activity against *C. albicans* (4 mg/mL MIC). *L. casei* and *L. plantarum* were sensitive to cinnamon and sweet flag extracts with 5 mg/mL, respectively. *E. coli* was susceptible to roselle extract (12 mg/mL MIC), while *S. aureus* was sensitive to ginger extract (5 mg/mL). *C. albicans* was more vulnerable to cinnamaldehyde and eugenol (0.02 mol/L MIC) than all tested bacteria. Alpha-asarone had strong antimicrobial activity against *C. albicans*, *L. casei* and *S. aureus*

at 0.31 mg/mL. Kaempferide strongly inhibited the growth of *L. plantarum* at 0.02 mg/mL MIC, whereas galangin mostly showed antimicrobial activity at 0.1 mg/mL MIC. Among all microorganisms tested, chlorhexidine dihydrochloride possessed the strongest antimicrobial effect against *S. aureus* at 0.08 mg/mL MIC (**Table 2**).

Table 2 Antimicrobial activity of plant extracts by agar dilution method.

Samples	Concentration (mg/mL)				
	C. albicans	***L. casei***	***L. plantarum***	***E. coli***	***S. aureus***
Plant extracts					
Sweet flag	10	20	5	40	10
Lesser galangal	20	10	40	40	10
Cinnamon	4	5	10	60	20
Roselle	60	10	40	12	20
Ginger	10	40	40	60	5
Compounds					
α-asarone	0.31	0.31	1.25	5	0.31
Cinnamaldehyde[a]	0.02	0.05	0.05	0.05	0.05
Eugenol[a]	0.02	0.04	0.04	0.04	0.04
Kaempferide	0.1	0.1	0.02	0.1	0.1
Galangin	0.1	0.1	0.2	0.1	0.1
Positive control					
chlorhexidine	0.63	0.31	0.16	0.16	0.08

[a]Concentration of cinnamaldehyde and eugenol is mol/L.

Antimicrobial activity of cinnamon extract may relate to the activity of cinnamaldehyde and eugenol. Cinnamaldehyde is hydrophobic and can penetrate into lipid bilayer of cytoplasmic membrane, leading to leakage of cellular organelles and finally cell death [32]. In the current study, sweet flag, and roselle extracts showed antimicrobial activity. Elshikh *et al.* [33] revealed that sweet flag (*A. calamus*) methanolic extract had antimicrobial activity against *Staphylococcus epidermis*, *Proteus vulgaris* and *Bacillus cereus* at 5 μg/mL. The presence of asarone, n-hexadecanoic acid, 9, 15-octadecadienoic acid, methyl ester, (z,z)- and 9,12-octadecadienoic acid (z,z) was reported. Antimicrobial activity of roselle extracts may relate to their acids. Mariod *et al.* [27] reported that roselle flowers contained several types of acids including organic acids such as citric, malic, tartaric and ascorbic acid.

Anti-oral cancer activity of plant extracts

Among all extract, only lesser galangal extract showed cytotoxic potency to oral cancer cell. Lesser galangal extract displayed up to 97.83 % cytotoxicity (13.99 μg/mL IC_{50}). However, other extracts were non-cytotoxic, except for the positive controls, ellipticine and doxorubicin at 5 μg/mL which showed 66.84 and 98.17 % cytotoxicity, respectively. Lesser galangal rhizome extract has been reported to possess anticancer activity [30]. Kaempferide and galangin may cause inhibitory effect on KB-oral cavity cancer cells. Anti-oral cancer activity of lesser galangal rhizome extract has never been reported, but this plant rhizome has been shown to decrease proliferation of cancer cell lines, such as human gastric, breast and lung cell carcinoma [30]. Thus, it is possible to use lesser galangal as a natural ingredient in anti-oral cancer drugs or any oral care products.

Detection of active compounds in *Alpinia officinarum* extract by HPLC and TLC analysis

The HPLC analysis of *A. officinarum* extract was performed to confirm the presence of active compounds. The HPLC chromatogram of peak 1 with 5.153 min retention time in the extract was

identified as kaempferide (**Figure 1(A)**), while peak 2 with 5.207 min retention time was identified as galangin (**Figure 1(B)**). This indicated that kaemperide and galangin were active components in lesser galangal extract, and were found 0.003 and 0.0029 % w/w in dried *A. officinarum* rhizome, respectively.

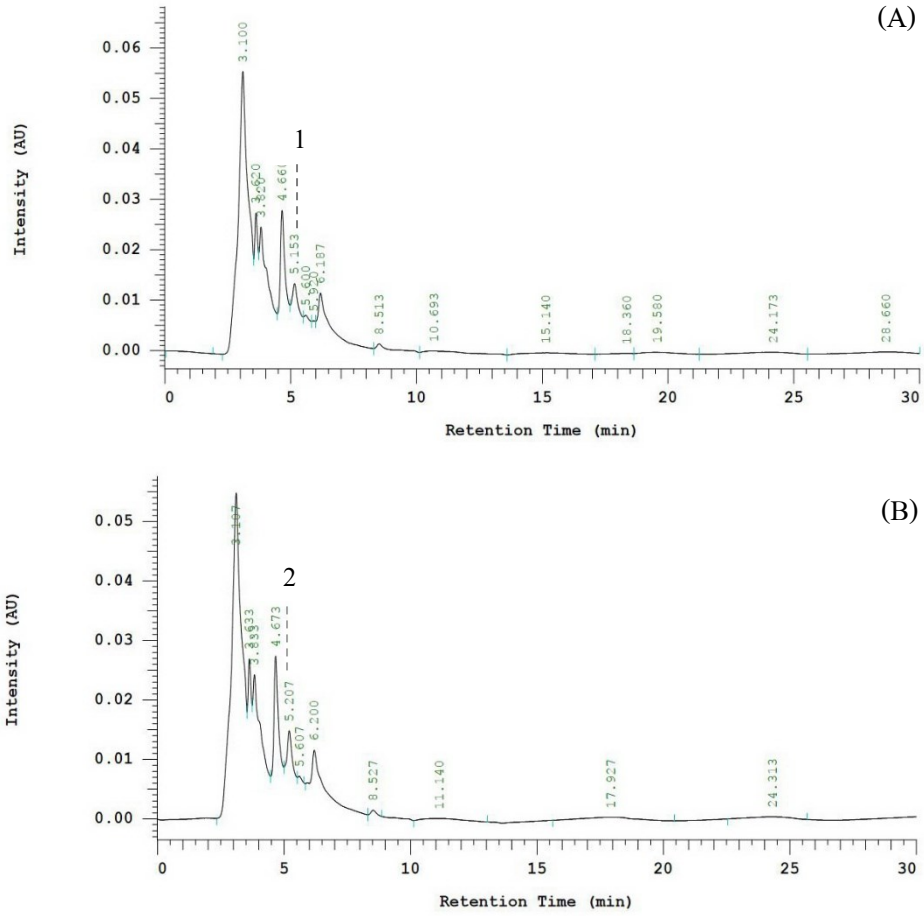


Figure 1 HPLC Chromatogram of active compounds in *Alpinia officinarum* extract; (A) extract injected (measured at 254 nm), peak 1: Kaempferide, and (B) extract injected (measured at 254 nm), peak 2: Galangin.

TLC analysis showed that lesser galangal (*A. officinarum*) extract and 2 reference compounds displayed overlapped zone of dark violet spots and violet yellow spots with slightly different Rf, 0.73 for sample (lesser galangal extract, lane 1 and 2), 0.75 for galangin standard (lane 3 and 4) and 0.78 for kaempferide standard (lane 5 and 6) (**Figure 2**). This indicated that lesser galangal extract possibly contained these 2 active compounds. They are flavonols, a subclass of flavonoids commonly found in food plants. These interesting flavonols include galangin, kaempferol, quercetin, myricetin, rhamnetin, morin, azaleatin, and their glycosyl derivatives [34]. Kaempferide is O-methylated flavonol, while galangin is 3,5,7-trihydroxyflavone. It has been reported to have antioxidant and antimutagenic activity. Most studies of galangin focused on its anticancer activity [35]. Galangin has been reported to possess anticancer effect including breast, ovarian, colon, cervical and lung cancer [36]. In the current study, galangin was found as an active compound in lesser galangal extract. Thus, anti-oral cancer activity of this plant may be due to the action of its galangin.

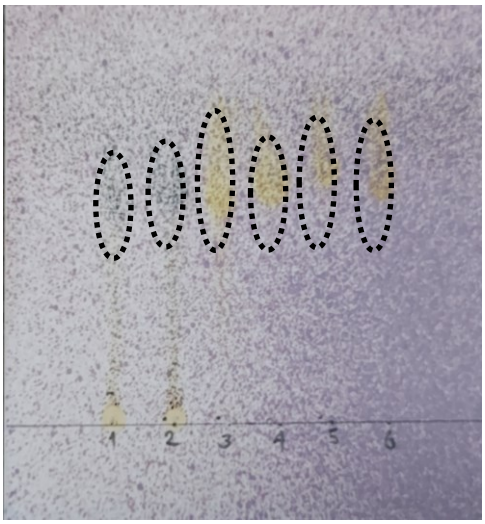
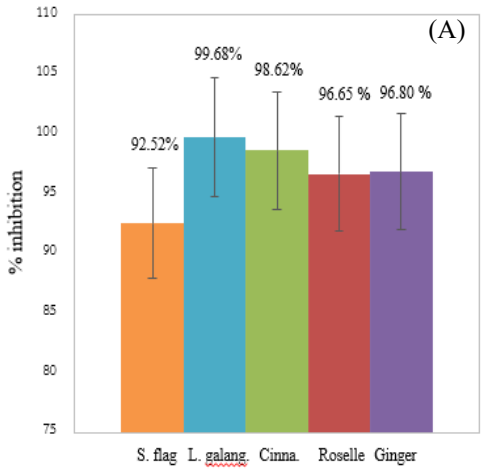


Figure 2 TLC Chromatogram showing the bands of active compounds in *Alpinia officinarum* (lesser galangal) extract: Lane 1 and 2, *A. officinarum* extract (average Rf, 0.73); Lane 3 and 4, galangin (average Rf, 0.75); Lane 5 and 6, kaempferide (average Rf, 0.78).

Anti-biofilm activity

Cinnamon and lesser galangal extracts could significantly inhibit biofilm formation by *C. albicans* (98.62 - 99.68 % inhibition) stronger than those of other extracts ($p < 0.05$). However, galangin and kaempferide had anti-biofilm activity against *C. albicans* at 49.97 and 49.18 % inhibition, while cinnamaldehyde and eugenol could inhibit biofilm formation at 35.29 and 45.92 % inhibition, respectively (**Figure 3**). Biofilm formation of *C. albicans* on biological surface should be eradicated. Previous reports have shown that cinnamon oil possessed anti-biofilm activity against *Candida* biofilm and affected exopolysaccharide layer of *Candida* after examining with scanning electron microscope [19]. Essid *et al.* [37] reported that cinnamon essential oil at half MIC value (31.25 µg/mL) exhibited inhibition of biofilm formation of *C. albicans* at 85.57 % and showed 92.79 % eradication of *C. albicans* mature biofilm at 2X MIC value. Lesser galangal extract has been shown to possess anti-biofilm activity against *C. albicans*, induce deformation of *C. albicans* cells and finally cell death [38].



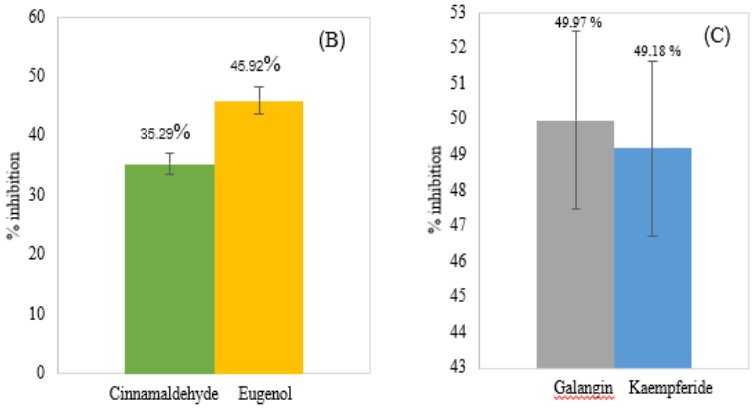


Figure 3 Anti-biofilm activity of plant extracts and active compounds against *Candida albicans*: (A) plant extracts (sweet flag, lesser galangal, cinnamon, roselle and ginger), 3.76 mg/mL; (B) cinnamaldehyde and eugenol, 0.01 mol/L; (C) kaempferide and galangin, 0.03 mg/mL.

Effect of cinnamon extract and cinnamaldehyde on *Candida albicans* time-kill curve

In all treatments added with cinnamon or cinnamaldehyde, *C. albicans* showed less viability as compared to the control ($p < 0.05$) (**Figure 4**). After 24-h incubation, cinnamon extract at 6X MIC and cinnamaldehyde at 4X MIC affected decreasing of the cell number (2.68 and 2.71 log decrease, respectively) as compared to other treatments ($p < 0.05$), while cinnamaldehyde at 2X MIC could decrease *C. albicans* cell number by 2.50 log cycle.

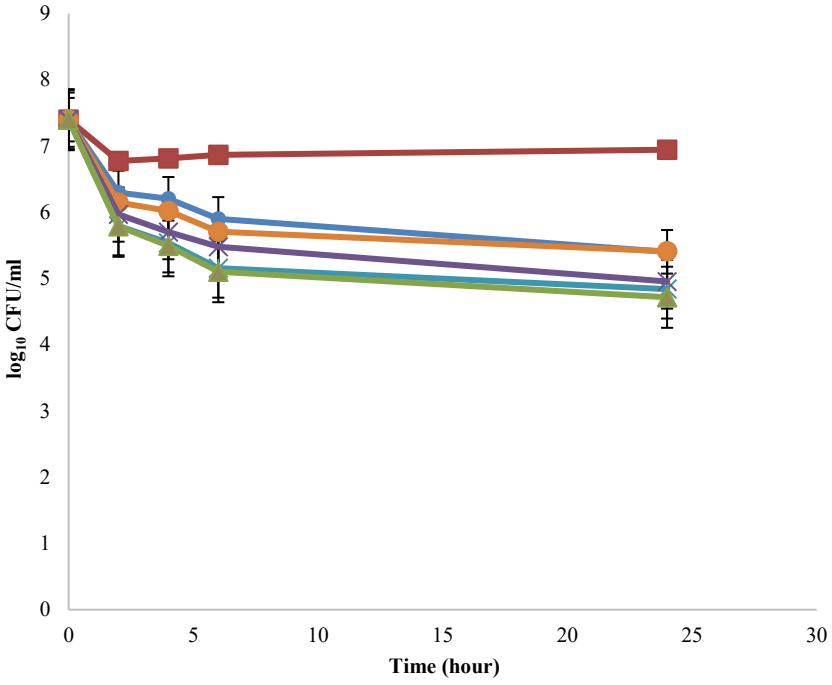


Figure 4 Effect of cinnamon extract and cinnamaldehyde on *Candida albicans* time-kill curve: ■, Control; ●, 1X MIC cinnamon extract (4 mg/mL); •, 4X MIC cinnamon extract (16 mg/mL); ×, 6X MIC cinnamon extract (24 mg/mL); ×, 2X MIC cinnamaldehyde (0.04 mol/L); ▲, 4X MIC cinnamaldehyde (0.08 mol/L)

Protein leakage

After 24-h incubation, protein leakage of all treatments with cinnamon extract or cinnamaldehyde was higher as compared to those at the beginning of the incubation period ($p < 0.05$) (**Table 3**). Of all, cinnamon extract at 6X MIC resulted in highest protein leakage (1.26 time of initial protein content). Increased concentration of cinnamaldehyde at 2X and 4X MIC affected 1.4 - 1.5 time of cellular protein leakage after 24-h incubation. Protein leakage assay in *C. albicans* indicated that cinnamon extract or cinnamaldehyde may damage the cell wall and membrane. Cinnamaldehyde, the main compound in cinnamon stem bark was found up to 66.28 - 81.97 % in cinnamon oil [32]. Zhang *et al.* [39] reported antifungal effect of cinnamon oil-mesoporous silica nanoparticles. There were obvious changes in membrane permeability and cell component leakage in *Mucor* sp.

Table 3 Effect of cinnamon extract and cinnamaldehyde on cellular protein leakage of *Candida albicans*.

Time (h)	Control	Protein leakage (μg) ± SD				
		Cinnamon extract			Cinnamaldehyde	
		1X MIC	4X MIC	6X MIC	2X MIC	4X MIC
0	677.83 ± 2.31^{dC}	696.17 ± 5.77^{cD}	707.50 ± 2.60^{bE}	728.50 ± 4.33^{aE}	675.67 ± 2.02^{dE}	667.00 ± 7.79^{eE}
2	687.83 ± 4.04^{eAB}	716.50 ± 6.93^{cC}	753.00 ± 1.73^{bD}	763.67 ± 4.62^{aD}	701.00 ± 2.59^{dD}	686.33 ± 6.64^{eD}
4	689.17 ± 5.77^{dA}	718.33 ± 4.04^{cBC}	765.50 ± 1.73^{bC}	795.17 ± 5.77^{aC}	712.50 ± 2.60^{cC}	719.67 ± 2.31^{cC}
6	664.33 ± 3.17^{eD}	728.83 ± 5.48^{dB}	811.50 ± 0.87^{bB}	829.00 ± 1.73^{aB}	739.33 ± 4.62^{cB}	735.50 ± 5.19^{cdB}
24	681.83 ± 2.02^{eBC}	775.50 ± 7.79^{cA}	875.00 ± 6.93^{bA}	916.67 ± 5.48^{aA}	775.83 ± 6.35^{cA}	760.00 ± 0.87^{dA}

Note: 1X, 4X and 6X MIC cinnamon extract were 4, 16 and 24 mg/mL concentration, while 2X and 4X MIC cinnamaldehyde were 0.04 and 0.08 mol/L concentration, respectively. ^{a,b,c}Different letters in different column of the same row indicated significant difference ($p < 0.05$). ^{A,B,C}Different letters in different row of the same column indicated significant difference ($p < 0.05$).

Antimicrobial activity of plant extracts in mouthrinse by agar diffusion method

Cinnamon, roselle and ginger extracts (10 %) significantly affected wider inhibition zone diameter against *C. albicans*, compared to those at lower concentration ($p < 0.05$). Of all, sweet flag extract (8 - 10 %) caused widest inhibition zone (13.33 - 16 mm) against *S. aureus*. Most extracts (10 %) inhibited the growth of *L. casei, S. aureus* and *E. coli* stronger as compared to those of base ingredient mixture without the plant extract. Almost all concentrations of plant extract at 6 - 10 %, except for 6 % roselle extract could significantly inhibit the growth of *L. casei* stronger than Listerine ($p < 0.05$). Moreover, lesser galangal and cinnamon extracts at 10 % showed stronger antibacterial activity against *L. plantarum*, compared to Listerine (**Table 4**).

Cinnamon, ginger and roselle showed strong antimicrobial activity in mouthrinse products. Some researchers reported similar results. Vonasorn *et al.* [22] used a plant in Zingiberaceae (*Bosenbergia pandurata*) as an ingredient in the mouthrise and revealed that this plant could inhibit the growth of *Streptococcus mutans*. Waty *et al.* [40] used *Cinnamomum burmannii* stem bark as an ingredient in the mouthrinse and found that this plant extract could inhibit most of isolated lactic acid bacteria (*Streptococcus* sp.). Barbieri *et al.* [26] reported that roselle possessed antimicrobial activity, and inhibited microbial attachment on teeth surface.

Table 4 Antimicrobial effect of plant extracts in mouthrinse formulations.

Plant extracts	pH	Mouth rinse color	Inhibition zone diameter (mm) ± SD				
			Candida albicans	***Lactobacillus casei***	***Lactobacillus plantarum***	***Escherichia coli***	***Staphylococcus aureus***
Sweet flag							
6 %	3.40 ± 0.01	Light brown yellow	6.00 ± 0.00^{g}	8.67 ± 0.58^{bc}	6.83 ± 0.58^{d}	10.67 ± 0.58^{bcd}	9.67 ± 1.53^{c}
8 %	3.47 ± 0.01	Brown yellow	6.00 ± 0.00^{g}	9.83 ± 0.29^{ab}	7.33 ± 0.29^{bcd}	11.33 ± 1.15^{bc}	13.33 ± 2.52^{b}
10 %	3.50 ± 0.01	Dark brown yellow	6.67 ± 0.05^{efg}	10.00 ± 0.50^{ab}	7.67 ± 0.58^{bcd}	12.67 ± 1.15^{ab}	16.00 ± 1.00^{a}
Lesser galangal							
6 %	4.51 ± 0.01	Light brown yellow	6.50 ± 0.50^{fg}	7.67 ± 0.58^{cd}	7.83 ± 1.04^{bcd}	10.00 ± 1.73^{cde}	7.00 ± 0.87^{efg}
8 %	4.51 ± 0.01	Brown yellow	7.00 ± 1.00^{def}	8.00 ± 0.50^{cd}	8.17 ± 0.76^{bcd}	10.67 ± 2.31^{bcd}	7.67 ± 1.04^{defg}
10 %	4.53 ± 0.01	Dark brown yellow	7.67 ± 0.58^{cd}	8.83 ± 0.29^{bc}	8.50 ± 0.50^{bcd}	14.33 ± 1.15^{a}	8.50 ± 0.50^{cdef}
Cinnamon							
6 %	3.77 ± 0.01	Light brown yellow	6.00 ± 0.00^{g}	8.67 ± 0.58^{bc}	7.67± 0.76^{bcd}	7.67 ± 0.58^{ef}	8.67 ± 0.28^{cde}
8 %	3.91 ± 0.01	Brown yellow	6.33 ± 0.29^{fg}	8.83 ± 0.76^{bc}	8.17 ± 0.76^{bcd}	8.67 ± 0.58^{def}	9.50 ± 0.50^{c}
10 %	3.89 ± 0.01	Dark brown yellow	7.00 ± 0.00^{def}	10.33 ± 0.58^{ab}	8.83 ± 2.08^{b}	9.67 ± 0.58^{cde}	9.83 ± 0.58^{c}
Roselle							
6 %	2.13 ± 0.01	Red	6.00 ± 0.00^{g}	6.50 ± 0.50^{d}	7.00 ± 0.50^{cd}	7.17 ± 0.29^{g}	6.83 ± 0.29^{fg}
8 %	2.10 ± 0.00	Dark red	6.17 ± 0.29^{fg}	9.00 ± 2.00^{abc}	7.83 ± 0.76^{bcd}	8.33 ± 0.58^{def}	7.17 ± 0.58^{efg}
10 %	2.08 ± 0.01	More Dark red	6.83 ± 0.29^{efg}	10.00 ± 2.00^{ab}	7.83 ± 0.29^{bcd}	8.67 ± 0.76^{def}	7.67 ± 0.58d^{efg}
Ginger							
6 %	5.37 ± 0.01	Light brown yellow	6.83 ± 0.29^{efg}	8.00 ± 2.00^{cd}	7.83 ± 0.76^{bcd}	10.00 ± 1.73^{cde}	8.67 ± 0.58^{cde}
8 %	5.40 ± 0.01	Brown yellow	7.50 ± 0.50^{de}	8.83 ± 0.00^{bc}	8.17 ± 1.26^{bcd}	10.67 ± 1.15^{bcd}	9.33 ± 0.76^{cd}
10 %	5.43 ± 0.01	Dark brown yellow	8.33 ± 0.58^{c}	9.33 ± 0.76^{abc}	8.67 ± 0.76^{bc}	14.00 ± 2.65^{a}	10.00 ± 0.50^{c}
base*	5.35 ± 0.02	Bight yellow	6.67 ± 0.29^{efg}	6.83 ± 0.29^{d}	8.00 ± 1.00^{bcd}	8.33 ± 0.28^{def}	6.50 ± 0.00^{g}
C − 20	5.46 ± 0.03	Light pink	11.33 ± 0.58^{a}	10.67 ± 1.53^{a}	11.67 ± 1.04^{a}	11.67 ± 0.58^{bc}	15.00 ± 1.00^{a}
Listerine	4.13 ± 0.03	Light yellow	9.5 ± 0.50^{b}	6.83 ± 0.29^{d}	8.33 ± 0.58^{bcd}	8.17 ± 0.29^{ef}	7.17 ± 0.76^{efg}

^{a,b,c}Different letters in different row of the same column indicate significant difference ($p < 0.05$). *Base is the basic ingredients, the mixture of tween 20, tartazine, menthol, salt, eucalyptus oil, sorbitol and ethanol, while C - 20 and Listerine are commercial mouth rinse products.

Conclusions

This study confirmed antimicrobial activity of cinnamon against *C. albicans* and *L. casei*. Evidence of anti-*C. albicans* biofilm and time-killing effect of cinnamon was revealed. Besides, lesser galangal was shown to be a prominent anti-oral cancer plant. Cinnamon, ginger and roselle exhibited antimicrobial action in mouthrinse products. However, one plant can not possess all strong pharmacological activities. Therefore, combination of different plants was suggested for development of novel oral care products with multifunctional activity to prevent oral diseases and cancer.

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