UNIVERSITY OF
BATH

University of Bath

Original article   http://dx.doi.org/10.1016/j.apjtb.2016.11.009

Anticaries and antimicrobial activities of methanolic extract from leaves of *Cleistocalyx operculatus* L.

Phuong Thi Mai Nguyen[1*], Nadin Schultze[2], Christin Boger[2], Zeyad Alresley[2], Albert Bolhuis[3], Ulrike Lindequist[2]

[1]*Department of Plant Biochemistry, Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Caugiay, Hanoi, Vietnam*

[2]*Institute of Pharmacy, University of Greifswald, Greifswald, Germany*

[3]*Department of Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom*

ARTICLE INFO

ABSTRACT

Objective: To investigate antimicrobial activities of methanolic extract of leaves of *Cleistocalyx operculatus* L. (*C. operculatus*) grown in Vietnam.
Methods: The methanolic extract of *C. operculatus* leaves was phytochemically screened and tested for its antimicrobial activity against six Gram-positive bacteria (three of which were antibiotic multiresistant *Staphylococcus* spp.), two Gram-negative bacteria, and one fungal species using an agar diffusion method. Anticaries activity was tested using pH drop and biofilm assays formed in 96-well plastic plates.
Results: Phytochemical screening revealed the presence of flavonoids and terpenes, in which flavonoid content was 6.8 mg/g dry material. Antibacterial activity of the *C. operculatus* extract was shown only against Gram-positive bacteria *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus mutans* GS-5 (*S. mutans*), and three multiresistant bacteria being *Staphylococcus epidermidis* 847, *Staphylococcus haemolyticus* 535 and *Staphylococcus aureus* North German epidemic strain. Interestingly, methanolic extract of *C. operculatus* leaves exhibited the anticaries activity against *S. mutans* in terms of inhibition of acid production and biofilm formation. Activity of two key enzymes responsible for acidogenicity of *S. mutans*, F-ATPase and phosphotransferase system were inhibited by the extract with IC_{50} of 51.0 and 98.0 µg/mL, respectively. Cytotoxicity of the extract against keratinocytes was found only for higher concentrations [IC_{50} = (119.98 ± 4.63) µg/mL].
Conclusions: The methanolic extract of *C. operculatus* leaves has the potential for development of antimicrobial preparations, especially anticaries products.

1. Introduction

The complex problems associated with the misuse and overuse of antibiotics have made infectious diseases a particularly urgent issue [1]. With the increasing prevalence of multiresistant bacteria, there is an increasing pressure to find novel effective alternatives. For years, plants have been recognized as natural sources of bioactive compounds with promising potentials in combating infectious diseases. Plants are in rich in secondary metabolites, many of which have been found to have antimicrobial activities *in vitro* [2–6].

Dental caries is one of the most common infectious diseases in humans today. *Streptococcus mutans* (*S. mutans*) is the main cause of dental decay. *S. mutans* is highly acidogenic and is capable of producing exopolysaccharides and forming biofilms (dental plaque), allowing it to adapt to and survive the environment of the oral cavity [7–10]. Thus, the control of cariogenic bacteria using antimicrobial agents capable of inhibiting their virulence will be an effective and advantageous chemotherapeutic strategy in controlling dental caries. However, long-term use of chemical agents at high concentrations, for example fluoride or chlorhexidine, may result in

*Corresponding author: Phuong Thi Mai Nguyen, Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Caugiay, Hanoi, Vietnam.
Tel: +84 917500965
Fax: +84 4 38360853
E-mail: phuong_nguyen_99@yahoo.com

undesirable side-effects. Therefore, natural anticaries, especially phytochemicals, have been attracting much attention as promising alternatives.

Cleistocalyx operculatus L. (*C. operculatus*) is a well-known perennial tree, widely distributed throughout China, Vietnam and several other tropical countries. Traditionally, the brewed leaves and buds of *C. operculatus* have been used as herbal tea for gastrointestinal disorders or as medicine such as antiseptic for dermatophytic infections [11]. Previous reports revealed that the *C. operculatus* buds had various biological activities *in vitro* and *in vivo* such as anticancer, antitumor, antihyperglycemic and cardiotonic action [11–15]. The bark contains oleanane-type triterpene [16], and sterols (*e.g.* β-sitosterol), while flavanones, chalcones and triterpene acids (*e.g.* ursolic acid) are present as the main constituents in the methanol extract of the *C. operculatus* buds [12,17–20]. Gas chromatography and gas chromatography–mass spectrometry analysis of essential oils from *C. operculatus* leaves has been also reported [21–23], but the chemical composition and biological activities of *C. operculatus* leaves have not been studied in detail.

In the present investigation, we examined the biological activities of a methanolic extract from the leaves of *C. operculatus* including antimicrobial, and anticaries activities.

2. Materials and methods

2.1. Plant materials

Leaves of *C. operculatus* were collected in Ha Tay Province, North of Vietnam in April, 2014 and identified by the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi. Voucher specimens were deposited at Department of Botany, Vietnam National University, Hanoi University of Science.

2.2. Test organisms

The following microorganisms were used as test organisms: *Staphylococcus aureus* (*S. aureus*) (ATCC 6538), *Bacillus subtilis* (ATCC 6059) (*B. subtilis*), *S. mutans* GS-5 (kindly provided by Dr. Robert Marquis, University of Rochester, New York, US), *Escherichia coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida maltosa* (SBUG 700) (*C. maltosa*), which were deposited at the strain collection of the Department of Biology of the University of Greifswald. In addition, three multiresistant *Staphylococcus* strains namely, *Staphylococcus epidermidis* 847, *Staphylococcus haemolyticus* 535 and *S. aureus* North German epidemic strain (kindly provided by the Institute of Hygiene of Mecklenburg-Vorpommern, Greifswald, Germany) were also applied. The bacteria were grown in Mueller–Hinton agar at 37 °C for 24 h. For growing of *S. mutans* GS-5, the organism was cultured statically in TYG (3% tryptone, 0.5% yeast extract, 1% glucose) for acidogenic assays or TYS (TY with 1% sucrose instead of glucose) for biofilm assays.

2.3. Extraction

Air-dried and powdered plant material (10 g) was extracted separately with 400 mL *n*-hexane for 8 h, followed by extraction with 400 mL methanol for another 8 h by using a Soxhlet apparatus. The extractions were repeated twice and dried to yield hexane and methanol residues using rotary vacuum.

2.4. Phytochemical screening and bioautography

The screening of chemical constituents was carried out with the methanol extracts using chemical methods. The extract was separated on thin-layer chromatography (TLC) plates using solvent system of toluene: ethyl acetate: acetone: formic acid (5:3:1:1). The presence of terpenes/steroids, alkaloids, flavonoids, and other organic compounds was investigated after spraying specific reagents for the compounds on TLC plates. Natural products-polyethylene glycol was used to detect flavonoids; anisaldehyde-sulfuric acid was used to detect terpenes; Dragendorff's reagent was used to detect alkaloids; 5% KOH was used to detect coumarine; and vanillin-sulfuric acid was used to detect organic compounds in general. Bioautography was done according to the methodology described by Wagner and Bladt [24] using TLC followed by determination of antimicrobial activity on agar plate.

2.5. Flavonoid determination

The total flavonoid content in the extracts was determined spectrophotometrically according to Subedi *et al.* [25] using a method based on the formation of a flavonoid-aluminum complex, which has an absorbance maximum at 430 nm. Briefly, 1 mL of sample was dissolved in 1 mL of 2% aluminum chloride methanolic solution. Following 15 min of incubation at room temperature, Milton Roy 601 UV–vis spectrophotometer was used to measure the absorbance of the reaction mixture at 430 nm. The flavonoid content was expressed in mg per gram of quercetin equivalent.

2.6. Antimicrobial activity assay

The disc-diffusion assay was used to determine the antimicrobial activity of the investigated extracts. Sterile nutrient agar (Oxoid, Basingstoke, UK) was inoculated with microbial cells (200 μL microbial cell suspensions at concentration of 10^6 CFU/mL) in 20 mL of agar medium and poured into a sterile Petri dish. Sterile filter paper disc (Merck, Germany) was impregnated with the solution containing 2 mg of the dried extract. The solvent in the filter disc was left to evaporate before placed onto the surface of the inoculated agar plates. Plates were then refrigerated for 2 h to enable diffusion of the extracts into the agar, followed by overnight incubation for 48 h at 37 °C for *S. mutans* GS-5 plates and at 28 °C for *C. maltosa*. Ampicillin, gentamicin and nystatin (Sigma, Deisenhofen, Germany) were used as positive controls. Negative controls were prepared by loading methanol into filter discs. The inhibition zones were measured after the incubation period.

2.7. pH drop assay

A simple and rapid method to measure acid production by *S. mutans* was described by Belli & Marquis [26]. In brief, washed *S. mutans* GS-5 cells were resuspended in 50 mmol/L KCl solution containing 1 mmol/L $MgCl_2$ to reach biomass concentration of 2 mg dry weight per milliliter. KOH was used to adjust the pH of the cell suspension to 7.2 before adding 1% glucose. The subsequent drop in pH from glycolytic acid production was monitored using a glass pH electrode.

2.8. Enzyme assays

F-ATPase activity was assayed in terms of the release of inorganic phosphate in Tris-maleate buffer, pH 7.0 containing $MgCl_2$ and permeabilized cells. The F-ATPase reaction was initiated by the addition of ATP. Samples were removed and assayed for inorganic phosphate liberated from cleavage of ATP with reagents from American Monitor Co. (Indianapolis, IN) [26]. Phosphotransferase system (PTS) activity was assessed in terms of pyruvate production from phosphoenolpyruvate in response to glucose addition. Pyruvate was assayed by use of lactic dehydrogenase and measurements of the change in absorbance of 340 nm light associated with oxidation of NADH. The permeabilized cells were prepared by subjecting cells to 10% toluene (v/v) followed by two cycles of freezing and thawing as previously described by Nguyen *et al.* [27].

2.9. Anti-biofilm assay

The assay was performed in flat-bottomed 96-well plates as described by Molhoek *et al.* [28]. Briefly, 50 μL of a 16 h *S. mutans* GS-5 culture, which was adjusted to an optical density at 590 nm of 0.02, was added into the wells containing 100 μL TYS medium and the test extract at different concentrations (ethanol as vehicle, negative control) was added. The plate was incubated for 24 h at 37 °C. After growing, the wells were emptied and washed twice with phosphate buffered saline to remove non-adherent bacteria. The biofilms that formed in the wells were then stained with 0.1% crystal violet for 10 min. Excess stain was removed by washing three times with phosphate buffered saline. Bound crystal violet was solubilized in 200 μL of 33% acetic acid per well. The absorbance at 590 nm was then measured. The mean of the six replicates was calculated after subtraction of the blank measurement and the results were expressed as a percentage of biofilm in relation to the untreated control. Each assay was carried out independently three times.

2.10. Confocal microscopy

S. mutans GS-5 was cultured overnight in TYG, and diluted to an optical density of 0.4–0.6 at 600 nm. Polyvinyl plastic coverslips (22 mm × 22 mm) were sterilized in 100% isopropanol, dried before placed in 3 wells of a six-well cell culture plate. An aliquot (2 mL) of the diluted bacterial suspension was added to each well followed by an aliquot (2 mL) of TYS. The plate was incubated at 37 °C for 24 h. The media was then aspirated and replaced with fresh TYS (4 mL). The test extract was pipetted to the wells. The plate was again incubated at 37 °C for another 24 h. After the second incubation, the media were removed and the coverslips were washed with sterile water (6 × 2 mL). The coverslips were stained with LIVE/DEAD BacLight mixture (50:50 v/v, 800 μL, 0.3% v/v of each supplied dye solution diluted in sterile water). The coverslips were left for 15 min in the dark prior to washing with sterile water (6 × 2 mL) before mounted on glass slides using nail varnish. Stained biofilms were observed using laser scanning confocal fluorescence microscopy (Olympus, Tokyo, Japan).

2.11. Cytotoxicity assay

Adherent human keratinocytes (HaCaT) were cultured in RPMI1640 medium (Sigma–Aldrich) supplemented with 1% v/v antibiotics (10.000 IU/mL penicillin; 10.000 mg/mL streptomycin) (Sigma–Aldrich) and 8% v/v heat inactivated fetal calf serum (Sigma–Aldrich) in a humidified atmosphere (37 °C, 5% CO_2). The cells were subcultivated twice a week. Cell viability was assessed using Luna™ automated cell counter (Logos Biosystems, Annandale, USA) by trypan blue (Sigma–Aldrich) exclusion. Cells were seeded in T25 cell culture flasks (0.1×10^6 cells/mL). The cell culture was regularly tested for *Mycoplasma* contamination.

The cell viability was determined by using the MTT-assay that is based on the reduction of a tetrazolium salt [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] to a formazan, which mainly depends on the cells' mitochondrial activity. After 24 h cultivation in a 96-well microtiter plate (8×10^3 cells/well) to allow cell attachment, medium was aspirated and cells were exposed to vehicle (0.5% MeOH), the positive control etoposide (0.99 μmol/L) or the extract at concentrations between 31.25 and 250.00 μg/mL for 72 h in six technical replicates. After the incubation period medium was removed and cells were washed with 200 μL Hanks balanced salt solution (Sigma–Aldrich). Then 100 μL of an MTT (Carl Roth, Karlsruhe, Germany) solution (end concentration 0.5 mg/mL) was added for 3 h. The supernatant was discarded and 200 μL dimethylsulfoxide (Carl Roth, Karlsruhe, Germany) was added to dissolve the colored formazan crystals. For a better dissolution of the crystals the plate was mixed on a shaker for further 30 min. The absorbance values of the solutions were measured at 550 nm and 620 nm using a plate reader (FLUO star Omega, BMG Labtech, Ortenberg, Germany). The IC_{50} was calculated by linear regression. The extract was tested for five times.

2.12. Statistical analysis

All the experiments were performed in triplicate. For each result, data are presented as the mean ± SD. Student's *t*-test was used to calculate the significance of the difference between the experimental and the control samples. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Phytochemical screening and flavonoid content of methanolic extract of C. operculatus leaves

The phytochemical screening using TLC showed positive results for flavonoids and terpenes in the methanolic extract of *C. operculatus* leaves. Flavonoids were the major constituents with a concentration of 6.8 mg/g dry material. Previous studies also indicated that flavonoids were the major constituents found in this plant [18,22,23]. Bioautography results revealed the activity
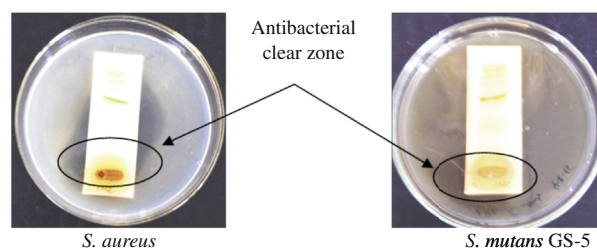

Figure 1. Bioautography of *C. operculatus* leaves extract.
The extract was resolved by TLC using solvent system toluene: ethyl acetate: acetone: formic acid (5:3:1:1). The TLC plates were then placed in an agar plate seeded with the test bacteria. The plates were incubated for 24 or 48 h for observation of inhibition zone.

of polar compounds in the extract (appeared as bands with low R_f value in TLC plates) against two pathogenic bacteria *S. aureus* and *S. mutans* GS-5 (Figure 1).

3.2. Antimicrobial activity of C. operculatus leaves extract

The investigated extract exhibited inhibitory activity mainly against the Gram-positive bacteria and the yeast *C. maltosa*. Interestingly, the multiresistant *Staphylococcus* strains showed more sensitivity to the extract compared to the antibiotic susceptible Gram-positive bacteria (Table 1). The hexane extract was also tested, but this did not exhibit any antimicrobial activity (data not shown).

Table 1
Antibacterial activity of *C. operculatus* leaves extract.

Tested microorganisms	Inhibition zone diameter (mm)	
	Extract	Antibiotics
S. aureus	12	40 (Ampicillin)
Escherichia coli	–	23 (Ampicillin)
B. subtilis	7	21 (Ampicillin)
Pseudomonas aeruginosa	–	12 (Gentamicin)
C. maltosa	12	20 (Nystatin)
S. mutans GS-5	8	30 (Ampicillin)
S. aureus North German epidemic strain	16	0 (Ampicillin)
Staphylococcus epidermidis 847	12	18 (Ampicillin)
Staphylococcus haemolyticus 535	14	0 (Ampicillin)

The data are representatives of three independent experiments.

3.3. Anticaries activity of C. operculatus leaves extract

3.3.1. Anti-acidogenic activity

S. mutans GS-5 is a strong acid producing and tolerant organism in dental plaque. It is able to perform glycolysis, even at a pH < 4.0 [7,8]. *S. mutans* GS-5 carcinogenicity can be related directly to acid production during glycolysis at low pH values. As shown in Figure 2, glycolysis by *S. mutans* GS-5 in
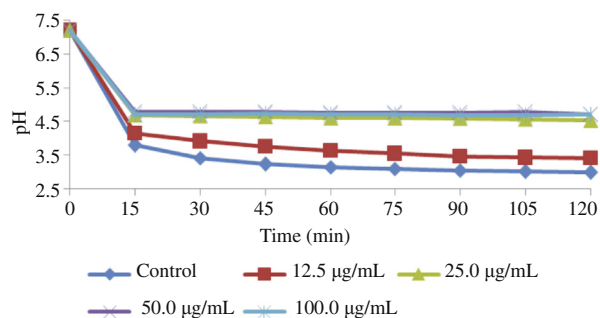

Figure 2. Effect of methanol extract of *C. operculatus* leaves on acid production by *S. mutans* GS-5.

planktonic form was clearly inhibited by methanol extract of *C. operculatus* leaves at concentration of 25 µg/mL. In this assay, the medium contained excess glucose (0.5% m/v or about 28 mmol/L) so that, as shown in the figure, glycolysis was not limited by catabolite supply. The obtained result suggested that F-ATPase may play a role.

The membrane-bound F-ATPase (H^+-translocating ATPase) is considered the primary determinant of the acid tolerance of *S. mutans* GS-5, as it translocates protons from the cytoplasm and maintains the pH across the cell membrane. Thus, the enzyme maintains the pH of the cytoplasm close to neutral and, under certain conditions, uses the proton gradient to generate ATP for growth and persistence [8,26,27]. As methanol extract of *C. operculatus* leaves inhibits acid production, we tested whether this was an effect on the F-ATPase activity. As shown in Figure 3A, F-ATPase activity was indeed strongly inhibited by the extract, with an IC_{50} of 51 µg/mL.

S. mutans GS-5 can take up and metabolize various sugars, resulting in the production of lactic acid. An important first step in the acidogenicity is the PTS, which is responsible for sugar uptake in oral streptococci. The coupled transport and phosphorylation of sugar involves a two-step reaction catalyzed by two enzymes, enzyme I and enzyme II, with the protein HPr as an intermediate phosphoryl donor. Consequently sugar is transported across the membrane to release a sugar phosphate in the cytoplasm. In this study, the glucose-PTS of permeabilized cells of *S. mutans* was inhibited in its phosphotransferase activity by the extract, with an average IC_{50} of 98 µg/mL (Figure 3B). A biological action of many phenolics is to bind tightly to and disrupt cell membranes. Although the exact nature of *C. operculatus* leaves extract in the inhibition of the membrane bound enzymes F-ATPase and PTS found in this study remains to be determined, our data indicated that the extract could be a potent inhibitor of membrane-located enzymes.

3.3.2. Anti-biofilm activity

Strong production of biofilm by *S. mutans* GS-5 is an important virulence factor of this organism. The effect of methanol extract of *C. operculatus* leaves on the ability of *S. mutans* GS-5 to form biofilms was measured in 96-well polystyrene plates. The extract was added into the culture medium at the beginning of growth to analyze activity against biofilm formation by *S. mutans* GS-5. The results presented in Figure 4 show a reduction in biofilm biomass up to 67% at concentrations of 3 µg/mL compared to the untreated biofilms. Lower concentrations were not effective on biofilm biomass of this organism. There was no bactericidal activity of the extract found at the treated concentrations (data not shown).
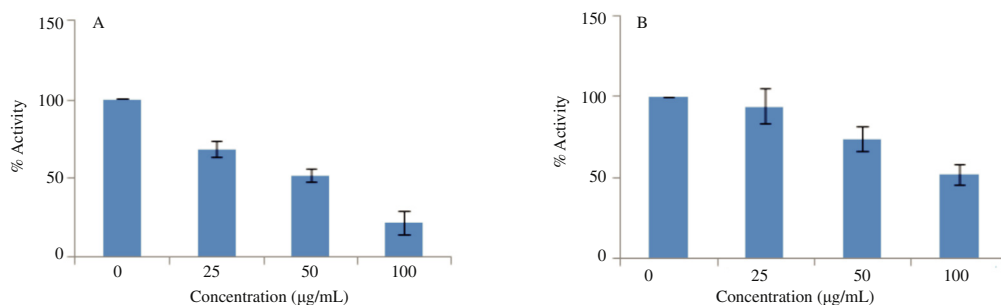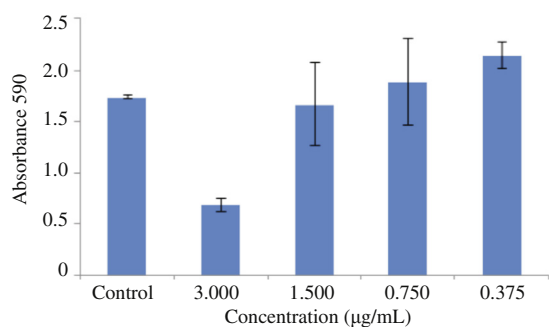

Figure 3. Effect of methanol extract of *C. operculatus* leaves on membrane bound enzymes of *S. mutans* GS-5.
A: F-ATPase; B: PTS. Values are represented as mean ± SD of three independent experiments.

Figure 4. Effect of methanol extract of *C. operculatus* leaves on biofilm formation by *S. mutans* GS-5.
Values are represented as mean ± SD of three independent experiments.

Figure 6. Cell viability of HaCaT cells after methanol extract of *C. operculatus* leaves treatment for 72 h.
Etop: 0.99 μmol/L of etoposide. Cell viability was measured using the MTT assay. *: $P < 0.01$ using student's *t*-test compared to the control. Values are represented as mean ± SD ($n = 5$) with six technical replicates each.

3.4. Confocal microscopy

Figure 5 presents the effect of the extract in killing bacteria. The treated biofilm clearly fluoresced red, which indicated that the bacteria were dead, whereas the fluorescence of the *S. mutans* GS-5 biofilm was significantly more green in the absence of the extract.
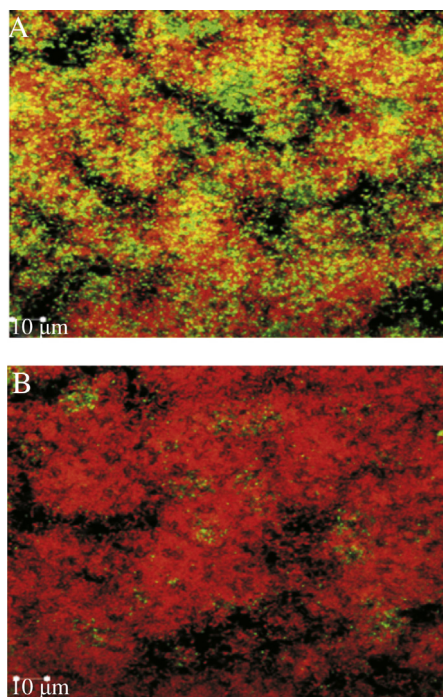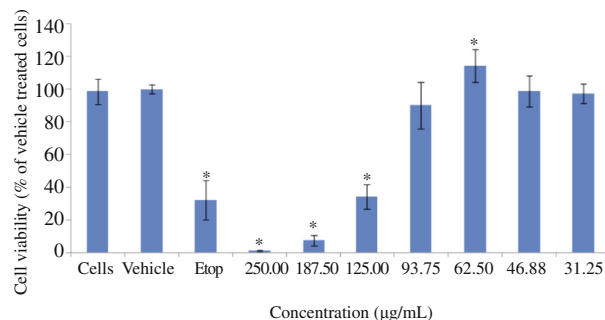

Figure 5. Confocal microscopy images of biofilms grown on coverslips. Pre-formed biofilms of *S. mutans* were incubated for 24 h in fresh TYS medium (A) or in TYS medium supplemented with 40 μg/mL methanol extract of *C. operculatus* leaves (B).

3.5. Cytotoxicity of methanol extract of C. operculatus leaves

A keratinocyte cell line (HaCaT cells) was exposed to increasing concentrations of the extract for 72 h, and toxicity was analyzed using the MTT assay. Only high concentrations between 125.00 and 250.00 μg/mL decreased cell viability. The IC_{50} value was calculated by linear regression compared to vehicle treated cells with a concentration of (119.98 ± 4.63) μg/mL. The extract at a concentration of 62.50 μg/mL stimulated cell viability (Figure 6).

4. Discussion

In this study, the antimicrobial activity, and anticaries of methanolic extract from leaves of *C. operculatus* were investigated. It was found that the extract showed very clear activity against Gram-positive bacteria including *S. aureus*, *B. subtilis* and *S. mutans* GS-5. Moreover, it also showed a remarkably inhibitory activity against *C. maltosa* and 3 methicillin-resistant *Staphylococcus* strains. Previous investigation indicated the presence of flavonoids, triterpenes and essential oil in the buds and bark of this plant which could be responsible for biological activity of plant [12,16–20]. Our phytochemical screening result also indicated the presence of these components, especially flavonoids (6.8 mg/g) in the leaves of *C. operculatus*. It is remarkably lower than that in the bud of *C. operculatus* grown in Thailand [28].

In this study, for the first time, we determined the anticaries activity of methanolic extract of the leaves of *C. operculatus* by testing its capacity of inhibiting acid production and acid tolerance of *S. mutans* GS-5 cells, as observed in the glycolytic pH-drop assay. The final pH values of extract, which were above critical pH (5.5) for tooth enamel demineralization, were significantly higher than those in the presence of the vehicle control. Based on data from the pH-drop curves, these effects can be attributed to proton permeability of the cell membrane.

Moreover, our study also focused on membrane bound enzymes F(H$^+$)-ATPase and the PTS, a possible primary locus for the inhibitory effects of the extract. Aciduricity of *S. mutans* GS-5 is coupled with an increase in the activity of the F_1F_0 H$^+$-translocating ATPase enzyme [26]. The F-ATPase maintains the cytoplasmic pH in a range where metabolic enzymes and transporters can function, and is thus important for acid tolerance. Acid production is linked to uptake and metabolism of sugars, and demineralization of teeth occurs when the pH in dental plaque drops below 4.0 as a result of acid production by *S. mutans* GS-5. Our data indicated that both F-ATPase and PTS activity were suppressed by methanolic extract of the leaves of *C. operculatus*, and consequently acid production was reduced.

In *S. mutans* GS-5, glycosyltransferases are responsible for exopolysaccharide production. The previous study with other antimicrobial agents such as fluoride or α-mangostin [7,29–31], indicated that this enzyme is a target for anti-biofilm formation by this organism. Thus, probably the inhibition of glycosyltransferases may cause the disruption in biofilm development when treated with the extract. The effect of methanolic extract of

C. operculatus leaves on quorum sensing system involving the biofilm formation by *S. mutans* GS-5 should also be examined to see if it could be another target of the extract. Cytotoxicity showed only weak effect in cell culture assays with keratinocytes, a predominant cell type in the epidermis, suggesting it is safe for topical use in health care products. Thus, the *C. operculatus* leaves extract seems to be a promising source of new anticaries agent because it possesses both anti-acidogenic and anti-biofilm activity.

In conclusion, the present study suggests that methanolic extract of *C. operculatus* leaves could be an interesting material for the development of antibacterial drugs against Gram-positive bacteria and anticaries agents. In future, work will be done to isolate bioactive constituents of the extract to identify the desired pharmacological agents, as well as mechanisms of action of potential compounds against *S. aureus*, including methicillin resistant *S. aureus*, and *S. mutans* GS-5.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

References

[1] World Health Organization. Antimicrobial resistance: global report on surveillance 2014. Geneva: World Health Organization;2014. [Online] Available from: http://www.who.int/drugresistance/documents/surveillancereport/en/ [Accessed on 10th June, 2016]

[2] Jeon JG, Rosalen PL, Falsetta ML, Koo H. Natural products in caries research: current (limited) knowledge, challenges and future perspective. *Caries Res* 2011; **45**: 243-63.

[3] Saranraj P, Sivasakth S. Medicinal plants and its antimicrobial properties: a review. *Glob J Pharmacol* 2014; **8**(3): 316-27.

[4] Ali NA, Wursterb M, Denkert A, Arnold N, Fadail I, Al-Didamony G, et al. Chemical composition, antimicrobial, antioxidant and cytotoxic activity of essential oils of *Plectranthus cylindraceus* and *Meriandra benghalensis* from Yemen. *Nat Prod Commun* 2012; **7**(8): 1099-102.

[5] Naz R, Bano A. Phytochemical screening, antioxidants and antimicrobial potential of *Lantana camara* in different solvents. *Asian Pac J Trop Dis* 2013; **3**: 480-6.

[6] Phuong NT, Cuong TT, Quang DN. Anti-inflammatory activity of methyl ferulate isolated from *Stemona tuberosa* Lour. *Asian Pac J Trop Med* 2014; **7S1**: S327-31.

[7] Nguyen PT, Falsetta ML, Hwang G, Gonzalez-Begne M, Koo H. α-Mangostin disrupts the development of *Streptococcus mutans* biofilms and facilitates its mechanical removal. *PLoS One* 2014; **9**(10): e111312.

[8] Lemos JA, Quivey RG Jr, Koo H, Abranches J. *Streptococcus mutans*: a new Gram-positive paradigm? *Microbiology* 2013; **159**(Pt 3): 436-45.

[9] Bowen WH, Koo H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Res* 2011; **45**: 69-86.

[10] Flemmig TF, Beikler T. Control of oral biofilms. *Periodontol 2000* 2011; **55**(1): 9-15.

[11] Loi DT. [*Vietnamese medicinal plants and herbs*]. Hanoi: Medicine Publishing House; 2014. Vietnamese.

[12] Giang PM, Phuong VT, Chinh TT. A new taraxastane-type triterpenoid from *Cleistocalyx operculatus*. *Nat Prod Commun* 2016; **11**(1): 29-30.

[13] Ha TK, Dao TT, Nguyen NH, Kim J, Kim E, Cho TO, et al. Antiviral phenolics from the leaves of *Cleistocalyx operculatus*. *Fitoterapia* 2016; **110**: 135-41.

[14] Hu YC, Luo YD, Li L, Joshi MK, Lu YH. *In vitro* investigation of 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone for glycemic control. *J Agric Food Chem* 2012; **60**(42): 10683-8.

[15] Min BS, Cuong TD, Lee JS, Woo MH, Hung TM. Flavonoids from *Cleistocalyx operculatus* buds and their cytotoxic activity. *Bull Korean Chem Soc* 2010; **31**(8): 2392-4.

[16] Ye CL, Lai YF. 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone, from buds of *Cleistocalyx operculatus*, induces apoptosis in human hepatoma SMMC-7721 cells through a reactive oxygen species-dependent mechanism. *Cytotechnology* 2016; **68**(2): 331-41.

[17] Zhang FX, Liu MF, Lu RR. Chemical constituents from the bud of *Cleistocalyx operculatus*. *Zhiwu Xuebao* 1990; **32**: 469-72.

[18] Ye CL, Lu YH, Wei DZ. Flavonoids from *Cleistocalyx operculatus*. *Phytochemistry* 2004; **65**: 445-7.

[19] Yu WG, He H, Yao JY, Zhu YX, Lu YH. Dimethyl cardamonin exhibits anti-inflammatory effects via interfering with the PI3K-PDK1-PKCα signaling pathway. *Biomol Ther (Seoul)* 2015; **23**(6): 549-56.

[20] Ye CL, Lai YF, Liu XG, Huang Q. [Study on mechanism of inducing apoptosis in human hepatoma SMMC-7721 cells by DMC, a chalcone from buds of *Cleistocalyx operculatus*]. *Zhongguo Zhong Yao Za Zhi* 2014; **39**(15): 2942-6. Chinese.

[21] Dung NX, Luu HV, Khoi TT, Leclercq PA. GC and GC/MS analysis of the leaf oil of *Cleistocalyx operculatus* Roxb. Merr. et Perry (Syn. *Eugenia operculata* Roxb.; *Syzygicum mervosum* DC.). *J Essent Oil* 1994; **6**: 661-2.

[22] Dung NT, Bajpai VK, Yoon JI, Kang SC. Anti-inflammatory effects of essential oil isolated from the buds of *Cleistocalyx operculatus* (Roxb.) Merr and Perry. *Food Chem Toxicol* 2009; **47**(2): 449-53.

[23] Nguyen TD, Kim JM, Kang SC. Chemical composition, antimicrobial and antioxidant activities of the essential oil and the ethanol extract of *Cleistocalyx operculatus* (Roxb.) Merr and Perry buds. *Food Chem Toxicol* 2008; **46**: 3632-9.

[24] Wagner H, Bladt S. *Plants drug analysis: a thin layer chromatography atlas*. 2nd ed. Berlin: Springer; 1996.

[25] Subedi L, Timalsena S, Duwadi P, Thapa R, Paudel A, Parajuli K. Antioxidant activity and phenol and flavonoid contents of eight medicinal plants from Western Nepal. *J Tradit Chin Med* 2014; **34**(5): 584-90.

[26] Belli WA, Marquis RE. Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. *Appl Environ Microbiol* 1991; **57**: 1134-8.

[27] Nguyen PT, Marquis RE. Antimicrobial actions of alpha-mangostin against oral streptococci. *Can J Microbiol* 2011; **57**: 217-25.

[28] Molhoek EM, van Dijk A, Veldhuizen EJ, Haagsma HP, Bikker FJ. Cathelicidin-2-derived peptide effectively impairs *Staphylococcus epidermidis* biofilms. *Int J Antimicrob Agents* 2011; **37**: 476-9.

[29] Maisuthisakul P, Suttajit M, Pongsawatmani R. Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. *Food Chem* 2007; **100**(4): 1409-18.

[30] Marsh PD. Contemporary perspective on plaque control. *Br Dent J* 2012; **212**(12): 601-6.

[31] Falsetta ML, Klein MI, Lemos JA, Silva BB, Agidi S, Scott-Anne KK, et al. Novel antibiofilm chemotherapy targets exopolysaccharide synthesis and stress tolerance in *Streptococcus mutans* to modulate virulence expression *in vivo*. *Antimicrob Agents Chemother* 2012; **56**: 6201-11.