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The effects of methanolic extract of *Uncaria gambir* against microflora of dental caries

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The antibacterial effects of *Uncaria gambir* extract have the potential to be expanded in dentistry, mainly in the management of dental caries and its sequelae. This preliminary study was conducted to investigate the *in vitro* antibacterial activity of methanolic extract of *U. gambir* against dental caries-related microflora: *Streptococcus mutans, Streptococcus sobrinus, Lactobacillus casei* and *Enterococcus faecalis.* The methanolic extract of *U. gambir* in powder form was dissolved and serially diluted in 1% dimethyl sulfoxide. The antibacterial effects of the extract were determined using the broth microdilution technique. A transmission electron microscope (TEM) was used to assess the effects of the extract on the morphology of the bacteria. A 0.12% chlorhexidine (CHX) and Man-Rogosa-Sharpe/brain heart infusion broth were used as positive and negative control respectively. Greatest antibacterial effects were seen on both Streptococci species with the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of 1.25 and 5 mg/mL respectively, followed by *Enterococcus faecalis* (MIC=2.5 mg/mL, MBC=10 mg/mL) and *Lactobacillus casei* (MIC=7.5 mg/mL, MBC=30 mg/mL) in ascending order. Cell wall damage of all bacteria at their respective MIC value was observed through the TEM analysis. Tukey's posthoc test showed no statistically significant difference in the antibacterial activity exerted by *U. gambir* extract and 0.12% CHX, with $P > 0.05$. Conclusively, *U. gambir* extract exhibits a good antibacterial effect against the microflora of dental caries and carries great potential for future development.

Keywords: Antibacterial, *Enterococcus faecalis*, *Lactobacillus casei*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Uncaria gambir*

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Introduction

Dental caries is a chronic, multi-factorial process of tooth surface destruction of bacterial origin. Under the constant acidic challenge, produced by the oral bacteria following fermentation of refined carbohydrates, the enamel structure undergoes a demineralisation process which leads to the formation of a cavity. Untreated dental caries can lead to toothache, dental infection, facial swelling and impaired quality of life. Poor academic performance secondary to frequent school absentees has also been reported[1].

Streptococcus mutans and *Streptococcus sobrinus* are two bacteria species that are well known for their role in caries initiation and are regarded as the early coloniser of dental plaque. Both of the Streptococci species have been frequently isolated from the carious tooth surface and saliva of children with dental caries[2]. *Lactobacillus casei* were regarded as secondary colonisers where the bacteria used to colonise, reside and proliferate in the low pH environment within the carious lesion[3], mainly at the deep dentinal and root surface caries. In addition, *Enterococcus faecalis* is regarded as the most resistant bacteria species, found predominantly in the case of root canal reinfection second to dental caries[4].

Although there has been a reduction in caries prevalence worldwide in the past few decades, there are not many changes seen in the developing and third world countries, mainly from the Asia and Africa continent[5]. This imbalance might exist due to cultural and geographical differences, as well as poor access to dental care, lifestyles and poor parental awareness. Thus, preventive measures have been identified as the best mechanism to close this gap, mainly through oral health education and promoting good oral hygiene practices.

Fluoride, in form of toothpaste, varnish or water fluoridation has been introduced as part of the public health measures in caries prevention with great success[6]. However, fluoride lacks bactericidal action

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as it only interferes with the bacterial enzymatic activity instead of killing it. Researchers are keen to combine the benefit of fluoride with other materials with a potent antibacterial effect. Natural product has come into mind due to its wide therapeutic effects, being readily available, cheap and most importantly producing minimal side effects[7].

Various natural-based products such as the extract of propolis, miswak, neem, aloe vera and green tea have been added into the formulation, with success[8]. However, most of the effects are directed against a specific bacteria species. It is beneficial to have a product that can target multiple bacterial species involved in caries formation. One of the natural products that have caught our attention is *Uncaria gambier* (Hunt) Roxb or also known as *Uncaria gambir (U. gambir)*.

This herbal plant is part of the Rubiaceae family in the plant taxonomy which can be found in the tropical forest of Africa, South America, and Southeast Asia, especially in the rainforest of Indonesia and Malaysia[9]. It has been used as traditional medicine for centuries to treat headaches, sore throat, skin lesions, and gastrointestinal-related problems[10], as well as remedies for toothache and gum problems[9]. These wide therapeutic effects are due to the presence of flavonoids and quinic acid as the plant's main secondary metabolite[11,12], which are well-known for their excellent antibacterial, anti-inflammatory, and antioxidant effects[13].

Realising its potential, *U. gambir* may have some role in dentistry, mainly in dental caries prevention. However, scientific evidence on the antibacterial effects of *U. gambir* extract against oral bacteria is scarce. Most of the studies were conducted against *Streptococcus mutans* whereas human's oral cavity harbours other pathogenic bacteria as well. The development of an antibacterial agent that affects a wide range of bacterial species will be beneficial and cost-effective. Hence, the present study was conducted to investigate the potential antibacterial activity of *U. gambir* extract against four oral bacteria that are commonly associated with dental caries: *Streptococcus mutans* (*S. mutans*), *Streptococcus sobrinus (S. sobrinus)*, *Enterococcus faecalis (E. faecalis)*, and *Lactobacillus casei (L. casei)*.

Materials and Methods

Collection and identification of the plant sample

The *U. gambir* leaves were collected from Bukit Diman, Ajil, Terengganu, Malaysia in mid-November 2018, during the period when the area received its highest average rainfall in a calendar year. Following verification by the botanist, Mohamad Nazrin Ahmad Azmi, the Assistant Director for Herbarium Management and Plant Conservation Section Putrajaya Botanical Garden, a voucher specimen number representing *U. gambir* (HTBP 4320) was deposited at the Herbarium Putrajaya Botanical Garden, Putrajaya, Malaysia.

Test bacteria

The test bacteria namely *Streptococcus mutans* ATCC®25175™ (ATCC, Manassas, Virginia, USA), *Streptococcus sobrinus* ATCC®33478™ (ATCC, Manassas, Virginia, USA), *Enterococcus faecalis* ATCC® 29212™ (ATCC, Manassas, Virginia, USA) and *Lactobacillus casei* ATCC®393™ (ATCC, Manassas, Virginia, USA) were obtained from the manufacturer and stored in cryogenic vials (Simport, Canada) at -80°C as instructed by the manufacturer until further use.

Preparation of the plant extract

The methanolic extraction of *U. gambir* was done following the methods described by Ibrahim *et al.*[14]. The leaves of *U. gambir* were washed, dried and ground into a fine powder before being weighted with a balance (Mettler, Toledo). The powder was macerated with distilled methanol at room temperature for 72 h. Following evaporation of methanol, 127.37 g of the extract in a powder form were obtained. The powder was freeze-dried by keeping it in the refrigerator at 4ºC until further use[14].

The stock solution of the extract was prepared by dissolving the freeze-dried powder in dimethyl sulfoxide (DSMO) (Merck, Sweden), using an ultrasonicator (Branson, USA). Following that, the extract was filtered through a 0.2 µm syringe filter (Sartorius Stedim, Germany) and underwent 2-fold serial dilution to obtain a series of concentrations ranging from 10 to 0.15625 mg/mL in 1% DMSO, except for the extract tested against *L. casei*. As the trial minimum inhibitory concentration (MIC) did not produce any result; a higher concentration ranging from 30 to 0.4687 mg/mL was used against the bacteria.

Preparation of the bacterial strain

Man-Rogosa-Sharpe (MRS) agar and broth (Oxoid, United Kingdom) were used as the medium for *L. casei* while other bacteria used brain heart infusion (BHI) agar and broth (Oxoid, United Kingdom) as the growth medium.

The frozen bacteria stocks were thawed at room temperature to revive the bacteria. Exactly 100 µL of the bacterial inoculum was pipetted out and streaked across the surface of the MRS/BHI agar using a sterile inoculating loop. Another 100 µL of the bacterial inoculum was pipetted into a sterile universal container containing 10 mL of MRS/BHI broth. Both the agar plate and container were incubated (Lab Companion, Korea) for 24 h at 37°C and 5% CO_2.

Post-incubation, the bacterial purity was confirmed by observing the growth of the bacterial colonies and through the Gram staining procedures[15]. Then, the bacterial cell density was standardized to 10^6 CFU/mL using the spectrophotometer (Jenway, UK) as described by Clinical and Laboratory Standard[16].

Minimum inhibition concentration (MIC)

The minimum inhibitory concentration (MIC) of the *U. gambir* extracts against the bacteria of interest was determined using the broth microdilution technique[16]. Different well plates were used for different bacterial strains.

Briefly, 50 µL of each of the following solutions: *U. gambir* extract in eight different concentrations ranging from 10 to 0.15625 mg/mL, 0.12% chlorhexidine gluconate (CHX) (positive control), and BHS/MRS broth (negative control) were added into the 96-well plate (Thermo Scientific, Germany) according to their respective group. Then, 50 µL of the *S. mutans* suspension was pipetted into the respective wells to give the final bacterial concentration of 10^5 CFU/mL in each well. In addition, the untreated control group consists of 50 µL of *U. gambir* extract for each concentration and 50 µL of BHI broths were pipetted into the plate. All test and control samples were done in triplicate and incubated anaerobically for 24 h at 37°C. Similar steps were repeated for *S. sobrinus*, *E. Faecalis*, and *L. casei*, where the latter used a higher concentration of *U. gambir* extract as mentioned earlier.

After incubation, the turbidity of the wells was observed using the naked eye. The MIC was quantitatively measured using the ELISA microplate reader (Varioskan Flash® Multimode Reader Thermo, USA). The optical density (OD) was set at 590 nm for *S. mutans*, *S. sobrinus* and *L. casei* and 625 nm for *E. faecalis*. The MIC was determined by comparing the mean absorbance value of the test wells with the corresponding control wells[17], where the lowest concentration of the extract with no visible bacteria growth was recorded as the MIC.

Minimum bacterial concentration (MBC)

The MBC was determined by sub-culturing 10 µL of the samples taken from the wells at MIC values onto the respective agar plate in triplicate. The agar plate was incubated at 37ºC for 24 h with 95% relative humidity. After 24 h, any bacterial growth on the agar plate was inspected with the naked eye. The lowest concentration that yielded no growth after sub-culturing was recorded as the MBC value[18].

Transmission electron microscope (TEM)

The bacterial sample at MIC value was pipetted out, harvested and prepared according to the methods described by Wang *et al.*[19]. The prepared specimens were then observed under the TEM (Philips CM12 120kV, The Netherlands; Thermo Scientific Talos L120C, USA).

Ethical approval

Ethical approval (UKM PPI/111/8/JEP-2018, dt. 26.10.2018) was obtained from the institution's Research Ethical Committee.

Data recording and analysis

The recorded MIC value of the extract against all bacterial strains was analysed and confirmed statistically. The mean OD value of the extract was compared with the positive (0.12% CHX) and negative (BHS and MRS broth) control using one-way Analysis of Variance (ANOVA) in Statistical Package for the Social Sciences (SPSS) Version 23 software (IBM, Armonk, NY, USA). The normality of the data was assessed using Shapiro-Wilk's test, while any violation of homogeneity of variance was assessed using Levene's statistic. Post-Hoc Tukey test was done to determine the significant difference between the test material and the control groups for each bacterium. The significance level for all tests was set at 0.05. The direct visualisation of MBC and TEM was descriptively documented.

Results

Determination of MIC

The MIC value of the *U. gambir* extract against all the bacterial strains was shown in Table 1. Both *S. mutans* and *S. sobrinus* showed the lowest MIC value

Table 1 — The MIC value of each bacteria after exposure with *Uncaria gambir* extract

Bacteria species	Minimum inhibitory concentration (mg/mL)
Streptococcus mutans	1.25
Streptococcus sobrinus	1.25
Enterococcus faecalis	2.5
Lactobacillus casei	7.5

(1.25 mg/mL) followed by *E. faecalis* (2.5 mg/mL) and *L. casei* (7.5 mg/mL) in ascending order.

A comparison of the mean OD value of the extract with the control groups were shown in Table 2. No statistically significant difference was observed when comparing the MIC value of *U. gambir* extract with 0.12% CHX for all bacterial strains, with *P*> 0.05 as opposed to the respective broth as the negative control (P=0.00).

Determination of MBC

Table 3 and 4 summarise the MBC value obtained for each bacterial species used in the study. *S. mutans* and *S. sobrinus* recorded the lowest MBC value at 5 mg/mL, followed by *E. faecalis* (10 mg/mL) and *L. casei* (30 mg/mL) respectively.

Morphological changes of treated bacterial species viewed under TEM

The ultrastructure of all bacteria viewed under TEM showed cell wall damage with alteration and leakage of the cytoplasmic content when treated with *U. gambir* extract at their respective MIC value (Fig. 1-4). The difference was apparent when compared to the negative control which showed smooth and intact cell wall and cytoplasmic content. Exposure to 0.12% CHX showed more damaging effects on the bacterial cell wall and shrinkage of the cytoplasm with the formation of intracellular cystic space. *L. casei* appeared to be the least affected bacteria when tested against the *U. gambir* extract and 0.12% CHX as compared to other bacterial species.

Discussion

The current findings are the authors' preliminary works in investigating the antibacterial activity of *U. gambir* extract. We postulate that the antibacterial effect exerted is due to the presence of two main components of *U. gambir*: catechin and quinic acid, identified through the High-Performance Liquid Chromatography of the plant's crude extract[9,12]. Both

Table 2 — Comparison of mean OD value between *Uncaria gambir* extract and control groups for all bacterial strains

Bacterial strain	Dependent variable	Mean OD value (±SD)	*P*-value Tukey's post hoc test		
			CHX	Negative control	*Uncaria gambir* (MIC)
Streptococcus mutans	Positive control (CHX)	0.020±0.01	-	0.000*	0.299
	Negative control (broth)	0.650±0.03	0.000*	-	0.000*
	Uncaria gambir (MIC)	0.056±0.35	0.299	0.000*	-
Streptococcus sobrinus	Positive control (CHX)	0.020±0.01	-	0.000*	0.199
	Negative control (broth)	0.597±0.04	0.000*	-	0.000*
	Uncaria gambir (MIC)	0.063±0.02	0.199	0.000*	-
Enterococcus faecalis	Positive control (CHX)	0.020±0.01	-	0.000*	0.760
	Negative control (broth)	0.660±0.02	0.000*	-	0.000*
	Uncaria gambir (MIC)	0.033±0.03	0.760	0.000*	-
Lactobacillus casei	Positive control (CHX)	0.056±0.03	-	0.000*	0.080
	Negative control (broth)	1.273±0.04	0.000*	-	0.000*
	Uncaria gambir (MIC)	0.020±0.01	0.080	0.000*	-

**P* is significant at α= 0.05

Table 3 — Determination of MBC of *Streptococcus mutans, Streptococcus sobrinus* and *Enterococcus faecalis*

Bacteria species	Concentration (mg/mL)								Positive (CHX)	Negative (BHI)
	10	5	2.5	1.25	0.625	0.3125	0.1562	0.0781		
Streptococcus mutans	-	-	+	+	+	+	+	+	-	+
Streptococcus sobrinus	-	-	+	+	+	+	+	+	-	+
Enterococcus faecalis	-	+	+	+	+	+	+	+	-	+

+ Bacterial growth observed; - No bacterial growth observed

Table 4 — Determination of MBC of *Lactobacillus casei*

Bacteria species	Concentration (mg/mL)								Positive (CHX)	Negative (MRS)
	30	15	7.5	3.75	1.875	0.9375	0.4688	0.2344		
Lactobacillus casei	-	+	+	+	+	+	+	+	-	+

+ Bacterial growth observed; - No bacterial growth observed

compounds can inflict damage on the bacterial cell wall. This can be confirmed through the TEM analysis of the bacteria following exposure to the *U. gambir* extract. Lysis of the cell wall and leakage of the cytoplasmic content, described as the ring phenomenon by Bai *et al.*[20] can be seen mainly in the image involving the *Streptococci* species and *E. faecalis*.

The mechanism of action of catechins on the bacterial cell wall is believed due to the ability of the compound to interpose itself into the lipid bilayer of the bacterial cell wall[21]. The penetration of molecules without the gallate group (catechin and epicatechin) into the lipid bilayer was said to be better. The process of surface binding was mediated by the ability

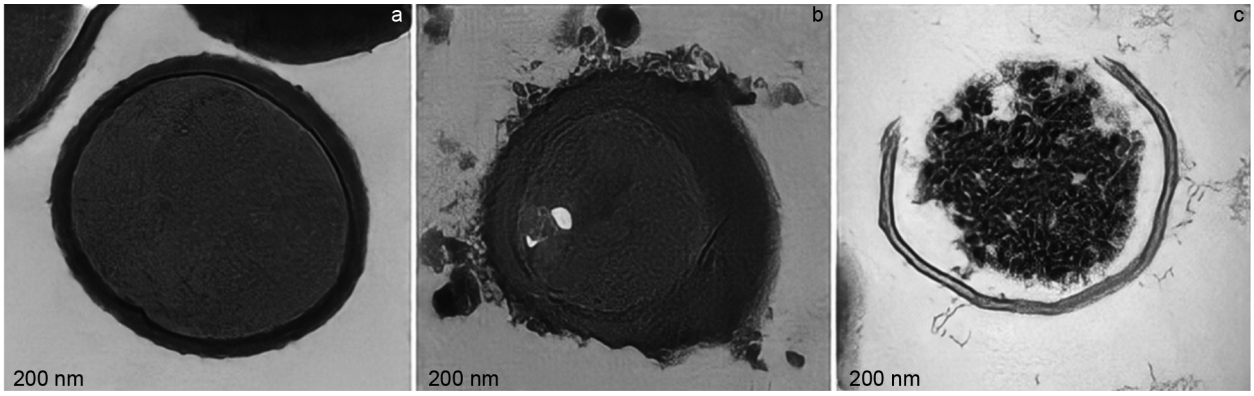

Fig. 1 — Ultrastructure of *Streptococcus mutans*: a) Negative control (without treatment); b) Treatment with 1.25 mg/mL of *Uncaria gambir* extract; and c) Treatment with 0.12% CHX (positive control). (CHX: Chlorhexidine).

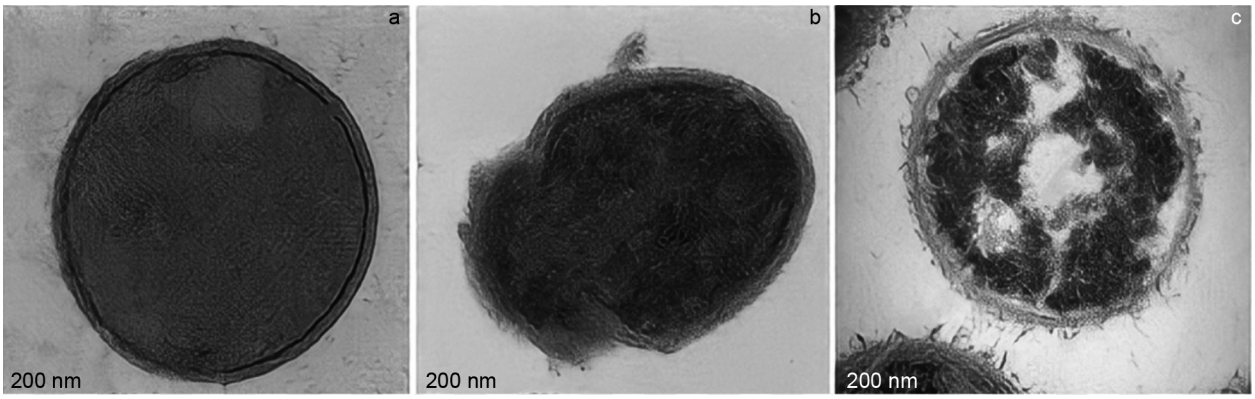

Fig. 2 — Ultrastructure of *Streptococcus sobrinus*: a) Negative control (without treatment); b) Treatment with 1.25 mg/mL of *Uncaria gambir* extract; and c) Treatment with 0.12% CHX (positive control). (CHX: Chlorhexidine).

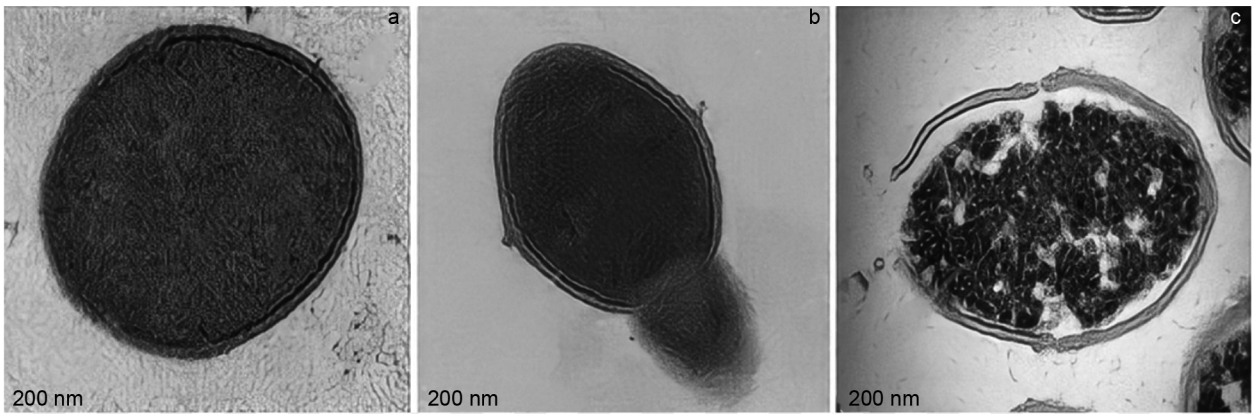

Fig. 3 — Ultrastructure of *Enterococcus faecalis*: a) Negative control (without treatment); b) Treatment with 2.5 mg/mL *Uncaria gambir* extract; and c) Treatment with 0.12% CHX (positive control). (CHX: Chlorhexidine).

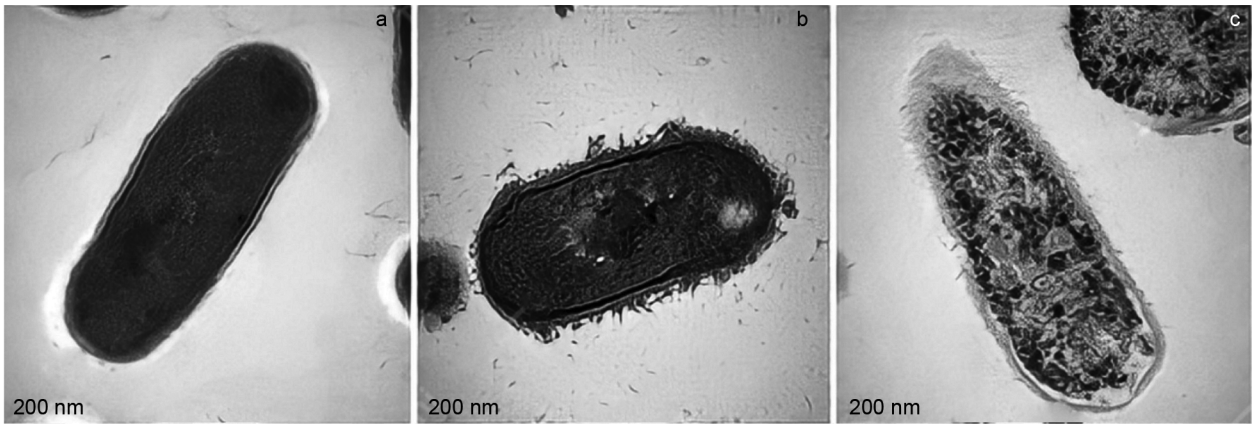

Fig. 4 — Ultrastructure of *Lactobacillus casei*: a) Negative control (without treatment); b) Treated with 7.5 mg/mL of *Uncaria gambir* extract; and c) Treated with 0.12% chlorhexidine gluconate (positive control). (CHX: Chlorhexidine).

of hydroxyl groups of the catechins to form hydrogen bonds with the oxygen atoms on the lipid side[22]. This causes partitioning of the tightly-packed cell membrane, decreases the lipid packing at the site of attachment and displaces the lipoteichoic acid from the cell membrane[23]. Further, lateral expansion of the membrane occurs which subsequently reduces the barrier activity of the membrane[24]. Continuation of the lateral displacement causes irreversible membrane disruption and leakage of its content[25].

The binding of the catechin on the lipid bilayer might also cause inhibition of the membrane-associated glycolytic enzyme, mediated by a group of enzymes on the cell membrane and those in the cytoplasm called PEP-PTS[26]. The enzyme acts as the transporter of sugar into the bacteria, where suppression of its activity will prevent the uptake of glucose into the bacterial cell. This will prevent acid production and suppress the metabolism of the bacteria leading to starvation of the biofilm[27] and suppressing its growth.

On the other hand, the effects of quinic acid on the bacterial cell wall are based on its ability to alter the bacterial cell membrane integrity and disrupt the cell membrane function. This has been studied in depth by Bai *et al.*[28]. The major component of the cell membrane of Gram-positive bacteria is phosphatidylglycerol[29]. Phosphatidylglycerol act as the regulator of the bacterial cell membrane by activating and controlling the lipid gate channel[30]. Quinic acid alters the lipid metabolites of the phosphatidylglycerol, causing loss of cell membrane function. Furthermore, it also suppresses the synthesis of L-lysine, a major precursor of peptidoglycan within the bacterial cell wall[28], which subsequently results in the failure of bacterial cell wall synthesis.

The results of the current study showed that different bacteria species were affected by the extract at different concentrations. The high susceptibility of the *Streptococci* species towards the extract may be due to the direct inhibition of the growth and proliferation of the bacteria exerted by catechin. Furthermore, catechin can prevent the adhesion of the bacteria to the enamel through the inhibition of the glucosyltransferase (gtf) activity[31]. The resistance of *E. faecalis* is associated with the presence of various virulence factors such as the toxic cytolysin, collagen-binding antigen and the lytic enzymes protease and gelatinase[32]. It has been suggested that damage to the cell membrane of *E. faecalis* is caused by the ability of catechin to produce hydroxyl radicals. This process takes place through a series of reactions where the generation of hydrogen peroxide by catechin is reduced by ferrous ion forming hydroxyl radicals, which later damage the proteins and lipid layer of the cells[33].

The 'resistance' of *L. casei* against *U. gambir* extract is down to the structural difference of the cell wall of the bacterial species itself. A similar observation was reported by Higuchi *et al.*[34] where they needed almost 10 folds concentration of green tea extract to kill *Lactobacillus spp.* as compared to other oral microbiota[34]. *L. casei*, categorized as lactic acid bacteria (LAB) has the cell wall of the Lys-type, where bacteria with this type of cell wall resisted the action of catechin more than those of Orn-type of the cell wall. The hydrophilicity of the extracellular polysaccharides of the LAB species also affects the absorption and attachment of catechin to the bacterial cell wall which is mediated by its hydrophobic surface[35].

In the current study, CHX was used as the positive control. CHX is a biguanide compound that possesses broad-spectrum antimicrobial properties. CHX is effective against both aerobic and anaerobic bacteria[36], which justifies its selection as the positive control. At a concentration between 0.1 to 0.2%, CHX as a mouthwash is effective in reducing the *S. mutans* bacterial load and dental plaque formation. Hence, it has been regarded as the gold standard mouthwash in adjunctive dental plaque control[37]. Further, CHX has been shown to successfully eliminate *E. faecalis* from the dentinal tubules when used as an endodontic irrigant, both *in vitro* and *in vivo*[38]. The bactericidal effects exerted by CHX are down to its cationic properties, which enable it to bind to the negatively charged bacterial cell wall. This leads to alteration in the cell membrane permeability and compromising its integrity, resulting in a leakage of the cytoplasmic component out of the bacterial cell. Further damage irreversibly causes cytoplasmic coagulation and precipitation, inhibits the intracellular enzyme and later lysis of the bacteria[36]. The TEM analysis in the present study was in agreement and confirms the bactericidal effects of CHX against the tested bacteria. The outcome also indirectly validates the methods and results from the present study.

Despite that, there are some concerning issues related to the prolonged usage of CHX as a mouthwash for dental plaque control. Teeth staining, altered taste sensation, salivary gland swelling, oral mucosa desquamation and allergic reaction all have been reported in the literature[39], casting some doubt on the long-term usage of CHX in the oral cavity. The recent guidelines by the European Federation of Periodontology stated that the usage of antiseptic solution is only as an adjunct to mechanical plaque control and interdental cleaning, and if needed the usage should be limited for a short period of time[40].

Based on the finding from the present study, we believe that *U. gambir* extract has the potential to be used in dentistry, mainly in the prevention of dental caries and root canal infections. The bactericidal activity of this extract is as effective as 0.12% CHX, a potent antibacterial solution. This is reflected by the statistically non-significant difference between the two materials, and the outcome from the TEM analysis. Thus, the extract can be proposed as an alternative natural-based material to CHX, considering the drawbacks of the latter solution as reported in the literature. Nevertheless, further research on its biocompatibility and antibacterial activity against other bacteria species through *in vitro* and *in vivo* studies is needed to strengthen the findings in this preliminary study.

Conclusion

The present study showed that the methanolic extract of *U. gambir* exhibits good antibacterial effects against *S. mutans*, *S. sobrinus*, *E. faecalis* and *L. casei* in ascending order. These results suggest that the extract can be exploited as a potential natural product in managing dental caries. However, further *in vivo* and clinical studies are required.

Conflict of interest

The authors declare that there is no conflict of interest.

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