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PHYTOCHEMICALS ISOLATION, ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF MALAYSIAN *Uncaria tomentosa* AGAINST ORAL STREPTOCOCCI

(Pengasingan Fitokimia, Aktiviti Antibakteria dan Antibiofilem *Uncaria tomentosa* dari Malaysia terhadap *Streptococci* Oral)

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Abstract

Uncaria tomentosa (Rubiaceae), locally known as 'kait-kait' has long been used for medicinal purposes to heal various diseases, including those related to bacterial infection. In an attempt to search for new antimicrobial agents, the present study was undertaken to evaluate the antibacterial and antibiofilm potentials of hexane (Hx), dichloromethane (DCM), and 80% ethanolic (EtOH) stem extracts of Malaysian *U. tomentosa* against five oral *streptococci* bacteria namely *S. salivarius*, *S. mitis*, *S. uberis*, *S. mutans* and *S. anginosus*. The antibacterial assay employed the agar-well diffusion method where cycloheximide was used as the positive control, while minimum inhibitory concentration (MIC) was determined through resazurin microtiter assay using 96-well microtiter plates. On the other hand, the antibiofilm activity was investigated using a crystal violet assay. The DCM stem extract was found to be active against all tested bacterial strains with ZOI ranging from 10.3±0.6 to 16.0±1.0 mm, and a MIC value of 234.4 µg/mL. Compared to average biofilm growth, the extract reduced biofilm formation by at least 50%. Phytochemical work on the DCM extract afforded four compounds elucidated as isopteropodine (**1**), pteropodine (**2**), β-sitosterol (**3**), and scopoletin (**4**). These four compounds have been previously reported to exhibit antibacterial activity against different bacterial strains. The findings provide evidence to support the plant's traditional use and could be the source of antibacterial and antibiofilm agents for further studies.

Keywords: *Uncaria*, dichloromethane extract, oral bacteria, streptococci, antimicrobial

Abstrak

Uncaria tomentosa (Rubiaceae), yang dikenali sebagai 'kait-kait' telah lama digunakan untuk tujuan perubatan dalam menyembuhkan pelbagai penyakit, termasuk yang berkaitan dengan jangkitan kuman. Dalam usaha mencari agen antimikrob baharu, kajian ini telah dijalankan untuk menilai potensi antibakteria dan antibiofilem ekstrak heksana (Hx), diklorometana (DCM) dan 80% etanol (EtOH) daripada batang *U. tomentosa* dari Malaysia terhadap lima bakteria *streptokokus* oral iaitu *S. salivarius, S. mitis, S. uberis, S. mutans* dan *S. anginosus*. Ujikaji antibakteria menggunakan kaedah ujian peresapan telaga agar di mana sikloheksimida digunakan sebagai kawalan positif, manakala kepekatan perencatan minimum (MIC) ditentukan melalui ujian mikrotiter resazurin dengan menggunakan plat mikrotiter 96 telaga. Ekstrak batang DCM didapati aktif terhadap semua strain bakteria yang diuji dengan ZOI dalam julat 10.3±0.6 - 16.0±1.0 mm, dan nilai MIC 234.4 µg/mL. Berbanding purata pertumbuhan biofilm normal, ekstrak ini dapat mengurangkan pembentukan biofilm sekurang-kurangnya 50%. Kajian fitokimia pada ekstrak DCM menghasilkan empat sebatian yang dikenalpasti sebagai isopteropodine (**1**), pteropodine (**2**), β-sitosterol (**3**), dan scopoletin (**4**). Empat sebatian ini sebelum ini telah dilaporkan mempamerkan aktiviti antibakteria terhadap strain bakteria yang berbeza. Penemuan ini memberikan bukti untuk menyokong penggunaan tradisional tumbuhan dan boleh menjadi sumber agen antibakteria dan antibiofilem untuk kajian lanjut.

Kata kunci: *Uncaria*, ekstrak diklorometana, bakteria oral, streptokokus, aktiviti antimikrob

Introduction

Oral bacteria are an example of an opportunistic human species capable of causing biofilm-related infections. Epithelial cells, dental surfaces, and orthodontic prostheses are some of the many surfaces in the oral cavity that are prone to the formation of multispecies biofilms [1]. In the form of supragingival and subgingival plaque, biofilm is the etiologic agent in dental caries (tooth decay) and periodontal (gum) disease [2]. Furthermore, studies have demonstrated an association between periodontitis and various systemic diseases and conditions in recent years, including cardiovascular disease, diabetes mellitus, respiratory disease, adverse pregnancy outcomes, obesity, pancreatic cancer, and Alzheimer's disease [3].

Uncaria (Rubiaceae) is a flowering woody climber plant. The genus comprises 34 species that distributed mainly in tropical regions, including Southeast Asia, Africa, and South America. Cultivation varies based on the species and region [4,5]. *Uncaria tomentosa* was given the name una de gato (cat's claw) because of hook-like thorns that resemble the claws of a cat. Phytochemical studies of the Peruvian *U. tomentosa* have led to the isolation of numerous constituents including quinovic acid glycosides, oxindole alkaloids, proanthocyanidins, polyphenols, triterpenes and numerous sterols [6-8]. Although there was no literature on the existence of the species in Malaysia, the species has been discovered in Malaysian forests.

The extracts of *U. tomestosa* is also widely studied compared to other species of the genus and they have been found to display multiple biological activities including anti-inflammatory, antioxidant, cytotoxicity, immunostimulant, and antibacterial [9,10]. So far, there is a lack of scientific reports indicating this plant's antimicrobial activities against oral pathogens. Moreover, no study has been published to indicate the potential antibiofilm activities of the plant. Hence, this study aimed to investigate the antibacterial and antibiofilm potentials of the hexane (Hx), dichloromethane (DCM), and 80% ethanol (EtOH) stem extracts of *U. tomentosa* against oral bacteria and to isolate its chemical constituents.

Materials and Methods

Plant material

The plant material was collected from Pasir Raja, Dungun, Terengganu in December 2011. The plant sample was identified by Dr. Shamsul Khamis of Universiti Putra Malaysia. The plant materials were air-dried in the dark at room temperature before being ground with a mechanical grinder.

Preparation of extracts

The dried samples were cut into small pieces and ground into a fine powder with a mechanical grinder. The fine powder of the stems (5 kg) was successively extracted with solvents ranging in different polarities: hexane (nonpolar), dichloromethane (medium polar) and 80% ethanol (polar) solvents by continuous hot extraction

technique using a Soxhlet extractor. The solvents were then evaporated under reduced pressure at a temperature lower than 40 ºC using a vacuum rotary evaporator. The dried extracts were stored in the refrigerator at a temperature of 4 °C until further analysis.

Antibacterial activity

The extracts of stems were prepared by dissolving in 10% dimethyl sulphoxide (DMSO) to a final concentration of 100 mg/mL. As a solvent for all the test samples, DMSO was first tested against bacteria to ensure that it did not contribute to the antibacterial activity of the samples. Then, the extracts were diluted to reach the concentration of 50, 35, and 15 mg/mL. These concentrations of test samples were subjected to an agar well-diffusion assay screening test. The lowest concentration of the test samples showed inhibition activity in the screening test and were chosen for further evaluation of MIC using the resazurin microtiter-plate dilution assay.

Bacterial strains and growth condition

A total of 5 oral bacteria were employed in the study, including *Streptococcus salivarius* (ATCC 13419), *Streptococcus mitis* (ATCC 6249), *Streptococcus uberis* (ATCC 19436), *Streptococcus mutans* (ATCC 25175) and *Streptococcus anginosus* (ATCC 33397). All bacterial strains were purchased from Oxoid (UK). The bacterial strains were grown in brain heart infusion broth (BHI) and were maintained on brain heart infusion agar (BHI) at 4 °C, and subcultures were freshly prepared before being subjected to bioassay.

Antibacterial screening

The agar well-diffusion assay was performed in this study to screen the antibacterial activity of the extracts against the bacteria tested. Briefly, 24 hours of bacterial suspension was adjusted to absorbance reading within the range of 0.08-0.10 at OD625 nm, equivalent to 1-2 x 10^8 CFU/mL [11] that matched the turbidity 0.5 McFarland standards [12]. Liquefied (25 mL) sterile brain heart infusion agar (BHI) at 45 °C was aseptically mixed with 1 mL standardized inoculum and poured immediately into previously labeled sterile petri dishes. Then, the plates were left to solidify in a laminar flow cabinet for 20 minutes. Once the agar hardened, four wells (8 mm) were bored using a sterile cork borer. A 100 µL volume of each extract was introduced into the wells. Chlorhexidine (CHX) of 0.12 % was used as a positive control, while a 10% DMSO was used as a negative control. The extracts were allowed to pre-diffuse at room temperature for 1 hour before the agar plates were incubated at 37 °C for 24 hours. The antibacterial activity was expressed as the average diameter of a clear zone to the nearest millimeter. The tests were performed in triplicate and repeated twice to reduce the possibility of a test error.

Determination of minimum inhibitory concentration (MIC)

Resazurin microtiter-plate assay was used to determine the MIC. The assay was performed according to the procedure described by Gahlaut and Chhillar [13] with some modifications. Under aseptic conditions, a flat bottom sterile 96-well polystyrene tissue culture plate was labeled, and each well was filled with 100 µL of BHI broth. Each extract (100 µL) was then added to the first well, and two-fold serial dilutions were performed so that each well had 100 µL of test extracts in serially descending concentrations. Then, the standardized bacterial culture suspension (50 µL) was added to each well. The final volume in each well was 150 µL. The following controls were prepared: wells containing broth only (sterility control), bacterial strain with no extract (inoculum viability), serial dilution of CHX (positive control), and DMSO (negative control). The plates were incubated at 37 °C overnight. A 30 µL resazurin solution was added to each well, and the plates were incubated for another one to two hours. The MIC was determined as the lowest sample concentration at which no pink color (signifying live growth) appeared.

Antibiofilm activity: Preparation of biofilm development

Preliminary work has been done to determine the optimum growth condition applied to the biofilm formation assay. This work was adopted according to the protocol designed by Stepanović et al. [14]. The standardized bacterial suspension (~1 x 10^8 CFU/mL) was vortexed, 50 µL of the cell solution was then diluted 1:100 by adding to 4950 µL of desired medium [BHI,

BHI+ 1% (w/v) glucose, BHI+ 1% (w/v) sucrose]. These diluted bacteria (~1 x 10^6 CFU/mL) were then vortexed for 30 s to 1 min and transferred to a flat bottomed 96-well polystyrene tissue culture treated plate (200 µL per well). The plates were prepared in triplicates and placed in an incubator shaker at 37 °C for 24 hours. After incubation, the attached biofilm was quantified by measuring the absorbance of the solution at 570 nm [15].

Determination of minimum biofilm inhibitory concentration

The crystal violet (CV) assay was used to investigate the effect of *U. tomentosa* extracts on biofilm formation by adopting the protocol by Stepanović et al. [14]. Briefly,100 µL of fresh BHI supplemented with 1% (w/v) sucrose was distributed into all the wells of the flat bottom 96-well tissue culture plate. In sequence, 100 µL of extracts in a concentration used in the antibacterial activity (MIC) were added to the wells. Serial dilution was performed and followed by adding 100 µL bacterial suspension (1 x 10^6 CFU/mL). The plates were then incubated at 37 °C for 24 hours. After 24 hours of incubation time, the supernatant was discarded. Then, the plates were gently washed thrice with phosphate buffer saline (PBS) solution to remove the remaining planktonic cells, followed by air drying for 30 minutes. The adherent biofilm in the plates was fixed with methanol for 20 minutes, the solution was then removed, and the plates were air-dried. The attached bacterial cells were then stained with 150 µL of 2% crystal violet and left at room temperature for 15 minutes. Following staining, the stains were discarded and rinsed off by placing the plates under running tap water. The plates were blotted on paper towels and air-dried at room temperature. The bound dye was extracted from the stained cells using 150 µL of 95 % EtOH and left at room temperature for 30 minutes. The content's optical density (OD) was measured at 570 nm using a microtiter plate reader. The intensity of staining is directly proportional to the number of biofilms adhering to the 96-well plate. The percentage biofilm inhibition was calculated by the following formula, according to:

$$\%\ inhibition = \frac{OD\ (mean)^{control} - OD\ (mean)^{test\ sample}}{OD\ (mean)^{control}} \times 100\% \quad (1)$$

Where OD control is the mean OD measured for 24 hours bacterial biofilm grown without the extracts, while ODtest samples is the mean OD measured for 24 hours bacterial biofilm grown in the presence of extracts. The minimum biofilm inhibitory concentration ($MBIC_{50}$) was defined as the lowest concentration of the tested extracts required to prevent the formation of ≥50% of the biofilm [16].

Phytochemical work: General procedures

Column chromatography was conducted using silica gel 60, 70-230 mesh ASTM (Merk 7734 packed by slurry packing method. Radial chromatography was carried out using a glass plate with Merck's silica gel (Kieselgel 60 PF254 Merck Art 7749). Preparative TLC was performed using glass-supported silica gel 60 F254 (1.0 mm thickness). TLC was used to monitor the eluates was performed using pre-coated aluminium-backed supported silica gel 60 F254 (0.2 mm thickness). Identification of the spots on the TLC plate was carried out by spraying Dragendorff's reagent for alkaloid and P-anisaldehyde-sulfuric acid for terpenes, sterols, and steroids. Spots and bands for compounds on TLC and PTLC were viewed individually under UV lights (254 and 365 nm). The mass spectrum was recorded with an Agilent1100 series LC/MS ion trap. The infrared (IR) data was obtained on a Perkin Elmer model FT-IR spectrometer as a KBr disc. ^1H and ^{13}C NMR spectra were recorded with a Bruker 300 Ultra shield NMR spectrometer measured at 300 and 75 MHz, respectively.

Isolation and elucidation of compounds

The DCM extract was subjected to an acid-base extraction yielded an alkaloid mixture (3g) and a non-alkaloid mixture (18g). The alkaloid and non-alkaloid mixture was then subjected to column chromatography over silica gel, eluted with Hx:DCM (1:0-0:1) and subsequently DCM:MeOH (1:0-0:1) yielding 30 and 9 fractions, respectively. Based on their TLC profiles, the fractions from the alkaloid mixture were combined together into 9 major subfractions (F1-F9). Subfraction

F7 (40 mg) was subjected to radial chromatography using DCM and EtOAc in an increasing polarity manner to give compounds **1** (20 mg) and **2** (15 mg). Column chromatography on the targeted fraction of non-alkaloid mixture using solvent system Hx:DCM (9.5:0.5) successfully yielded compound **3** (9.4 mg). Another targeted fraction of the mixture using preparative TLC on silica gel using solvent system DCM: EtOAc (8.0:2.0) afforded 2.7 mg of compound **4**. The structure of isolated compounds was elucidated as isopteropodine (**1**), pteropodine (**2**), β-sitosterol (**3**), and scopoletin (**4**) based on spectroscopic data analysis as well as by comparing with the literature values.

Statistical analysis

All the experiments were run in triplicates and were repeated at least twice. The values obtained were analyzed using IBM SPSS Statistics v.22. The replicate absorbance readings were calculated as the mean of individual experiments in triplicate. The significant difference between the means of the test samples and the control group was performed using a Bonferroni post-test. A difference was considered significant when p values ≤ 0.05.

Result and Discussion

Antibacterial activity

The antibacterial activity possessed by extracts of increasing polarity against five oral bacteria is reported in Table 1. From the results obtained, the antibacterial activity of the extracts was greatly affected by solvent polarity. Out of the three extracts, the DCM extract exerted the most pronounced activity. This extract exhibited an inhibitory effect against all tested bacterial strains (*S. mutans*, *S. mitis*, *S. salivarius*, *S. anginosus*, and *S. uberis*). Meanwhile, Hx and 80% EtOH extracts were effective against only two strains (*S. salivarius* and *S. uberis*). The results obtained from this study indicate that the extracts showed varying degrees of antibacterial activity as the extracts were prepared by successive extraction of different polarities. Separating the constituents into nonpolar (Hx), medium polar (DCM), and polar fractions (80% EtOH) is expected to concentrate each extract's antibacterial constituents and enhance their inhibitory ability. The screening test using the agar well-diffusion assay was not a definitive method in detecting the inhibition of the test bacterial because many of the antimicrobial compounds in plant extracts are relatively nonpolar, and these compounds do not diffuse well into the surrounding agar around the wells [17]. This could be the reason for the negative activity shown by the Hx extract.

The test samples were then subjected to the microplate dilution method with resazurin as an indicator to determine the extracts' minimum inhibitory concentration (MIC) because it is more sensitive and provides reproducible results. The results are shown in Table 2. The blue color or the color before pink in the microtitre plates after adding the resazurin dye indicator was determined as the lowest concentration that completely inhibited the microorganism's growth. The MIC values obtained in this study from the extracts tested ranged from 234.4 µg/mL to 937.5 µg/mL. The antimicrobial potential of plant extracts is classified as follows: MIC < 100 µg/mL – strongly active; MIC 100-500 µg/mL – moderately active; MIC 500-1000 µg/mL – weakly active and MIC > 1000 µg/mL – inactive [18]. Based on the criteria given, generally, the extracts of this plant had moderate to low antibacterial activity against the bacteria tested. The strongest (MIC = 234.4 µg/mL) and the weakest (MIC = 937.5 µg/mL) antibacterial activity was shown by the DCM against *S. mutans*, and *S. anginosus*, respectively. In addition, the Hx and DCM extracts showed similar growth inhibitory activity against *S. salivarius*, *S. mitis* and *S. uberis* at a MIC value of 468.8 µg/mL. Meanwhile, no antibacterial activity was observed with the Hx extract against *S. mutans* and *S. anginosus*, and none by the 80% EtOH extract against either bacterial tested.

The difference in the MIC values obtained suggests a varying degree of susceptibility of the microorganisms, as was determined by comparing the MIC values of the test extracts against each bacterial tested. The lower the MIC value, the more susceptible the bacteria tested to the antimicrobial agent and vice-versa. Of the five bacterial tested, *S. mutans* was the most susceptible, as indicated by the lowest MIC values obtained from the DCM extracts (234 µg/mL). The trend in susceptibility to the extracts was *S. mutans* > *S. salivarius*, *S. mitis*, *S. uberis* > *S. anginosus*. In contrast, *S. anginosus* was

found to be less sensitive as only inhibited by the DCM extract. The activity of the extract tested can be ranked as DCM > Hx > EtOH 80% extracts. These results indicated considerable variation in antibacterial activity between extracts obtained with the different polarities of extracting solvents.

Table 1 Inhibition zone diameter of stem extracts against oral bacteria

Extract Type	**Conc. (mg/mL)**	**Zone of Inhibition (mm)**				
		S. mutans	**S. mitis**	**S. salivarius**	**S. uberis**	**S. anginosus**
Hx	50	0	0	12.0±0.0	11.0±0.0	0
	35	0	0	12.0±0.0	10.7±0.6	0
	15	0	0	12.0±0.0	10.3±0.6	0
DCM	50	12.0±1.0	11.0±0.0	15.0±0.0	16.0±1.0	11.3±0.6
	35	11.3±0.6	10.7±0.6	14.3±0.6	13.0±1.0	11.0±0.0
	15	11.0±0.0	10.3±0.6	14.0±1.0	12.3±0.6	10.7±0.6
80% EtOH	50	0	0	15.0±0.0	14.7±0.6	0
	35	0	0	15.0±0.0	14.3±0.6	0
	15	0	0	13.5±0.6	14.0±1.0	0
DMSO	0	0	0	0	0	0
CHX	29.0±1.0	27.0±1.0	29.7±0.6	27.7±0.6	25.3±0.6	29.0±1.0

Table 2. Minimum inhibitory concentration (MIC) of extracts against oral bacteria

Extract Type	**Minimum Inhibitory Concentration (µg/mL)**				
	S. mutans	**S. mitis**	**S. salivarius**	**S. uberis**	**S. anginosus**
Hx	-	468.8	468.8	468.8	-
DCM	234.4	468.8	468.8	468.8	937.5
80% EtOH	-	-	-	-	-

Different solvent extracts showed a different activity level, with DCM extract showing the most pronounced activity against all tested bacteria. It seems that extracts with an intermediate polarity had a much higher antibacterial activity than more polar (80% EtOH) or nonpolar (Hx) extracts. This agrees with the study by Eloff et al. reported that *Melianthus comosus* extract from the intermediate polarity solvent (acetone) had the best activity against fungal pathogens [19]. This has been attributed to more polar compounds being either less or completely inactive against the bacteria tested. It is worth notifying that DMSO (negative control) used as diluents did not affect the bacteria tested. Reports are available on the antibacterial activity of different concentrations of *U. tomentosa* extracts against several microorganisms [20-22]. But a few studies have

investigated the antibacterial activity of *U. tomentosa* against microorganisms frequently found in the human oral cavity. Ccahuana-Vasquez and co-workers employed MIC assay to determine the antimicrobial activity of 0.25% to 5% of micropulverized *U. tomentosa* extract on different strains of microorganisms isolated from the human oral cavity [23]. The authors reported that concentrations of 3% *U. tomentosa* inhibited 52 % of the *S. mutans* strain, which agrees with our findings. On the other hand, different authors reported 2% CC (cat's claw) gel prepared from freeze-dried extract inhibited *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans* which are microorganisms commonly found in infected root-filled teeth [24].

Antibiofilm activity

The effects of adding alternative carbohydrates, glucose, and sucrose, in the BHI media frequently used to grow oral biofilm were examined to determine the best medium for the subsequent biofilm assays. For this purpose, three different culture media, BHI, BHI + 1% glucose, and BHI + 1% sucrose, was employed in a static biofilm setup to evaluate the best-suited medium for oral biofilm development under in vitro conditions. Biofilm formation of the oral *Streptococci* was assayed and categorized based on the A575 of the crystal violet-stained biofilm: OD570 >2.0, a good biofilm former; OD570 = 1.0 to 2.0, moderate biofilm former and OD570< 1.0, poor biofilm former [15].

As shown in Figure 1, when culture media supplemented with or without glucose were used, the biofilm of the bacteria tested grew poorly (OD570 < 1.0). The results point to the optimal media for simultaneous growth of at least three bacteria to be BHI with 1% sucrose, hence was used for all subsequent experiments. This result is in line with previous findings where sucrose is considered the most cariogenic carbohydrate not only it is being fermented by oral bacteria, but is also a substrate for the synthesis of extracellular polysaccharides which will then promote changes in the composition of the biofilm's matrix [25]. Out of the five bacteria tested, only *S. mutans* was found to be a good biofilm former. Thus, this bacterium was then subjected to a biofilm inhibition assay. The three remaining bacteria, which were found to be poor biofilm formers, were all of the following strains: *S. uberis*, *S. mitis*, and *S. anginosus*.

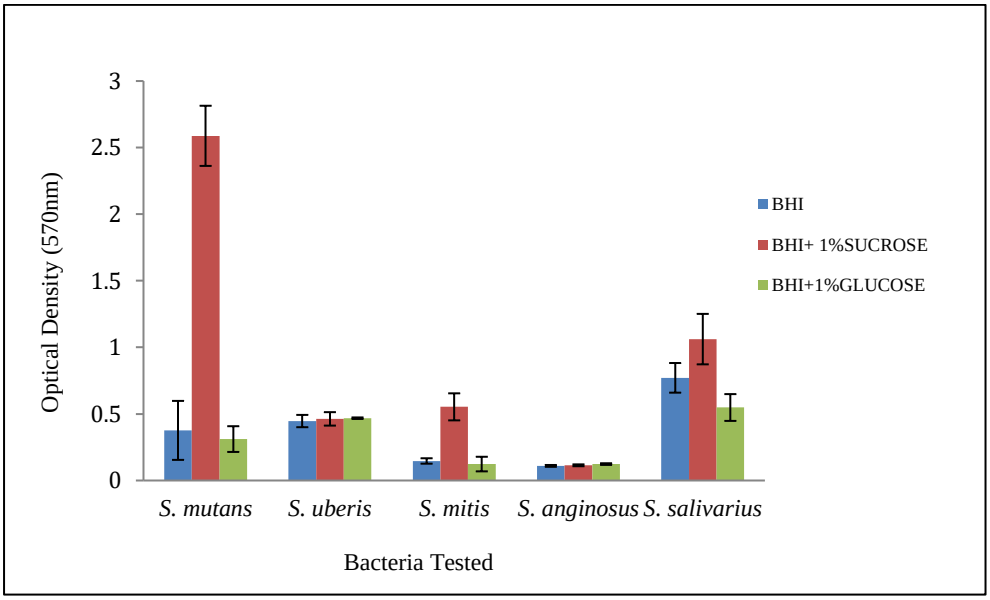

Figure 1. Results from Growth Optimization Assay. Blue, red, and green bars represent bacteria grown in different growth conditions. All assays were performed triplicate, and the data represent the average of 3 or more wells with standard deviations denoted by the error bars

Due to the highest antibacterial activity recorded by the DCM extract, it was further subjected to antibiofilm activity produced by *S. mutans*. As shown in Figure 2, *U. tomentosa* DCM extract showed an increasing ability to inhibit the biofilm by increasing its concentrations evidenced by the reduced absorbance values compared to the control biofilm (no extracts). Inhibition was expressed as percentages, with the extracts exhibiting good antibiofilm activity ≥ 50% inhibition. In this study, the lowest $MBIC_{50}$ reported was at a concentration of 58.6 µg/mL, indicating moderate antibiofilm activity. Higher MBIC (93.21% and 95.36%) was observed for the extract at the higher tested concentrations of 234.4 µg/mL and 468.8 µg/mL, respectively. Perusal the literature value, there are several ways in which natural products execute their antibiofilm activity which include preventing the formation of the polymer matrix, suppressing cell adhesion, interrupting extracellular matrix (ECM) generation, and reducing the production of virulence factors. This, in turn, blocks the quorum sensing (QS) network and hinders biofilm development [26]. The activities here could be contributed by some moderate polarity compounds that work synergically or singularly in the extract. The stem of the plant has been reported to contain a moderate polarity of compounds including coumarins, terpenes, flavonoids and alkaloids that may be responsible for the *U. tomentosa's* medicinal effects [9]. While these compounds may be responsible for the antibacterial activities reported in this study, only flavonoids and an alkaloid had been reported to possess good antibacterial activity [27,28]. In order to have more insight on the possible chemical constituents of the active extract, the DCM extract was subjected to phytochemical study.

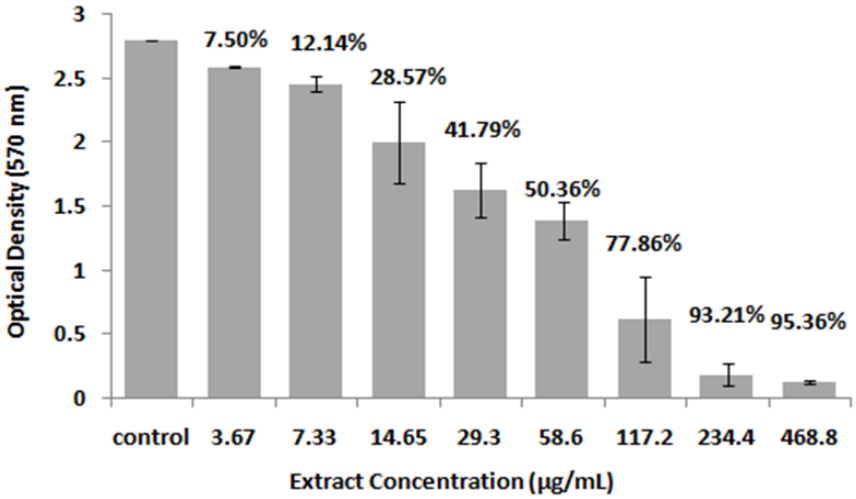

Figure 2. Inhibitory activity towards *S. mutans* biofilm formation by DCM extract. Percent biofilm formation inhibition, shown at the top of each column, was calculated by comparing the absorbance values of test samples with the absorbance of the control biofilm

Phytochemical work

Repeated chromatographic techniques and purification on the DCM extract of the stems of *U. tomentosa* afforded four compounds where two from the alkaloidal [isopteropodine (**1**) and pteropodine (**2**)] and two from the non-alkaloidal [β-sitosterol (**3**) and scopoletin (**4**)]. The structures were determined by spectroscopy particularly extensive NMR analyses as well as by comparison of their spectral data with the literature data [29-32]. The significant spectroscopic characteristics of the compounds are as follows, while their structures are shown in Figure 3.

Isopteropodine (**1**): ESIMS m/z 368 $[M+H]^+$ for $C_{21}H_{24}N_2O_4$; IR (KBR) υ_{max}: 3368 (NH), 1718 (ester C=O), 1657 (amide C=O) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ ppm: 8.30 (1H, *s*, N-H), 7.43 (1H, *s*, H-17), 7.29 (1H, *d*, *J* = 7.5 Hz, H-9), 7.20 (1H, *ddd*, *J* = 7.5, 1.2 Hz,

H-11), 7.03 (1H, *ddd*, *J* = 7.5, 1.2 Hz, H-10), 6.89 (1H, *d*, *J* = 7.5 Hz, H-12), 4.36 (1H, *m*, H-19), 3.60 (3H, *s*, OCH_3), 3.33 – 3.20 (2H, *m*, H-21β, H-5β), 2.60 – 2.30 (5H, *m*, H-15, H-21α, H-6β, H-5α, H-3), 2.00 (1H, *m*, H-6α), 1.60 (2H, *m*, H-14α, H-20), 1.43 (3H, *d*, *J* = 6 Hz, H_3-18), 0.89 (1H, *m*, H-14β); ^{13}C NMR ($CDCl_3$, 75 MHz) δ ppm: 181.25 (N-C=O), 167.64 (O-C=O), 155.00 (C-17), 140.96 (C-13), 133.69 (C-8), 127.58 (C-11), 124.07 (C-9), 122.04 (C-10), 109.85 (C-16), 109.43 (C-12), 72.16 (C-19), 71.34 (C-3), 57.09 (C-7), 54.15 (C-5), 53.53 (C-21), 50.99 (OCH_3), 37.90 (C-20), 34.86 (C-6), 30.49 (C-15), 30.19 (C-14), 18.65 (CH_3).

Pteropodine (**2**): ESIMS m/z 368 $[M+H]^+$ for $C_{21}H_{24}N_2O_4$; IR (KBR) υ_{max}: 3206 (NH), 1721 (ester C=O), 1629 (amide C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm: 8.66 (1H, *s*, N-H), 7.49 (1H, *s*, H-17), 7.22 (1H, *d*, *J* = 7.5 Hz, H-9), 7.19 (1H, *ddd*, *J* = 7.5, 1.2 Hz, H-11), 7.07 (1H, *ddd*, *J* = 7.5, 1.2 Hz, H-10), 6.83 (1H, *d*, *J* = 7.5 Hz, H-12), 4.57 (1H, *m*, H-19), 3.63 (3H, *s*, OCH_3), 3.37 – 3.19 (2H, *m*, H-21β, H-5β), 2.50 – 2.31(5H, *m*, H-15, H-21α, H-6β, H-5α, H-3), 2.03 (1H, *m*, H-6α), 1.74 (1H, *m*, H-14α), 1.61 (1H, *m*, H-20), 1.51 (1H, *m*, H-14β), 1.43 (3H, *d*, *J* = 6 Hz, H_3-18); ^{13}C NMR ($CDCl_3$, 75 MHz) δ ppm: 181.0 (N-C=O), 167.77 (O-C=O), 155.29 (C-17), 140.48 (C-13), 133.41 (C-8), 127.98 (C-11), 123.27 (C-9), 122.74 (C-10), 109.25 (C-12), 109.22 (C-16), 74.42 (C-3), 72.23 (C-19), 56.08 (C-7), 55.17 (C-5), 53.63 (C-21), 50.93 (OCH_3), 37.88 (C-20), 34.59 (C-6), 30.98 (C-15), 29.59 (C-14), 19.0 (CH_3).

β-sitosterol (**3**): ESIMS m/z 414 $[M+H]^+$ for $C_{29}H_{50}O$; IR (KBR) υ_{max}: 3381 (OH), 2934 - 2833 (aliphatic C-H), 1668 (C=C), 1450 (H-C-H) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm: 5.37 (1H, *t*, *J* = 5.4 Hz, H-6), 3.54 (1H, *m*, H-3), 2.24 (1H, *m*, H-4), 2.00 (2H, *m*, H-7, H-4), 1.83 (1H, *m*, H-25), 1.75 – 1.60 (4H, *m*, H-7, H-20, H-15, H-16), 1.55 – 1.45 (6H, *m*, H-2, H-12, H-11, H-28, H-24, H-17), 1.44 – 1.40 (2H, *m*, H-9, H14), 1.35 – 1.30 (5H, *m*, H-1, H-15, H-16, H-12, H-2), 1.25 – 1.00 (5H, *m*, H-11, H-22, H-23, H-1, H-8), 1.02 (3H, *s*, H_3-19), 0.93 (3H, *d*, *J* = 6.0 Hz, H3-21), 0.94 (3H, *t*, *J* = 6.6 Hz, H_3-29), 0.88 (3H, *d*, *J* = 6.3 Hz, H_3-27), 0.85 (3H, *d*, *J* = 6.3 Hz, H_3-26), 0.75 (3H, *s*, H_3-18); ^{13}C NMR (CDCl3, 75 MHz) δ ppm: 140.76 (C-5), 121.73 (C-6), 71.80 (C-3), 56.76 (C-14), 56.04 (C-17), 50.12 (C-9), 45.82 (C-24), 42.31 (C-13), 42.27 (C-4), 39.76 (C-12), 37.24 (C-1), 36.49 (C-10), 36.14 (C-20), 33.93 (C-22), 31.89 (C-7), 31.89 (C-8), 31.64 (C-2), 29.13 (C-25), 28.24 (C-16), 26.05 (C-23), 24.30 (C-15), 23.05 (C-28), 21.07 (C-11), 19.82 (C-26), 19.39 (C-19), 19.02 (C-27), 18.77 (C-21), 11.98 (C-29), 11.85 (C-18).

Scopoletin (**4**): ESIMS m/z 192 $[M+H]^+$ for $C_{10}H_8O_4$; IR (KBR) υ_{max}: 3350 (OH), 1670 (C=O), 1629 (amide C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm: 7.86 (1H, *d*, *J* = 9.3 Hz, H-4), 7.12 (1H, *s*, H-5), 6.79 (1H, *s*, H-8), 6.22 (1H, *d*, *J* = 9.3 Hz, H-3), 6.20 (1H, *br s*, OH), 3.92 (3H, *s*, OCH_3); ^{13}C NMR (CDCl3, 75 MHz) δ ppm: 161.46 (C=O), 150.25 (C-6), 149.70 (C-9), 144.01 (C-7), 143.30 (C-4), 113.40 (C-3), 111.49 (C-10), 107.49 (C-5), 103.19 (C-8), 56.41 (OCH_3).

As has been mentioned previously, these alkaloids (**1** and **2**), coumarin **4**, and terpene **3** have been reported to exhibit antibacterial activity against different bacterial strains [9,27,28]. Thus, these compounds are presumably responsible for the antibacterial and antibiofilm activities of the DCM extract in the present study. This is the first report of antibiofilm activity of *U. tomentosa* extracts to the best of our knowledge. The findings provide evidence to support the usage of *U. tomentosa* in microbial infections and could be the source of antibacterial and antibiofilm agents for further studies.

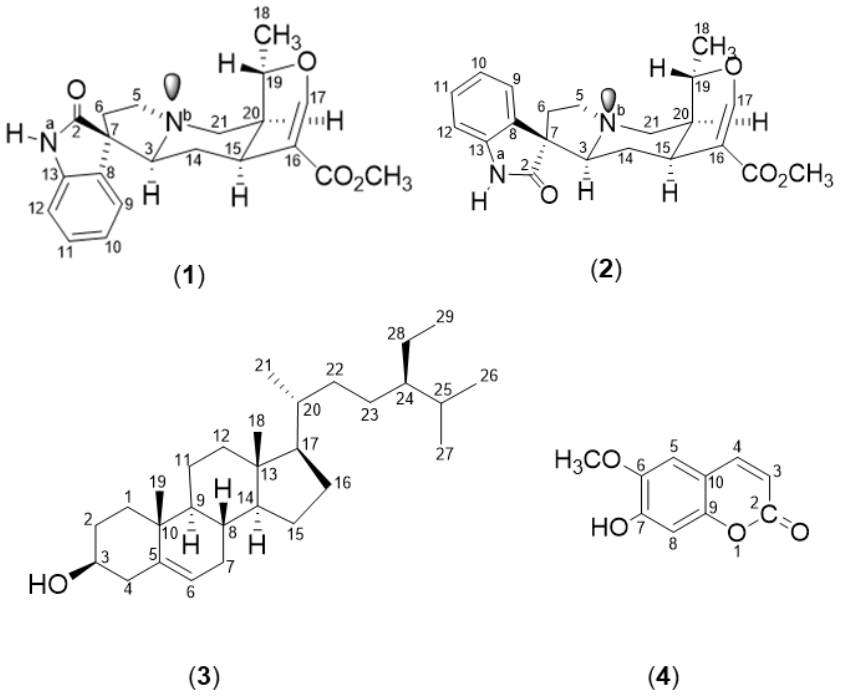

Figure 3. Structure of the isolated compounds: (**1**) pteropodine, (**2**) isopteropodine, (**3**) β-sitosterol and (**4**) scopoletin

Conclusion

The present data suggest that Malaysian *U. tomentosa* extracts exhibited antibacterial and antibiofilm activities against oral bacteria. The isolated phytochemicals namely isopteropodine, pteropodine, and β-sitosterol, scopoletin, are presumably responsible synergically and singularly for the activities of the active DCM extract. However, further research is needed to evaluate these compounds to ensure their contribution. The findings provide evidence to support the plant's traditional use and could be the source of antibacterial and antibiofilm agents for further studies.

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