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Antibiofilm Activity of Chemical Constituents from the Roots of *Lindera subumbelliflora* Kosterm (Lauraceae)

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The genus *Lindera*, part of the Lauraceae family, is found throughout Asia and Midwestern America and has been used in traditional medicine to treat various health issues, including pain, cold, and gastrointestinal and urinary conditions. This study focusses on analysing the phytochemicals from *Lindera subumbellifora* root extracts and assessing their antibiofilm activity. The phytochemicals were obtained using column chromatography techniques and characterised by spectroscopy methods (IR, MS, and NMR) and comparison with literature. Antibiofilm activity was determined using a semiquantitative static biofilm assay. The compounds were extracted by cold extraction with solvents (*n*-hexane, ethyl acetate, methanol) and isolated by column chromatography. Spectroscopy (IR, MS, NMR) identified eight compounds, including 5,6-dehydrokawain (**1**), pinostrobin (**2**), β-sitosterol (**3**), pinocembrin (**4**), ferulic acid (**5**), quercetin (**6**), syringic acid (**7**) and apocynin (**8**). The methanolic root extract showed strong inhibition (99.2%) against *Streptococcus mutans*, and quercetin demonstrated significant inhibitory effects against *Streptococcus mutans* (I: 98.4%) and *Candida albicans* (I: 45.7%). Molecular docking studies further examined the binding interactions of these compounds with lanosterol 14α-demethylase (Erg11), supporting their potential antifungal benefits. These findings highlight the effectiveness of *Lindera* extracts in preventing oral biofilm formation and suggest the potential for therapeutic applications in drug development.

Keywords: *Lindera subumbelliflora*; Lauraceae; phytochemical; antibioflm; *Streptococcus mutans*

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Biofilms are an ordered and arranged group of microorganisms living within an extracellular polymeric substance (EPS) matrix produced by them. This matrix enables microorganisms to adhere to both living and non-living surfaces, forming robust communities [1]. Biofilms can develop on various surfaces, including medical devices, natural water bodies, and within the human body. Microorganisms within a biofilm exhibit cooperative behaviours, such as nutrient sharing and enhanced resistance to environmental stressors. Antibiofilm refers to strategies, agents, or treatments designed to prevent the formation of biofilms or to disrupt and eradicate existing biofilms. These strategies aim to inhibit the initial adhesion of microorganisms to surfaces, thus preventing biofilm establishment, or disrupt the structure of biofilms [2]. Preventive measures include surface coatings and antibiofilm agents that reduce microbial attachment. By targeting the formation and persistence of biofilms, antibiofilm approaches improve the effectiveness of treatments and improve the results in combating biofilm-related issues [3]. In recent years, the search for natural compounds with antibiofilm properties has gained momentum.

The *Lindera* genus is a member of the Lauraceae family, which consists of approximately 100 species that are widely distributed in tropical and subtropical areas throughout the world. Lindera are evergreen or deciduous trees or shrubs. Most Lindera plants, particularly *L. aggregata*, are a well-known traditional Chinese medicine that has important medicinal value and health benefits [4]. A review of the literature showed that several species of the genus *Lindera* have been investigated for their chemical compounds and biological activities. Previous phytochemical investigations on *Lindera* species led to the isolation of sesquiterpenoids (dimeric and trimeric), alkaloids, flavonoids, butanolides,

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lucidones, and phenylpropanoids. Despite the fact that *Lindera* plants have a wide range of pharmacological and biological qualities, numerous studies have focussed on its analgesic, anti-inflammatory, anticancer, and antihypertensive effects [4].

The genus *Lindera* is a lesser known member of this family but has traditionally been valued in various cultures for its medicinal benefits. One of the most common members of the Lauraceae family is cinnamon essential oil, which has already been proven to possess a wide spectrum of biological activities, including the antibiofilm effect [5]. Furthermore, cinnamon bark has shown potential as a promising agent to treat oral infections involving *Candidia albicans* with the ability to attenuate the growth of biofilm formation and the adherence properties of *Candidia albicans* with minimum inhibitory concentrations and minimum fungicidal concentrations in the range of 0.039 to 0.078% [6]. Meanwhile, the essential oil of the genus *Lindera* also promises antibiofilm properties. The fruit oil of *L. communis* exhibited antifungal and antibiofilm properties as the main components are bis(2-hydroxyethyl)lauramide and *n*-carpic acid [7]. Additionally, *L. neesiana* fruit oil exhibited antimicrobial activity against *Staphylococcus aureus* and *Candida albicans* as components have been characterized, which are (*Z*)-citral, (*E*)-citral, eucalyptol, citronellal, α-pinene and β-pinene which attributes to the discovery of lead compounds to develop antibacterial drugs [8]. Therefore, essential oils and extracts from *Lindera* species have traditionally been used for their therapeutic effects, suggesting their potential utility in modern medical applications, including antibiofilm strategies against oral pathogens.

Lindera subumbelliflora Kosterm (syn. *Litsea subumbelliflora* (Blume) Ng) is a shrub or small tree up to 5 m high. It is distributed in hill forests up to 900 m asl and is found mainly in Malaysia [9]. Recently, we have reported the chemical components of leaf oil of *L. subumbelliflora* [10]. The analysis of essential oil revealed the presence of 28 components, accounting for 99.6% of the total oil. The main components of essential oil were β-eudesmol (14.6%), *cis*-α-bergamotene (11.0%), α-copaene (8.5%), dodecen-1-ol (8.5%), and (*E*)-nerolidol (8.3%). The essential oil exhibited activity against *Candida albicans* and *Streptococcus mutans* with MIC values of 250 and 500 µg/mL, respectively. The essential oil increased the biofilm of *Candida albicans* by 38.25%, however, decreased the biofilm of *Streptococcus mutans* by 47.89% when treated with 500 µg/mL.

As a continuation of our study, the primary objective of this research was to evaluate the antibiofilm efficacy of *L. subumbelliflora* extracts and isolated compounds against *Candida albicans* and *Streptococcus mutans.*

EXPERIMENTAL

Plant Material

The roots of *L. subumbelliflora* were collected from Fraser Hill, Pahang, in January 2023, and identified by Shamsul Khamis from the University of Kebangsaan Malaysia (UKM). The voucher specimen (SK156) was deposited at UKMB Herbarium, Faculty of Science and Technology, UKM

General Experimental Procedures

A cold extraction technique was applied to extract phytochemicals from the dried roots using different polarity solvents (*n*-hexane, ethyl acetate, and methanol). All reagents used (*n*-hexane, ethyl acetate, dichloromethane, chloroform, diethyl ether, and methanol) were of analytical grade (Merck). Column chromatography (CC) was performed using Merck silica gel 60 (70-230 mesh) as the stationary phase. Thin layer chromatography (TLC) analysis was performed on Merck precoated silica (SiO_2) gel F_{254} plates (0.22 mm thickness) to detect and monitor the presence of compound samples. The spots were visualized under UV light (254 and 365 nm) and included with spraying reagent vanillin sulphuric acid in MeOH followed by heating. Melting points were measured by comparing them with other literature. The ^{1}H NMR (500 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Avance 500 Spectrometer. Chemical shifts were reported in ppm and $CDCl_3$ as solvent. The residual solvent was used as an internal standard. The IR spectra were recorded on the Perkin Elmer ETR and 1600 spectrophotometer series as KBr discs or thin film of NaCl discs. Mass spectral data were obtained from Orbitrap Exploris 240 Mass Spectrometer.

Extraction and Isolation

The dried roots of *L. subumbelliflora* (500 g) were ground into powder and sequentially extracted with *n*-hexane, ethyl acetate and methanol sequentially by cold extraction. The extract was concentrated using rotary evaporation to afford the crude extracts. The *n*-hexane extract (LSRH, 8.1 g) was fractionated by VLC and eluted with *n*-hexane: DCM: EtOAc to afford 7 fractions (LSRH A-G). The LSRH-A was purified by CC and eluted with *n*-hexane: DCM to afford compound (**1**) (10.0 mg) and compound (**2**) (12.6 mg). The combined fraction LSRH-E was purified followed by PTLC to obtain compound (**3**) (5.5 mg) and compound (**4**) (8.7 mg). Purification of the EtOAc extract (LSRE, 11.3 g) by CC eluted with *n*-hexane: EtOAc: MeOH yielded 5 fractions (LSRE A-E). The LSRE-E and LSRE-B was by PTLC and achieved compound (**5**) (10.9 mg) and compound (**7**) (13.9 mg), respectively. Purification of the MeOH extract (LSRM, 10.3 g) by CC eluted with *n*-hexane: EtOAc: MeOH yielded 5 fractions (LSRM A-E). The combined fraction LSRM C was purified by PTLC with CHCl3:

MeOH to obtain compound (**6**) (7.6 mg). The combined fraction LSRM D-E was purified and washed with Et_2O to produce compound (**8**) (15.1 mg).

5,6-Dehydrokawain (**1**). Pale yellow solid (10.0mg); The R_f value of 0.55 was obtained using *n*-hexane:EtOAc (7:3). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.85 (3H, s, OCH_3), 5.52 (1H, d, *J* = 2.1 Hz, H-3), 5.97 (1H, d, *J* = 2.1 Hz, H-5), 6.59 (1H, d, *J* = 16.0 Hz, H-7), 7.36-7.51 (5H, m, H-10 – H-14), 7.53 (1H, d, *J* = 16.0 Hz, H-8); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 55.9 (OCH_3), 88.9 (C-3), 101.4 (C-5), 118.7 (C-7), 127.5 (C-10/C-14), 128.9 (C-11/C-13), 129.5 (C-12), 135.2 (C-9), 135.8 (C-8), 158.7 (C-4), 164.1 (C-6), 171.1 (C-2); EIMS: *m/z* 228 [M^+, $C_{14}H_{12}O_3$].

Pinostrobin (**2**). Colourless crystalline needles (12.6 mg); The R_f value of 0.48 was obtained using *n*-hexane:EtOAc (6:4). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.84 (1H, dd, *J* = 2.9 and 14.8 Hz, H-3a), 3.10 (1H, dd, *J* = 13.0 and 15.0 Hz, H-3b), 3.84 (3H, s, OCH_3), 5.44 (1H, dd, *J* = 2.9 and 13.0 Hz, H-2), 6.10 (1H, d, *J* = 2.3 Hz, H-6), 6.11 (1H, d, *J* = 2.3 Hz, H-8), 7.42-7.48 (5H, m, H-2′ – H-6′), 12.05 (1H, s, OH); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 43.4 (C-3a/C-3b), 55.7 (OCH_3), 79.3 (C-2), 94.3 (C-8), 95.2 (C-6), 103.2 (C-10), 126.2 (C-2′/C-6′), 128.9 (C-3′/C-4′/ C-5′), 138.4 (C-1′), 162.8 (C-9), 164.2 (C-5), 168.0 (C-7), 195.8 (C-4); EIMS: *m/z* 270 [M^+, $C_{16}H_{14}O_4$].

β-Sitosterol (**3**). White solid (5.5 mg); The R_f value of 0.85 was obtained using *n*-hexane:EtOAc (7:3). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.70 (3H, s, H-18), 0.83 (3H, d, *J* = 6.8 Hz, H-27), 0.85 (3H, d, *J* = 6.8 Hz, H-26), 0.86 (3H, d, *J* = 7.8 Hz, H-29), 0.94 (3H, d, *J* = 6.5 Hz, H-21), 1.02 (3H, s, H-19), 1.20-2.33 (29H, m, overlapping CH and CH_2), 3.55 (1H, m, H-3), 5.37 (1H, d, *J* = 5.2 Hz, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 11.8 (C-29), 11.9 (C-18), 18.7 (C-21), 19.0 (C-27), 19.3 (C-19), 19.7 (C-26), 21.0 (C-11), 23.1 (C-28), 24.3 (C-15), 26.1 (C-23), 28.2 (C-16), 29.2 (C-25), 31.6 (C-2), 31.9 (C-8/C-7), 33.9 (C-22), 36.1 (C-20), 36.5 (C-10), 37.5 (C-1), 39.8 (C-12), 42.3 (C-4/C-13), 45.8 (C-24), 50.1 (C-9), 56.0 (C-17), 56.7 (C-14), 71.8 (C-3), 121.7 (C-6), 140.7 (C-5); EIMS: *m/z* 414 [M^+, $C_{29}H_{50}O$].

Pinocembrin (**4**). White solid (8.7 mg); The R_f values that obtained is 0.36 with the ratio of *n*-hexane:EtOAc (7:3). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.74 (1H, dd, *J* = 3.0 and 17.2 Hz, H-3a), 3.0 (1H, dd, *J* = 13.0 and 17.2 Hz, H-3b), 5.35 (1H, dd, *J* = 3.0 and 13.0 Hz, H-2), 5.93 (1H, d, *J* = 2.2 Hz, H-6), 5.94 (1H, d, *J* = 2.2 Hz, H-8), 7.36-7.42 (5H, m, H-2′-H-6′), 11.97 (1H, s, 5-OH); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 43.4 (C-3a/C-3b), 79.3 (C-2), 95.5 (C-8), 96.8 (C-6), 103.3 (C-10), 126.2 (C-2′/C-6′), 128.9 (C-3′/C-4′/C-5′), 138.3 (C-1′), 163.1 (C-9), 164.4 (C-7), 164.5 (5-OH), 195.8 (C-4); EIMS: *m/z* 256 [M^+, $C_{15}H_{12}O_4$].

Ferulic acid (**5**). Brownish-yellow solid (10.9 mg); The R_f value of 0.27 was obtained using *n*-hexane:EtOAc (6:4). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.97 (3H, s, OCH_3), 6.31 (1H, d, *J* = 15.0 Hz, H-2′), 6.96 (1H, d, *J* = 8.9 Hz, H-5′), 7.08 (1H, d, *J* = 1.9 Hz, H-2′), 7.13 (1H, dd, *J* = 1.9 and 8.2 Hz, H-6′), 7.73 (1H, d, *J* = 15.0 Hz, H-3); ^{13}C NMR ($CDCl_3$, 500 MHz): δ 56.0 (OCH_3), 109.5 (C-6′), 114.2 (C-3′), 114.8 (C-2), 123.7 (C-2′), 126.6 (C-1′), 146.8 (C-3), 147.3 (C-5′), 148.5 (C-4′), 171.3 (C-1); EIMS: *m/z* 194 [M^+, $C_{10}H_{10}O_4$].

Quercetin (**6**). Yellow solid (7.6 mg); The R_f value of 0.44 was obtained using EtOAc:MeOH (9.5:0.5). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.26 (1H, d, *J* = 2.0 Hz, H-6), 6.51 (1H, d, *J* = 2.0 Hz, H-8), 6.98 (1H, d, *J* = 8.5 Hz, H-5′), 7.68 (1H, dd, *J* = 2.1 and 8.5 Hz, H-6′), 7.82 (1H, d, *J* = 2.1 Hz, H-2′), 12.16 (1H, s, 5-OH); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 93.6 (C-8), 98.3 (C-6), 103.2 (C-10), 114.9 (C-5′), 115.3 (C-2′), 120.6 (C-6′), 122.9 (C-1′), 135.9 (C-3), 145.0 (C-3′), 146.1 (C-2), 147.5 (C-4′), 156.9 (C-9), 161.4 (C-5), 164.2 (C-7), 175.7 (C-4); EIMS: *m/z* 302 [M^+, $C_{15}H_{10}O_7$].

Syringic acid (**7**). White crystalline solid (13.9 mg); The R_f value of 0.36 was obtained using EtOAc:MeOH (9:1). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.95 (6H, s, 3′/5′-OCH_3), 5.97 (1H, s, 4′-OH), 7.38 (2H, s, H-2′/H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 56.5 (3′/5′-OCH_3), 107.3 (C-2′/C-6′), 120.0 (C-1′), 140.0 (4′-OH), 146.7 (C-3′/C-5′), 170.7 (C-1); EIMS: *m/z* 198 [M^+, $C_9H_{10}O_5$].

Apocynin (**8**). White crystalline solid (15.1 mg); The R_f value of 0.58 was obtained using *n*-hexane:EtOAc (7.5:2.5). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.56 (3H, s, CH_3), 3.91 (3H, s, 3′-OCH_3), 6.31 (1H, s, 4′-OH), 6.95 (1H, d, *J* = 8.7 Hz, H′-5), 7.52 (1H, s, H-2′), 7.53 (1H, d, *J* = 8.7 Hz, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 26.2 (CH_3), 56.0 (3′-OCH_3), 110.1 (C-2′), 114.1 (C-5′), 124.2 (C-6′), 129.6 (C-1′), 146.5 (C-3′), 150.8 (4′-OH), 196.9 (C-1); EIMS: *m/z* 166 [M^+, $C_9H_{10}O_3$].

Antibiofilm Activity

The antibiofilm activity of crude extracts and isolated phytochemicals was investigated using a semiquantitative static biofilm assay [11]. Standardized cultures of *Candida albicans* and *Streptococcus mutans* (OD_{620nm}) were used in the biofilm procedure, with concentrations of 500 µg/mL for the sample and 3×10^4 cells/mL for 23 microbes. Negative controls contained only inoculated broth, while positive controls contained 0.12% chlorhexidine. The suspensions were mixed thoroughly using a vortex mixer for 30 sec and pipetted into each well of a sterile 96-well plate, which was then incubated for 72 h at 37°C aerobically, and the medium was replenished aseptically every 24 h. The experiment was performed in three biological replicates and three technical replicates to ensure

reproducibility. After incubation, the crystal violet (CV) assay was performed according to the previous protocol to quantify biofilm biomass [12]. Initially, the wells containing biofilms were washed twice with sterile PBS to remove the non-adherent cells. Later, the biofilms in the wells were fixed by adding 200 µL of MeOH and incubating for 15 min at 25°C. The supernatant was discarded, and the plate was air-dried for 45 min. Then 200 µL of 0.1% (w/v) CV solution was added to each well and incubated for 20 min at 25°C. The plate was washed gently twice using sterile distilled water to remove the unbound stain. Subsequently, the biofilms were detained with 200 µL of 33% (v/v) acetic acid for 5 min at room temperature. Finally, 100 µL of the acetic acid solution was transferred to a new sterile 96-well plate and the absorbance was measured at the 620 nm optical density (OD) wavelength (OD_{620nm}) using a microtiter plate reader. Each assay was performed three times and the mean absorbance values were used to measure the inhibition of biofilm formation as follows:

$$\frac{\text{Mean } OD_{620nm} \text{ of positive control} - \text{Mean } OD_{620nm} \text{ of experimental}}{\text{Mean } OD_{620nm} \text{ of positive control}} \times 100$$

Molecular Docking Studies

Molecular docking studies were performed to investigate the interactions between the isolated phytochemicals and the lanosterol 14α-demethylase enzyme (Erg11; PDB ID: 4ZE3). This enzyme is a crucial component of the fungal cytochrome P450 system, specifically from *Saccharomyces cerevisiae*, and is categorized under the CYP51 family (CYP51F1) [13]. Protein preparation involved the use of the UCSF Chimera, where steps such as the removal of water molecules, the addition of hydrogen atoms, and charge assignment were undertaken. The docking process was carried out using AutoDock Vina through the PyRx interface. For the docking site, we utilised the binding pocket where the native ligand fluconazole is typically located. Finally, the interactions and binding modes of the ligands were analysed and visualized using Discovery Studio.

RESULTS AND DISCUSSION

In this study, we successfully isolated eight compounds from the roots of *L. subumbelliflora*, which were characterised as dihydrochalcones, steroid, phenolics, and flavonol. They were all identified by analysing their spectroscopic data and comparing them with the reported literature. Their chemical structures are shown in Figure 1.

Compound (**1**) revealed the presence of a methoxy group at 4-OCH_3 were observed at δ 3.85 in ^{1}H NMR spectrum. Besides, two doublets observed at δ 6.59 (J = 16.0 Hz) and 7.53 (J = 16.0 Hz) were assigned to olefinic protons H-7 and H-8, respectively. The large coupling constant, J = 16.0 Hz suggested that these protons were in a *trans*-orientation. Another two sets of doublets that appeared at δ 5.52 and 5.97 (J = 2.1 Hz) were attributed to olefinic protons H-3 and H-5, pyran-2-one, respectively. The aromatic protons (H-10 to H-14) were observed as multiplet signals at δ 7.36-7.51. The ^{13}C NMR and DEPT spectra revealed the presence of fourteen carbons, consisting of one methoxy, nine methine, three quaternary carbons, and one carbonyl carbon.

The compound (**2**) supported the presence of hydroxyl group that was represented by a singlet signal at δ 12.05 in the ^{1}H NMR spectrum. Another singlet peak was observed at δ 3.84, assigned to a methoxy group (7-OCH_3). The meta-coupled signals appeared at δ 6.10 and 6.11 (J = 2.3 Hz), which were attributed to aromatic protons H-6 and H-8, respectively. In addition, three set doublet of doublet signal, each integrated for one proton were observed at δ 2.84 (J = 2.9 Hz and 14.8 Hz), 3.10 (J = 13.0 Hz and 15.0 Hz) and 5.44 (J = 2.9 Hz and 13.0 Hz,) were assigned for H-3a, H-3b and H-2, respectively. Furthermore, a multiplet signal resonated at δ 7.42-7.48 integrating for five protons of aromatic protons, H-2′-6′ of ring B. The carbon signal of the methoxy group (7-OCH_3) was clearly assigned at δ 55.7, while the carbonyl carbon was also observed at δ 195.8.

Compound (**3**) displayed two singlet signals at δ 0.70 (H-18) and 1.02 (H-19) in the ^{1}H NMR spectrum. Four doublets that resonated at δ 0.83, 0.85, 0.94, and 0.86 were attributed to four methyl groups of H-27, H-26, H-21, and H-29, respectively. Another doublet signals resonated at δ 5.37 (J = 5.2 Hz) and were attributed to H-6. The H-3 signal resonated as a multiplet at δ 3.55. The presence of 29 carbon atoms was confirmed by its ^{13}C NMR and DEPT spectra. Compound (**4**) was almost identical to compound (**2**) for its ^{1}H NMR spectrum. The only difference between both spectra was the absence of a methoxy signal in C-7 with additional proton OH in the ^{1}H NMR spectrum of compound (**2**). The ^{13}C NMR exhibited the presence of fifteen carbons, comprising one methylene, eight methine, five quaternary and one carbonyl carbon.

Compound (**5**) displayed a singlet signal δ 3.97 corresponding to a methoxy group in the ^{1}H NMR spectrum. The signals of aromatic protons were observed as doublets at δ 6.96 (J = 8.9 Hz, H-5′) and 7.08 (J = 1.9 Hz, H-2′) as well as doublet of the doublet signal at δ 7.13 (J = 1.9 and 8.2 Hz, H-6′). ^{13}C NMR showed the presence of 10 signals, consisting of 6 aromatic carbon and 4 aliphatic chains. Compound (**6**) showed a doublet signal at δ 6.26 (H-6) and δ 6.51 (H-8) in the ^{1}H NMR spectrum. The other three aromatic signals at δ 7.82 (d, J = 2.1 Hz), δ 7.68 (dd, J = 8.5 and 2.1 Hz), and 6.98 (d, J = 8.5 Hz) were assigned to protons H-2, H-6, and H-5', respectively. The ^{13}C NMR spectrum indicated the presence of 15 carbons, comprising of ten quaternary and 5 methine carbons.

Compound (**7**) supported the ^{1}H NMR spectrum by the presence of three singlet signals that appeared at δ 7.38 (H-2′ and H-6′) and δ 3.95 (3′/5′-OCH_3). Another singlet peak at δ 5.97 was assigned to a hydroxyl group. The carbon signal from the methoxy group was clearly assigned at δ 56.5, while carbonyl carbon was observed at δ 170.7 in the ^{13}C NMR spectrum. Compound (**8**) supported the presence of methoxy groups at 3.91 in the ^{1}H NMR spectrum. The ortho-coupled appeared as doublet signals at δ 7.53 and δ 6.95 with (J = 8.7 Hz) attributed to protons H-6′ and H-5′, respectively. In addition, three singlet signals were observed at δ 6.31, 2.56, and 7.52 and were complemented with hydroxyl, methyl groups, and H-2′, respectively. The ^{13}C NMR spectrum indicated the presence of nine carbons including one carbonyl, one methoxy, one methyl, three methine, and three quaternary carbon atoms.

To the best of our knowledge, all compounds were isolated from *L. subumbelliflora* for the first time. Compounds (**1**–**8**) have been previously reported from several *Lindera* species. Compounds (**1**), (**2**), and (**4**) have previously been isolated from *L. umbellata* [14], whereas compound (**5**) was isolated from *L. benzoin* [15]. Compounds (**3**), (**6**), and (**8**) were previously reported from *L. aggregata* [16], and compound (**7**) from *L. glauca* [17].

In this study, the antibiofilm activity of the root extracts and isolated compounds was determined for their antibiofilm activity using a semiquantitative static biofilm assay. Table 1 presents the inhibition rates (%) of root extracts on the biofilm activity of *Streptococcus mutans* and *Candida albicans* at a concentration of 500 µg/mL. All extracts included using *n*-hexane, ethyl acetate, and methanol as solvents showed significant differences in inhibition rates compared to the positive control of 0.12% chlorhexidine against *Streptococcus mutans* and *Candida albicans* at 99.5% and 51.9%, respectively. The ethyl acetate and methanol root extracts showed the highest inhibition rates against *Streptococcus mutans* with 97.6% and 99.2%, respectively, and *Candida albicans* with 48.1% and 49.2%, respectively. The results could be due to the abundance of secondary metabolites such as polyphenols, alkaloids, flavonoids, and terpenoids, which can exhibit various biological activities, including antibiofilm properties [18]. They can effectively penetrate the hydrophilic extracellular matrix of biofilms, disrupt microbial adhesion, and kill biofilm-embedded cells. For instance, polyphenolic compounds such as flavonoids can chelate metal ions by destabilizing biofilm matrix formation [19]. In addition, this may also be due to the synergistic interactions between different bioactive compounds within plant extracts that can contribute to their enhanced antibiofilm activity compared to individual components. Synergistic combinations penetrate the biofilm matrix and cause the death of cells. The complex mixture of phytochemicals present in the extracts can target multiple pathways involved in biofilm formation and maintenance, resulting in a broader spectrum of antimicrobial action and reduced likelihood of developing microbial resistance [21].

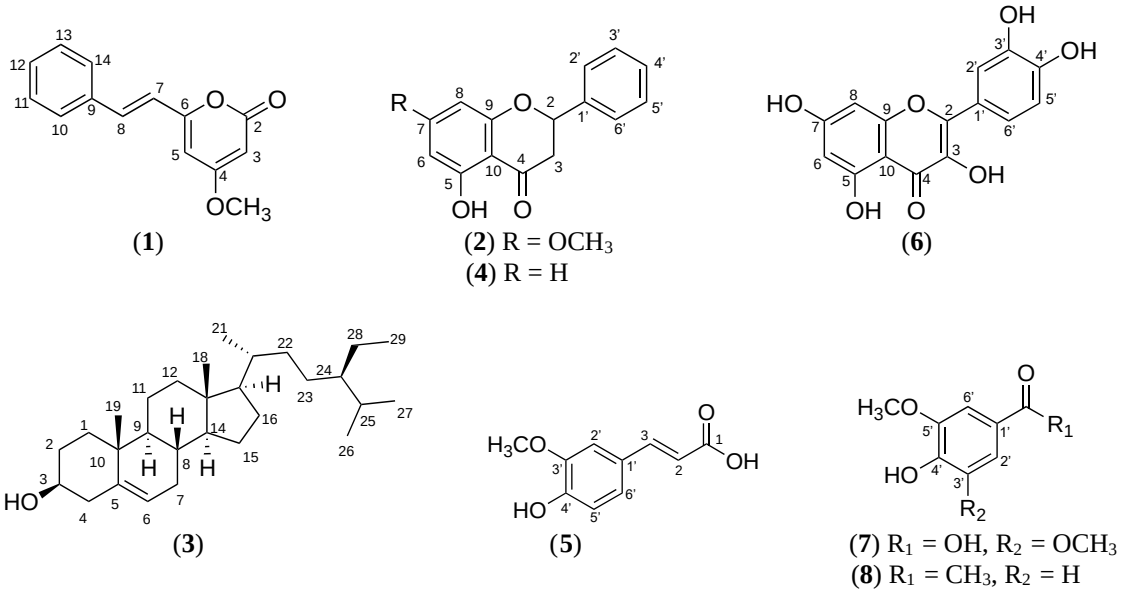

Figure 1. Chemical structures of constituents from *L. subumbelliflora*

Table 1. Antibiofilm activity of constituents of *L. subumbelliflora*

Samples/microbes	Inhibition rate (%) at 500 µg/mL	
	Streptococcus mutans	***Candida albicans***
LSRH	79.5 ± 0.24	35.7 ± 0.08
LSRE	97.6 ± 0.02	48.1 ± 0.01
LSRM	99.2 ± 0.01	49.2 ± 0.02
5,6-Dehydrokawain (**1**)	73.9 ± 0.28	35.1 ± 0.02
Pinostrobin (**2**)	89.3 ± 0.04	39.1 ± 0.02
β-Sitosterol (**3**)	91.3 ± 0.04	35.2 ± 0.03
Pinocembrin (**4**)	98.0 ± 0.01	43.7 ± 0.02
Ferulic acid (**5**)	93.0 ± 0.06	41.8 ± 0.02
Quercetin (**6**)	98.4 ± 0.04	45.7 ± 0.07
Syringic acid (**7**)	98.2 ± 0.01	40.1 ± 0.05
Apocynin (**8**)	92.7 ± 0.01	40.8 ± 0.03
Chlorhexidine 0.12 %	99.5 ± 0.01	51.9 ± 0.01

LSRH - *L. subumbelliflora* roots *n*-hexane extract; LSRE - *L. subumbelliflora* roots ethyl acetate extract; LSRM - *L. subumbelliflora* roots methanol extract

Among the isolated compounds, quercetin (**6**) stands out with notably high inhibition rates, 98.4% against *Streptococcus mutans* and 45.7% against *Candida albicans*. Chemically, quercetin is a flavonoid that can disrupt microbial cell membranes, inhibit enzyme activity, and inhibit nucleic acid synthesis. Quercetin has five hydroxyl (OH) groups attached to its benzene rings. These hydroxyl groups are crucial for their antibiofilm properties. They can donate hydrogen atoms to free radicals, stabilising them and preventing oxidative damage to cells. Microbial cells often rely on defences against oxidative stress for survival. However, under certain conditions, quercetin can enhance oxidative stress due to its interactions; thus, microbial membranes can be disrupted, making them more susceptible to damage and death [21]. Besides, quercetin is known to interfere with the pathways involved in bacterial quorum sensing, thereby preventing bacterial adhesion [22]. In other studies, quercetin and are active against the virulence properties of *S. mutans* and biofilm formation through inhibiting bacterial growth and metabolism, inhibiting acid production and inhibiting glucan synthesis, which contributes to the formation of extracellular biofilm matrix formation [23]. It should also be noted that the efficacy inhibitions against *Streptococcus mutans* do not have the same level of inhibition against *Candida albicans.* Syringic acid (**7**) exhibited a high inhibition rate against *Streptococcus mutans* (98.2%) but a comparatively lower rate against *Candida albicans* (40.1%). Ferulic acid (**5**) and apocynin (**8**) also exhibited a high inhibition rate against *Streptococcus mutans* compared to *Candida albicans.* Since these compounds have methoxy groups, it can influence their lipophilicity. Compounds with higher lipophilicity tend to interact more readily with lipid-based structures, especially in bacterial cell membranes. *Streptococcus mutans*, being a bacterium with a cell wall primarily composed of lipids and peptidoglycan, might be more susceptible to compounds with enhanced lipophilicity [24].

The molecular docking studies of the isolated phytochemicals were verified against lanosterol 14α-demethylase (Erg11; PDB ID: 4ZE3) revealed distinct binding affinities and specific residue interactions, underscoring the potential of these compounds as antifungal agents (Figure 2). Chlorhexidine, used as a control in the experimental assays, exhibited the strongest binding affinity of -10.9 kcal/mol in docking studies and showed the highest inhibition rate in the biofilm assays, with 99.5% inhibition against *S. mutans* and 51.9% against *C. albicans* at 500 µg/mL. This strong correlation between docking affinity and experimental activity reinforces chlorhexidine's efficacy as an antifungal agent. Pinostrobin (**2**) and β-sitosterol (**3**) with binding affinities of -9.4 and -9.2 kcal/mol, respectively, also demonstrated strong experimental biofilm inhibition, pinostrobin showing 89.3% inhibition against *S. mutans* and 39.1% against *C. albicans* and β-sitosterol showing 91.3% and 35.2%, respectively. Pinostrobin's docking interactions include π-π stacking with Phe241 and π-alkyl interactions with Leu95, Pro238, Tyr126, and Met509, stabilizing the flavonoid rings within the binding pocket, which may contribute to its high bioactivity. Pinocembrin (**4**) (-9.2 kcal/mol) forms a π-π stacked interaction - with Phe241 and π-alkyl interactions involving Pro238, Met509 and Leu95, which stabilize both ring A and ring C.

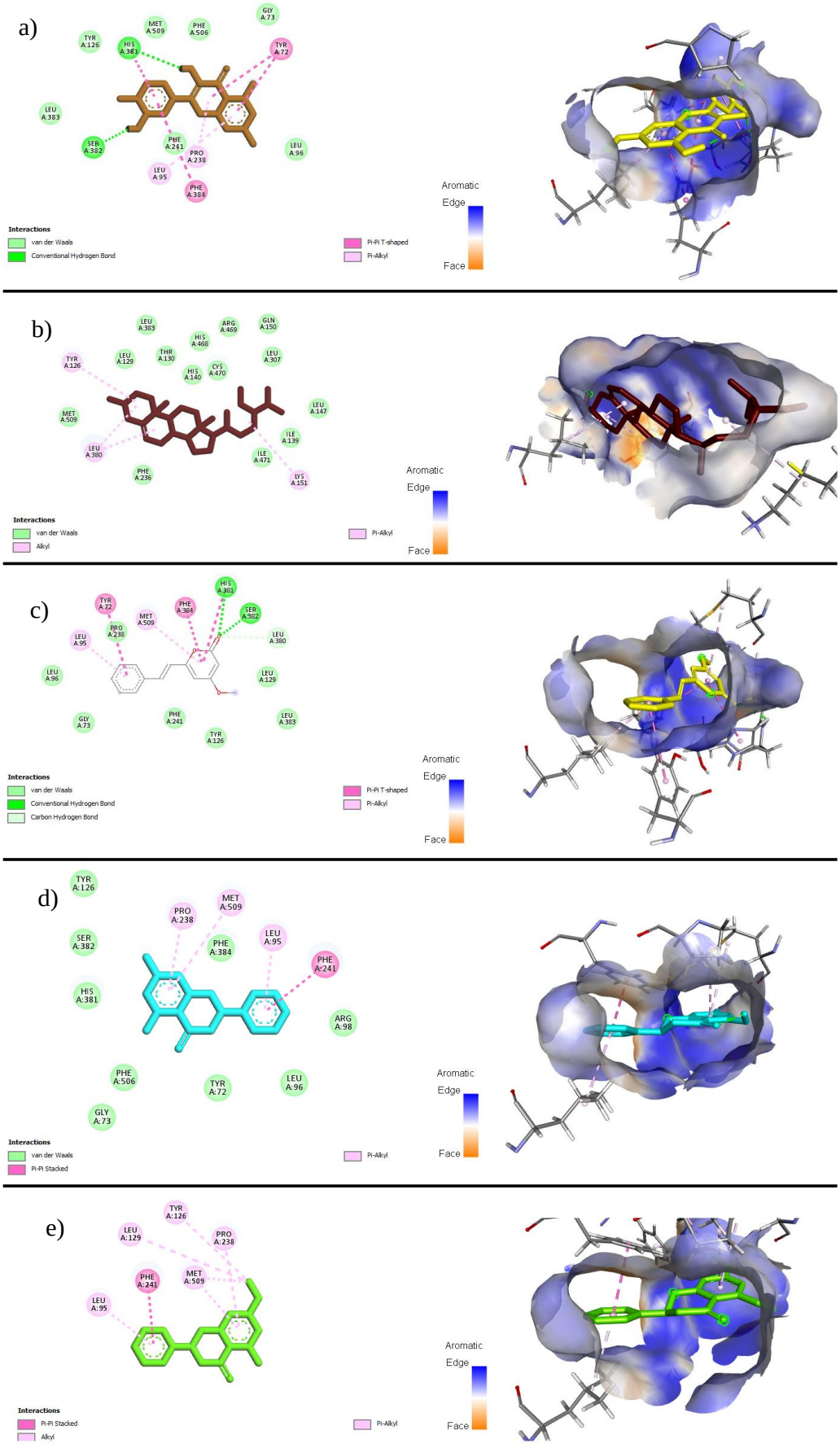

Figure 2. 2D and 3D conformation view of quercetin (**a**), β-sitosterol (**b**), 5,6-dehyrokawain (**c**), pinocembrin (**d**), pinostrobin (**e**) superimposed into Erg11 complex

Its experimental inhibition rates are 98.0% against *S. mutans* and 43.7% against *C. albicans*, correlating well with its strong docking affinity. Quercetin (**6**) (-9.0 kcal/mol) exhibited an intricate network of interactions, including hydrogen bonds with His381 and Ser382, dual π-π T-shaped interactions with Tyr72 involving rings A and B, and π-π interactions with His381 and Phe384. Experimentally, Quercetin showed a 98.4% inhibition against *S. mutans* and 45.7% against *C. albicans*, demonstrating its potent bioactivity, which is consistent with its complex and stabilising interactions within the enzyme. 5,6-Dehydrokawain (**1**) (-8.0 kcal/mol) showed moderate docking affinity and corresponding experimental inhibition rates of 73.9% against *S. mutans* and 35.1% against *C. albicans*. The compound exhibited interactions (Fig. 1) including a T-shaped π-π interaction with Tyr72, and π-alkyl interactions with Leu95 and Met509, which may explain its moderate bioactivity. In contrast, syringic acid (**7**) and apocynin (**8**) with lower binding affinities (-5.8 and -6.1 kcal / mol, respectively) still showed relatively high biofilm inhibition rates in experimental assays, with syringic acid showing 98.2% inhibition against *Streptococcus mutans* and 40.1% against *C. albicans* and apocynin showing 92.7% and 40.8%, respectively. Ferulic acid (**6**) with a low binding affinity of -6.7 kcal/mol, indicated a poor fit within the binding pocket, but managed to show significant inhibition rates of 93.0% and 41.8% against *S. mutans* and *C. albicans*, respectively.

These results suggest that while molecular docking provides valuable insights into potential binding affinities and modes of interaction, the actual bioactivity observed in experimental assays can be influenced by various factors, including compound solubility, cellular uptake, and metabolism [25]. Nevertheless, the strong correlation between docking results and biofilm inhibition for many of the compounds underscores the relevance of docking studies in predicting bioactivity and guiding the development of antifungal agents.

CONCLUSION

In conclusion, the antibiofilm activity of *Lindera* extract against *Streptococcus mutans* and *Candida albicans* shows promise for the development of new dental hygiene products. These findings underscore the need for further research on the active constituents, their mechanisms of action, and optimised extraction methods. Exploring these areas could provide valuable information on the properties of *Lindera* constituents, paving the way for therapeutic agents to target biofilm-associated infections, such as periodontal disease, endodontic infections, and other oral health conditions.

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