Antimicrobial activity of *Ruta angustifolia* L. Pers against periodontal pathogen: *Porphyromonas gingivalis* (#106059)

First revision

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Antimicrobial activity of *Ruta angustifolia* L. Pers against periodontal pathogen: *Porphyromonas gingivalis*

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Background. *Porphyromonas gingivalis* is widely recognised as a periodontal pathogen. In recent years, there has been growing interest in the use of medicinal plant extracts as alternative treatments for periodontitis to combat the emergence of antibiotic-resistant bacteria. *Ruta angustifolia* L. Pers has been traditionally used to treat various ailments, including oral bacterial infections. However, the antimicrobial potential of *R. angustifolia* extracts against the periodontal pathogen *P. gingivalis* remains unexplored. Hence, the aim of this study was to investigate the antimicrobial activity of *R. angustifolia* extracts against *P. gingivalis*. **Methods.** The antimicrobial activity of *R. angustifolia* extracts (crude methanol, hexane and chloroform fractionated extracts) against *P. gingivalis* was evaluated using the well diffusion method. Additionally, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. Biofilm biomass assessment and live/dead cell viability assays were performed to analyse the effect of *R. angustifolia* extracts. Ultrastructural morphological changes in *P. gingivalis* cells were determined using field emission scanning electron microscopy (FE-SEM). **Results.** It was found that *P. gingivalis* was susceptible to *R. angustifolia* extracts, with the chloroform fractionated extract exhibiting the highest inhibition zones. The MIC and MBC of chloroform fractionated extract were determined to be 6.25 mg/mL which substantially reduced *P. gingivalis* biofilm biomass. Live/dead cell viability assays showed the highest percentage of dead *P. gingivalis* cells after 48 hours of incubation. FE-SEM confirmed that the chloroform fractionated extract effectively damaged the bacterial cell wall and altered the ultrastructural morphology of *P. gingivalis*. **Conclusion.** The results indicated that extracts of *R. angustifolia* has the potential to be used as an alternative treatment in addition to conventional periodontal therapies.

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2 **against periodontal pathogen: *Porphyromonas***
3 ***gingivalis***

4

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18

19 **Abstract**

20

21 **Background.** *Porphyromonas gingivalis* is widely recognised as a periodontal pathogen. In
22 recent years, there has been growing interest in the use of medicinal plant extracts as alternative
23 treatments for periodontitis to combat the emergence of antibiotic-resistant bacteria. *Ruta*
24 *angustifolia* L. Pers has been traditionally used to treat various ailments, including oral bacterial
25 infections. However, the antimicrobial potential of *R. angustifolia* extracts against the
26 periodontal pathogen *P. gingivalis* remains unexplored. Hence, the aim of this study was to
27 investigate the antimicrobial activity of *R. angustifolia* extracts against *P. gingivalis*.
28 **Methods.** The antimicrobial activity of *R. angustifolia* extracts (crude methanol, hexane and
29 chloroform fractionated extracts) against *P. gingivalis* was evaluated using the well diffusion
30 method. Additionally, the minimum inhibitory concentration (MIC) and minimum bactericidal
31 concentration (MBC) were determined. Biofilm biomass assessment and live/dead cell viability
32 assays were performed to analyse the effect of *R. angustifolia* extracts. Ultrastructural
33 morphological changes in *P. gingivalis* cells were determined using field emission scanning
34 electron microscopy (FE-SEM).
35 **Results.** It was found that *P. gingivalis* was susceptible to *R. angustifolia* extracts, with the
36 chloroform fractionated extract exhibiting the highest inhibition zones. The MIC and MBC of
37 chloroform fractionated extract were determined to be 6.25 mg/mL which substantially reduced
38 *P. gingivalis* biofilm biomass. Live/dead cell viability assays showed the highest percentage of
39 dead *P. gingivalis* cells after 48 hours of incubation. FE-SEM confirmed that the chloroform

40 fractionated extract effectively damaged the bacterial cell wall and altered the ultrastructural
41 morphology of *P. gingivalis*.
42 **Conclusion.** The results indicated that extracts of *R. angustifolia* has the potential to be used as
43 an alternative treatment in addition to conventional periodontal therapies.
44
Introduction
46
47 According to the World Health Organisation (WHO), nearly 3.5 billion people worldwide are
48 affected by oral diseases, with three out of four affected individuals living in middle-income
49 countries. Globally, severe periodontal disease is estimated to affect around 19% of the global
50 adult population, representing more than 1 billion cases (Santhosh et al., 2023). The global oral
51 care market has witnessed a significant rise, primarily due to the increasing prevalence of oral
52 health issues such as periodontal diseases, dental caries, plaque-induced gingivitis, and oral
53 cancer (Amil et al., 2024). Specific pathogenic bacteria, such as *Porphyromonas gingivalis*, are
54 considered predominant causes of periodontitis due to their ability to disrupt the homeostatic
55 balance of the oral microbiota and dysregulate host immune responses. The consequences
56 include gum inflammation, breakdown of periodontal tissues, bone loss, and the formation of
57 periodontal pockets (Howard et al., 2021).
58
59 The growing awareness of oral hygiene has prompted individuals to invest in oral care
60 products for themselves and their families. As a result, the oral care market has experienced
61 significant revenue growth, with an estimated cumulative annual growth rate of 8.1%. Among
62 the diverse range of products available, mouthwashes have generated the highest revenue,
63 closely followed by toothpaste (Siripipatthanakul & Sixl-Daniell, 2021). Concerns regarding
64 potential chemical toxicity and adverse effects associated with prolonged exposure to chemical
65 ingredients in oral care products have fuelled the demand for natural alternatives, particularly
66 herbs and spices known for their antibacterial and chemopreventive properties (Böhle et al.,
67 2022). Furthermore, the rising concern of about antimicrobial resistance necessitates the
68 development of alternative, targeted therapies for oral health, particularly periodontal diseases.
69 Thus, finding safe and effective therapeutic agents for oral health is deemed necessary.
70
71 Historically, plants and their extracts have been utilised for therapeutic purposes across
72 various cultures, with traditional medicine practices highlighting their benefits. The appeal of
73 herbal extracts lies in their perceived safety, cost-effectiveness, and efficacy, as well as their role
74 as alternatives or complements to conventional treatments. Herbal extracts have been employed
75 for centuries to address a variety of health conditions, including oral health issues such as
76 gingivitis, periodontitis, and dental caries. The recent popularity of herbal medicines and
77 products is evident among dental patients, professionals and a wider range of consumers and
78 healthcare practitioners, due to their potential benefits for oral health and alveolar bone
79 protection. One study demonstrated that the systemic administration of curcumin, a compound

80 derived from turmeric, significantly reduced alveolar bone loss in a ligature-induced rat model,
81 suggesting its potential as an adjunct for initial periodontal therapy (Akpinar et al., 2017).
82 Another study revealed that extracts from *Cinnamomum zeylanicum* and *Salvadora persica*
83 exhibit antibacterial activity against various periodontal pathobionts such as *P. gingivalis*,
84 *Treponema denticola*, *Tannerella forsythia*, and *Actinobacillus actinomycetemcomitans* (Saquib
85 et al., 2019). The Rutaceae family, particularly the genus Ruta, such as *Ruta angustifolia* L. Pers,
86 commonly referred to as "garuda", has been traditionally used in Indonesia for the treatment of
87 jaundice and liver disorders. Additionally, it is cultivated in Java and Vietnam, where its
88 decoction is commonly applied to relieve cramps, bloating, and fever (Bailly, 2023).
89
90   Previous studies have reported that various extracts of *R. angustifolia* exhibit a wide
91 range of biological activities, including strong antiviral effects (Wahyuni et al., 2014) and
92 significant cytotoxic activity against several cancer types, such as lung cancer (A549), colon
93 cancer (HCT-116 and HT29), and cervical cancer (CaSki) (Richardson et al., 2016; Wu et al.,
94 2003). Additionally, *R. angustifolia* has demonstrated antibacterial properties, attributed to
95 naturally occurring compounds like flavonoids, alkaloids, and coumarins (Bailly, 2023). These
96 compounds have shown various medicinal effects, particularly against microbial infections
97 (Nahar et al., 2021). For instance, chalepin, isolated from the leaves of *R. angustifolia*, exhibited
98 weak antiparasitic activity against *Trypanosoma cruzi*, the pathogen responsible for Chagas
99 disease (Pavão et al., 2002). Furthermore, essential oil extracted from *R. angustifolia* has shown
100 significant antifungal activity against *Fusarium oxysporum* and *Botrytis cinerea*. However, there
101 is a lack of comprehensive research on the antibacterial effects of *R. angustifolia* extracts against
102 oral bacteria associated with periodontal diseases. Therefore, this study aims to investigate the
103 antimicrobial efficacy of *R. angustifolia* extracts against *P. gingivalis*, a prominent periodontal
104 pathogen, by assessing biofilm biomass, cell viability, and morphological characteristics.
105

Materials & Methods

107
Preparation of plant extracts
109
110 The plant extraction method was performed based on the method described by Suhaimi et al.,
111 (2017), with slight modifications. A total of 100.00 g of powdered leaves of *R. angustifolia* was
112 soaked three times in methanol (MeOH), yielding a greenish crude methanol extract (28.80 g,
113 28.87%). Hexane was used to extract the crude MeOH (30.00 g), resulting in a hexane insoluble
114 extract (28.85 g, 96.17 %) and a hexane fractionated extract (1.15 g, 3.83 %). The hexane
115 insoluble extract underwent further extraction with chloroform, producing an aqueous residue
116 and a chloroform fractionated extract (9.05 g, 30.2%). The resulting crude MeOH and
117 fractionated extracts (hexane and chloroform) were evaporated using a rotary evaporator
118 (BÜCHI, Switzerland) at 40°C under reduced pressure to remove the solvents completely

119 (Seidel, 2012; Suhaimi et al., 2017). Prior to assessing antimicrobial activity, the extracts of *R.*
120 *angustifolia* were reconstituted in 1% dimethyl sulfoxide (DMSO).

121

122 **Bacterial strains and culture conditions**

123

124 *P. gingivalis* ATCC 33277 was grown from stock culture in BHI-T broth (Tryptone Soya Broth
125 & Brain Heart Infusion broth), supplemented with hemin (5.00 mg/mL), menadione (5.00
126 mg/mL) & cysteine (0.50 g/mL), for 48 to 72 hours at 37°C under anaerobic conditions. The oral
127 bacterial strain was harvested from the broth by centrifugation (10,000 X g, 5 min, 4°C) and
128 washed three times with phosphate-buffered saline (PBS) prior to the experiments. The optical
129 density at 600 nm (OD_{600}) of the bacterial suspensions was adjusted to 0.1 using a UV-VIS
130 spectrophotometer (equivalent to 10^8 colony-forming units per millilitre, CFU/mL) for *P.*
131 *gingivalis* in all experiments. Fresh cultures of *P. gingivalis* were gram stained for validation and
132 used in all relevant experiments (Kobatake et al., 2019; Paranagama et al., 2020).

133

134 **Well diffusion assay**

135

136 Antimicrobial activity was determined using the well diffusion method (Michaylova et al.,
137 2021). A volume of 100.00 μL of a 10^8 CFU/mL suspension of *P. gingivalis* suspension (OD_{600}
138 adjusted to 0.1) was evenly spread over the surface of the BHI-T agar using a sterile cotton swab
139 (Zou et al., 2022).

140

141 Using a sterile cork borer, wells measuring 6.00 mm in diameter were punched in the
142 agar and filled with 30.00 μL of crude MeOH and fractionated extracts of *R. angustifolia* at
143 concentration of 50.00 mg/mL & 100.00 mg/mL, respectively. The plates were incubated for 24
144 to 72 hours at 37°C, based on optimised growth observations (Michaylova et al., 2021). A 0.12%
145 chlorhexidine gluconate (CHX) were used as the positive control, while 1% DMSO served as the
146 negative control. The final concentration of DMSO in the test wells did not exceed 1% (v/v). The
147 inhibition zone diameter was measured using a 0-150 mm METR-ISO Electronic Digital Caliper
148 6", manufactured by J.P. Selecta company in Spain. All data presented are the mean ± standard
149 deviation (SD) of triplicates obtained from three independent experiments.

150

151 **Determination of the minimum inhibitory concentration (MIC) & minimum bactericidal**
152 **concentration (MBC)**

153

154 The two-fold microdilution broth method was employed to obtain the MIC value (Nordin et al.,
155 2013). In this test, eight concentrations were tested, with triplicate measurement taken for each
156 concentration. The concentrations tested were 50.00, 25.00, 12.50, 6.25, 3.13, 1.56, 0.78 and
157 0.39 mg/mL. The bacterial cells of *P. gingivalis* were treated with the chloroform fractionated
158 extract of *R. angustifolia* using two-fold serial dilutions. Briefly, 100.00 μL of BHI-T broth was

159   dispensed into the wells of a 96-microtiter plate, labelled from Well 1 (W1) to Well 8 (W8).

160   Following this, 50.00 mg/mL of chloroform fractionated extract of *R. angustifolia* was added

161   into W1, and two-fold serial dilution were performed for the remaining wells. Hence, the final

162   concentrations of the extract ranged from 50.00 to 0.39 mg/mL.

163

164   A 0.12% CHX was used as the positive control. The chloroform fractionated extract of *R.*

165   *angustifolia* at different concentrations, without the addition of bacterial culture served as the

166   blank. The negative control consisted of wells containing solely a mixture of BHI-T broth and *P.*

167   *gingivalis* suspension with 1% DMSO. Then, 20.00 μL of *P. gingivalis* suspension (10^8

168   CFU/mL) was added to W1 through W8. After overnight incubation at 37°C, bacterial growth

169   (turbidity) was assessed visually and confirmed using a spectrophotometer at an OD of 600 nm.

170   The methods for conducting the MIC and MBC tests were according to the Clinical and

171   Laboratory Standard Institute guidelines (CLSI, 2020). The MIC was defined as the lowest

172   concentration of the extract in a well without turbidity (Madhloom et al., 2022). All data

173   presented are the mean ± standard deviation (SD) of triplicates obtained from three independent

174   experiments.

175

176   For the determination of MBC, 50.00 μL was taken from the MIC assay wells and spread

177   onto fresh BHI-T agar plates. The plates were incubated under anaerobic conditions at 37°C for

178   24 to 72 hours, or until visible growth appeared (Fadare et al., 2022). The MBC was then defined

179   as the minimum concentration of the chloroform fractionated extract from *R. angustifolia* at

180   which no visible microbial growth was observed on the agar plate. To ensure accuracy and

181   reproducibility, all antimicrobial assessments of the extracts were conducted in triplicate at three

182   separate times.

183

184   Crystal violet biofilm assay

185

186   The crystal violet staining method was used to analyse the biomass of biofilms using a broth

187   microdilution method in a 96-well microtiter plate, with some modifications (Luan et al., 2022).

188   One hundred microliters of 10^8 CFU/mL *P. gingivalis* suspension and 100.00 μL of two-fold

189   serial dilutions of the chloroform fractionated extract of *R. angustifolia* were added to each well

190   in the microtiter plate. The plates were incubated for 48 hours under anaerobic condition at 37°C.

191   The final concentrations of the extract were 12.50, 6.25, 3.13 and 1.57 mg/mL. The wells

192   containing solely the BHI-T broth and *P. gingivalis* suspension mixture served as the negative

193   control. All data presented are the mean ± standard deviation (SD) of triplicates obtained from

194   three independent experiments.

195

196   The supernatant was removed, and the wells were washed three times with PBS.

197   Subsequently, the adhering biofilms were stained with 0.04% (w/v) crystal violet for 15 minutes

198   after incubation with methanol. For detection, 95% ethanol was added after washing with

199   deionised water. The OD values were measured at a wavelength of 550 nm (Sharma & Saharan,
200   2016). The percentages of biofilm inhibition against *P. gingivalis* at different concentrations of
201   the chloroform fractionated extract of *R. angustifolia* were calculated as:
202
203   % biofilm inhibition = [1 − (A_C / A_0)] × 100
204   where A_C represents the absorbance of the well with treated *P. gingivalis* (with extract) and A_0
205   the absorbance of the control well, untreated *P. gingivalis* (without extract).
206

Live/dead cell viability assay using fluorescence microscopy

207
208
209   Fresh cultures of *P. gingivalis*, incubated for 48 hours at 37°C, were adjusted to an OD_{600} of 0.1
210   (equivalent to 10^8 CFU/mL) by diluting them in supplemented BHI-T broth using a UV-VIS
211   spectrophotometer. The *P. gingivalis* suspension and chloroform fractionated extract of *R.*
212   *angustifolia* were then mixed at a ratio of 1:1 (v/v) (Widyarman et al., 2019). *P. gingivalis*
213   biofilms were grown as described by Luan et al., (2022) with slight modifications.
214
215   Two sets of experiments were conducted, incubating the biofilms at 37°C for 24 and 48
216   hours, respectively. Following incubation under anaerobic conditions, 3.00 µL of the
217   LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012, Thermo Fisher Scientific, Waltham,
218   MA, USA) dye mixture was added per mL of bacterial suspension. The stained samples were
219   then incubated at room temperature in the dark for 15 minutes. A 5.00 µL aliquot of the stained
220   suspension was placed onto a microscope slide. The morphological changes of untreated and
221   treated *P. gingivalis* biofilms with the chloroform fractionated extract of *R. angustifolia* were
222   observed and recorded using a fluorescence microscope (Nikon Eclipse Ti-E Inverted
223   Microscope).
224

Field Emission Scanning Electron Microscopy (FE-SEM) to examine cell morphology

225
226
227   Briefly, *P. gingivalis* cultures were incubated for 48 hours at 37°C and adjusted to an OD_{600} of
228   0.1 to achieve cell concentration of 10^8 CFU/mL. The *P. gingivalis* suspension was then mixed
229   with the chloroform fractionated extract of *R. angustifolia* (6.25 mg/mL) at a ratio of 1:1 (v/v)
230   (Luan et al., 2022; Widyarman et al., 2019).
231
232   After 24-hour incubation under anaerobic condition at 37°C, the bacterial suspensions
233   were fixed in 4% glutaraldehyde for over 4 hours (Arvizu & Murray, 2021). The samples were
234   then washed twice in buffer for 10 mins each, followed by fixation in 1% osmium tetroxide for 1
235   hour. After two 10-minute washes in double-distilled water (DDH_2O), the samples were
236   subjected to a series of dehydration steps in ascending alcohol concentrations from 30, 50, 70,
237   80, 90, 95 and 100% (twice), with 15 minutes each step. The samples were further dehydrated
238   with three different ratios (3:1, 1:1, and 1:3) of ethanol-acetone mixtures for 15 mins each step,

239 before being fixed in pure acetone for 20 mins (twice). Finally, the samples were critical point
240 dried (CPD) for 1 to 3 hours, mounted on stubs with carbon adhesive, sputter-coated with gold,
241 and viewed using a scanning electron microscope (FE-SEM).
242
243 **Statistical analysis**
244
245 All data are presented as the mean ± standard deviation (SD) of triplicates obtained from three
246 independent experiments. Statistical analyses were conducted using SPSS software (IBM Corp.,
247 version 27.0, Armonk, NY, USA). The normality of the data for biofilm biomass inhibition of *P.*
248 *gingivalis* was assessed using the Shapiro–Wilk test, which indicated that the data followed a
249 normal distribution ($p > 0.05$). Data evaluation between groups was analysed using one-way
250 ANOVA, and post-hoc Tukey's HSD test was applied for multiple comparisons. The results of
251 inhibitory activities for *R. angustifolia* extracts against *P. gingivalis* were subjected to the
252 Shapiro-Wilk test to assess normality ($p < 0.05$), which indicated that the data distribution was
253 not normal. Statistical analysis between groups was performed by applying the non-parametric
254 Kruskal-Wallis test, followed by Dunn's post-hoc test. The significance level was set at 0.05 for
255 all analyses, with p-values < 0.05 considered statistically significant.
256
257 # Results
258
259 **Inhibitory effects of *R. angustifolia* extracts against *P. gingivalis***
260
261 Based on the well diffusion test presented in **Figure 1**, all *R. angustifolia* extracts effectively
262 suppressed the growth of *P. gingivalis* in a concentration-dependent manner. Inhibition zone
263 measurements indicated that the highest antimicrobial activity occurred at a concentration of
264 100.00 mg/mL for the chloroform fractionated extract of *R. angustifolia*, as shown in **Table 1**.
265 Among the three extracts tested, the chloroform fractionated extract exhibited the largest
266 inhibition zone, followed by the crude methanol extract (9.30 ± 0.26 mm) and the hexane
267 fractionated extract (8.19 ± 0.27 mm). These results demonstrate that the chloroform fractionated
268 extract of *R. angustifolia* most effectively inhibited the growth of *P. gingivalis*, while the
269 negative control (1% DMSO) showed no inhibition.
270
271 In comparison, the inhibition zone for 0.12% CHX was 13.34 ± 0.73 mm, while the
272 chloroform fractionated extract at 100.00 mg/mL demonstrated an inhibition zone of $11.65 \pm$
273 0.62 mm. Thus, these findings suggest that its efficacy is comparable to that of 0.12% CHX, a
274 widely used antibacterial agent for oral diseases. Although CHX exhibited a slightly larger zone,
275 the difference is not substantial, indicating that *R. angustifolia* may serve as an effective
276 alternative or adjunct in managing oral infections.
277
278

Determination of MIC and MBC

Given that *P. gingivalis* is a significant pathogen involved in the development and pathophysiology of periodontal infections, the primary aim of this study was to further assess the antimicrobial activity of the extracts against this oral bacterium. Based on the initial antimicrobial screening, the chloroform fractionated extract of *R. angustifolia* demonstrated the most potent inhibitory effect against *P. gingivalis*. Therefore, this extract was selected for all subsequent analyses in the study.

The MIC and MBC values of the chloroform fractionated extract of *R. angustifolia* (**Table 2**) were determined using serial microdilution, with concentrations ranging from 0.39 mg/mL to 50.00 mg/mL. The antimicrobial activity of the extract was compared against untreated *P. gingivalis* bacterial cells in 1% DMSO (which served as the negative control). The MIC was found to be at 6.25 mg/mL, as this concentration resulted in a significant reduction in *P. gingivalis* growth compared to the untreated sample. The negative control exhibited no inhibition, confirming its suitability as a baseline comparison. Further analysis of the MBC test showed bacterial growth at 3.13 mg/mL, indicating that MBC was not detected at this concentration. Therefore, the MBC of the chloroform fractionated extract of *R. angustifolia* against *P. gingivalis* was determined to be 6.25 mg/mL, as no bacterial growth was observed at this level.

Effect of chloroform fractionated extract of *R. angustifolia* against *P. gingivalis* biofilm biomass

As shown in **Figure 2**, the overall biomass of *P. gingivalis* biofilms treated with various concentrations of chloroform fractionated extract of *R. angustifolia* was performed using crystal violet staining. The extract demonstrated notable antibiofilm effects by suppressing the formation of biofilm in *P. gingivalis*. A clear, concentration-dependent decrease in absorbance was observed, indicating the efficacy of the extract in reducing the biofilm biomass. Notably, the biofilm biomass of *P. gingivalis* was remarkably reduced by up to 91% at the highest concentration of chloroform fractionated extract of *R. angustifolia*. This result was consistent with the MIC determined in **Table 2**, confirming that the chloroform fractionated extract of *R. angustifolia* effectively disrupted the formation of *P. gingivalis* biofilm compared to the control.

Live/dead cell viability assay using fluorescence microscope

LIVE/DEAD® BacLight™ viability staining was conducted to evaluate the impact of the chloroform fractionated extract of *R. angustifolia* on *P. gingivalis* biofilm (**Figure 3**). In the absence of the extract, the untreated *P. gingivalis* biofilm displayed a dense and highly uniform structure after 24 hours of incubation. In contrast, increasing concentrations of the extract led to

319 marked sparsity and dispersion of the biofilm, indicating a significant reduction in viable cells.
320 As a result, biofilm formation was substantially inhibited, with a notable decrease in bacterial
321 presence at higher concentrations of the chloroform fractionated extract of *R. angustifolia*.
322
323   Similar results were observed at 48 hours, where the untreated *P. gingivalis* biofilm
324 remained dense and uniform, while higher extract concentrations led to increased biofilm
325 disruption and cell death. Significantly, an abundance of lysed cells and cellular debris was
326 visible at the higher concentration of 6.25 mg/mL. These findings demonstrate that the
327 chloroform fractionated extract of *R. angustifolia* is capable of disrupting *P. gingivalis* biofilm
328 formation and viability in a concentration-dependent manner.
329
330 **The effects of the *R. angustifolia* extract on *P. gingivalis* cell morphology via FE-SEM**
331
332 FE-SEM imaging (**Figure 4**) was conducted to examine morphological alterations in *P.*
333 *gingivalis* exposed to the chloroform fractionated extract of *R. angustifolia*. Untreated *P.*
334 *gingivalis* exhibited a characteristic of smooth cell surface that appeared as tiny grape-like
335 clusters with intact cell membranes. In contrast, the *P. gingivalis* biofilm treated with 6.25
336 mg/mL of the chloroform fractionated extract of *R. angustifolia* displayed significant changes in
337 cellular morphology. The treated cells displayed an unravelled, crater-like appearance of the cell
338 walls, as along with swollen, elongated, and lysed forms that aggregated into large clusters of
339 cellular debris. Therefore, the FE-SEM analysis conclusively demonstrated that the chloroform
340 fractionated extract of *R. angustifolia* completely inhibited *P. gingivalis* cells. At this MIC
341 concentration, the integrity of the *P. gingivalis* bacterial cell membrane was compromised,
342 resulting in extensive cell lysis and disruption of cellular morphology.
343

Discussion

345
346 The present study reveals that the crude methanol extract, hexane, and chloroform fractionated
347 extracts of *R. angustifolia* exhibit inhibitory activity against *P. gingivalis*. Among these, the
348 chloroform fractionated extract of *R. angustifolia* was found to be the most effective in inhibiting
349 the growth of *P. gingivalis,* leading to its selection for further investigation. This report is the
350 first to document the antimicrobial susceptibility of *R. angustifolia* extracts against *P. gingivalis*.
351 The antimicrobial effect of the extracts was dose-dependent, with higher concentrations of the
352 chloroform fractionated extract of *R. angustifolia* displaying greater bactericidal activity. A
353 previous report identified twelve components isolated from the chloroform fractionated extract
354 of *R. angustifolia* (Richardson et al., 2017). These compounds were graveoline, kokusaginine,
355 bergapten, moskachan B, moskachan D, chalepensin, rutamarin, arborinine, chalepin and
356 neophytadiene. Most of these compounds belong to the classes of alkaloids, furanocoumarins,
357 and dihydrofuranocoumarins, which possess significant antiviral, anti-inflammatory, antioxidant,
358 and antiproliferative properties. Specifically, rutamarin and chalepin are categorized as

359   furanocoumarins. Current research indicates that coumarin derivatives, recognized for their
360   antioxidant, antifungal, and antibacterial properties, hold promise as therapeutic agents across
361   various medical applications. Structural modifications, such as the incorporation of nitrogen
362   atoms and azomethane groups, are thought to enhance antibacterial activity by increasing
363   membrane permeability, thereby contributing to potent bactericidal effects. These compounds
364   likely benefit from increased lipophilicity, which enhances their ability to penetrate bacterial
365   membranes. This lipophilic characteristic may also interfere with bacterial metabolic processes
366   by inactivating essential enzymes and denaturing cellular proteins, ultimately leading to bacterial
367   cell death (Betti et al., 2024). Additionally, recent studies have revealed that coumarin
368   derivatives exhibit significant cytotoxicity towards bacterial DNA, potentially due to their
369   modification of bacterial membrane components, including lipopolysaccharides (LPS). This
370   modification may activate enzymes such as topoisomerases and helicases, destabilizing the DNA
371   structure and obstructing replication, which culminates in bacterial cell apoptosis (Koszelewski
372   et al., 2023).
373
374   Furthermore, graveoline, the most abundant compound in *R. angustifolia* (Richardson et al.,
375   2016), exhibits antibacterial, anticancer, antifungal, and spasmolytic effects. Graveoline is a
376   quinolone alkaloid that interacts with bacterial cell membranes, affecting their function and
377   causing cell lysis. Alkaloids can suppress bacterial growth by interfering with nucleic acid,
378   protein synthesis, and metabolism. Additionally, these compounds may disrupt essential
379   metabolic processes and energy sources for various enzymatic reactions in bacteria, such as
380   adenosine triphosphate (ATP), thereby inhibiting growth. Alkaloids can also promote the
381   generation of reactive oxygen species (ROS), which damage bacterial cells (Yan et al., 2021).
382   Hence, *R. angustifolia* harbours numerous bioactive compounds, showcasing significant
383   potential for therapeutic applications in various diseases, including periodontal diseases.
384
385   The periodontal pathogen *P. gingivalis* is known as one of the late colonisers of the oral
386   cavity, which possess a range of virulent characteristics and robust biofilm-forming capabilities
387   that facilitate persistent infection at the gingival site (Xu et al., 2020). These late colonisers
388   consist of slower-growing, obligate anaerobes, and Gram-negative species, such as *P. gingivalis*,
389   along with other members of the red complex, *T. forsythia* and *T. denticola*, which are typically
390   found at elevated levels in diseased conditions (Siddiqui et al., 2022).
391
392   Gram-positive and Gram-negative bacteria are primarily distinguished by the thickness of
393   their cell walls and the presence of an outer membrane, which is unique to Gram-negative
394   bacteria. The thickness of the cell walls of Gram-positive and Gram-negative bacteria ranges
395   from 20 to 80 nm and 1.5 to 10 nm, respectively (Bismelah et al., 2022). Despite having thicker
396   cell walls, Gram-positive bacteria can absorb foreign chemicals, such as bioactive compounds,
397   making them more susceptible to elimination. In contrast, the effectiveness of some antibiotic
398   treatments against Gram-negative bacteria is impaired by the presence of an outer membrane

composed of lipopolysaccharides and phospholipids (Fivenson et al., 2023). This additional membrane layer functions as an effective permeable barrier, preventing antibiotic molecules from entering the bacterial cell.

The presence of virulence factors in various periodontopathic bacteria poses significant challenges in treating infections. For example, *P. gingivalis* and *T. forsythia* are characterised by high proteolytic and peptidolytic activities, which can hydrolyse antibiotics, leading to the inactivation of their antimicrobial properties (Bismelah et al., 2022). These factors may explain why higher concentrations of plant extracts are required to effectively inhibit *P. gingivalis* in susceptibility tests. Therefore, the extract of *R. angustifolia* could serve as an adjunctive treatment for periodontitis, as it effectively targets the growth of late colonisers, thereby helping to prevent the progression of periodontal infections.

Correspondingly, the chloroform fractionated extract of *R. angustifolia* showed promising results for MIC and MBC, completely inhibiting *P. gingivalis* at a concentration of 6.25 mg/mL. Furthermore, the biofilm assay provided evidence that the chloroform fractionated extract of *R. angustifolia* exhibits inhibitory effects on the formation of *P. gingivalis* biofilms. This significant discovery suggests that the extract has potential as a viable method for mitigating the metabolic activity of *P. gingivalis* within biofilms. Biofilms are complex three-dimensional structures composed of microorganisms bound together by an extracellular matrix (Mitchell, 2011). Once microorganisms form biofilms, they become increasingly challenging to control and eradicate. According to Raut & Karuppayil (2016), biofilms are associated with over 80% of microbial illnesses in humans. Since periodontal disease involves the formation of biofilms on gingival tissues, disrupting the biofilm formed by *P. gingivalis* is critically important (Bustamante et al., 2020). The ability of the chloroform fractionated extract of *R. angustifolia* to interfere with biofilm formation at varying concentrations is crucial for preventing *P. gingivalis* from recolonising the periodontal pocket, as this treatment eliminates the protective layer of the subgingival biofilm (Bostanci & Belibasakis, 2012).

Moreover, the outcomes of our investigation closely correlate with a recent study that have demonstrated the ability of *R. angustifolia* extracts in diminishing the formation of biofilm by *S. mutans* at concentrations of 20%, 50%, and 100% (Noer, 2021). In general, the antimicrobial activities of plants are attributed to their secondary metabolites. Previous studies has confirmed that *R. angustifolia* extract contains qualitative steroids, flavonoids, tannins, and quinones, suggesting its potential as an antibiofilm agent (Lu et al., 2019). Compounds such as flavonoids in *R. angustifolia* extract have been extensively studied for their bioactivity in inhibiting both Gram-positive and Gram-negative bacteria through the inactivation of extracellular proteins (Bismelah et al., 2022). Recent studies indicate that the antibacterial activities of plant flavonoids against Gram-negative bacteria are related to their lipophilicity. The cell envelope of Gram-negative bacteria contains an inner lipophilic membrane and an outer hydrophilic membrane,

439   while Gram-positive bacteria possess only a cell membrane. Consequently, it is suggested that
440   plant flavonoids employ multiple mechanisms against Gram-negative bacteria, with the cell
441   membrane serving as a critical site of action, in contrast to Gram-positive bacteria, which are
442   primarily affected at the cell membrane. Furthermore, DNA gyrase is another important target of
443   plant flavonoids against Gram-negative bacteria. Thus, plant flavonoids can damage the cell
444   membrane and inhibit DNA gyrase, representing a dual mechanism of action against Gram-
445   negative bacteria (Yan et al., 2024).
446

447   Live/dead cell viability assays demonstrated that the chloroform fractionated extract of *R.*
448   *angustifolia*, at both sub-MIC and MIC levels, induced a significant reduction in the viability of
449   *P. gingivalis* compared to the control group. The biofilm formation of *P. gingivalis* was
450   markedly inhibited by the chloroform fractionated extract of *R. angustifolia*, which led to a
451   greater dispersion of biofilm structures and an increase in cell death, particularly at a
452   concentration of 6.25 mg/mL after 48 hours of incubation. Moreover, non-viable/dead (red
453   fluorescence) cells were evidently in greater proportion than viable/live (green fluorescence)
454   cells. These findings suggest that the chloroform fractionated extract of *R. angustifolia* exhibits
455   biocidal effects on the *P. gingivalis* biofilm, as evidenced by the visualization of dead
456   microorganisms. Similar results were reported from a previous study by He et al., (2020), which
457   investigated the impact of quercetin on *P. gingivalis* aggregation and biofilm formation.
458

459   FE-SEM images revealed morphological changes in the cell structure of *P. gingivalis* after
460   treated with the chloroform fractionated extract of *R. angustifolia.* The results indicated that the
461   extract caused damage to the cell membrane, leading to subsequent cell death. Active bioactive
462   compounds, such as graveolinine, flavonoid, chalepin, and rutamarin, have been identified in *R.*
463   *angustifolia* extracts and are known to contribute to alterations in cell morphology in cancer cells
464   (Kamal et al., 2021; Richardson et al., 2017). Rutamarin and chalepin were reported to exhibit
465   similar mechanisms, resulting in cell shrinkage, the formation of apoptotic bodies, and rounding
466   of cancer cells (Richardson et al., 2016; Suhaimi et al., 2017). Furthermore, flavonoids have been
467   shown to damage the cytoplasmic membrane, inhibit nucleic acid production, and disrupt energy
468   metabolism of bacterial cells (Tsuchiya & Iinuma, 2000). These factors may collectively
469   contribute to the cell death of *P. gingivalis*. In contrast, untreated control cells grew
470   exponentially while maintaining an intact structure. He et al., (2020) observed similar effects,
471   where quercetin-treated *P. gingivalis* exhibited cell elongation from its coccobacillus shape,
472   followed by cell shrinkage and eventual lysis due to deformed cell walls. Notably, the
473   mechanism of action of the extract resembled that of chlorhexidine, in which low concentrations
474   of chlorhexidine disrupt the integrity of the cell wall, ultimately resulting in damage.
475   Consequently, chlorhexidine penetrates the cell, inducing leakage of the cytoplasm that leads to
476   cell death (He et al., 2020).
477

478   The initial findings on the antimicrobial activity of the *R. angustifolia* extracts against the
479   monospecies bacterium *P. gingivalis* are promising, indicating potential effectiveness in
480   inhibiting this essential periodontal pathogen. However, since *P. gingivalis* is only one species
481   within the complex oral microbiome, these results may not fully reflect the impact of the extracts
482   on other oral microorganisms that contribute to oral health and disease. Therefore, to enhance the
483   robustness and generalizability of the findings, future studies should encompass a broader range
484   of oral bacterial species, such as *T. denticola, T. forsythia, S. mutans,* and *Fusobacterium*
485   *nucleatum*. This approach will provide a better understanding of the antimicrobial potential
486   across the diverse microbial community in the oral cavity and enhance the relevance of the
487   findings.
488
489   In this present study, 0.12% Chlorhexidine (CHX) was used as the positive control.
490   Chlorhexidine (CHX) is a widely used antibacterial agent in dentistry, known for its broad-
491   spectrum antibacterial properties against various pathogens, including periodontal bacteria such
492   as *P. gingivalis*. The concentration of 0.12% CHX is frequently incorporated into mouthwashes
493   and other oral care products, establishing it as a standard concentration commonly used in
494   numerous studies as a positive control in antibacterial tests. Utilizing 0.12% CHX as a positive
495   control provides researchers with a reliable benchmark to compare the antibacterial effectiveness
496   of natural products or extracts against a well-established and clinically validated antibacterial
497   agent. This approach ensures that the findings are relevant to clinical practice.
498
499   Furthermore, while the *in vitro* results are encouraging, it is essential to thoroughly evaluate
500   the biocompatibility of the antimicrobial agents to ensure their safety for human use. This
501   evaluation should include testing for potential cytotoxicity and assessing the effects on normal
502   human oral cells such as gingival and periodontal cells. Additionally, clinical efficacy should be
503   explored through further studies utilizing human cellular models to replicate real-life conditions.
504   Moreover, in addition to *in vitro* studies, *in vivo* research using animal models, such as rodents,
505   is crucial for evaluating the effectiveness of the extracts in a living organism. This evaluation
506   should consider factors such as bioavailability, immune response, and long-term safety.
507   Ultimately, these additional studies will offer a more comprehensive understanding of the
508   therapeutic potential of the extracts while ensuring their safety and effectiveness for future
509   application in human oral healthcare.
510

Conclusions

512
513   In conclusion, this *in vitro* study provides new evidence that *R. angustifolia* exhibits
514   antimicrobial activity against periodontal pathogens, notably *P. gingivalis*. These findings
515   suggest that *R. angustifolia* has the potential to be used as an alternative or adjunct treatment for
516   periodontal disease and warrants further investigation.
517

518 **Acknowledgements**

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525

526 **References**

527

528 Akpinar, A., Calisir, M., Cansın Karakan, N., Lektemur Alpan, A., Goze, F., & Poyraz, O.
529   (2017). Effects of curcumin on alveolar bone loss in experimental periodontitis in rats: A
530   morphometric and histopathologic study. *Int J Vitam Nutr Res*, 87(5-6), 262-270.
531   https://doi.org/10.1024/0300-9831/a000243
532 Amil, M. A., Rahman, S., Yap, L. F., Razak, F. A., Bakri, M. M., Salem, L. S. O., Lim, X. Y.,
533   Reduan, N. A., & Sim, K. S. (2024). Antimicrobial and antiproliferative effects of
534   zingiberaceae oils: A natural solution for oral health. *Chem Biodivers*, 21(3),
535   e202301836. https://doi.org/10.1002/cbdv.202301836
536 Arvizu, I. S., & Murray, S. R. (2021). A simple, quantitative assay for the detection of viable but
537   non-culturable (VBNC) bacteria. *STAR Protoc*, 2(3), 100738.
538   https://doi.org/10.1016/j.xpro.2021.100738
539 Bailly, C. (2023). *Ruta angustifolia* Pers. (narrow-leaved fringed rue): Pharmacological
540   properties and phytochemical profile. *Plants*, 12(4), 827. https://www.mdpi.com/2223-
541   7747/12/4/827
542 Betti, N., Shia, J. S., Kadhum, A. A. H., & Al-Amiery, A. A. (2024). Harnessing coumarin
543   chemistry: Antibacterial, antifungal, and antioxidant profiling of novel coumarin
544   derivatives. *Journal of Medicinal and Pharmaceutical Chemistry Research*, 6(10), 1530-
545   1546. https://doi.org/10.48309/jmpcr.2024.447556.1138
546 Bismelah, N. A., Ahmad, R., Mohamed Kassim, Z. H., Ismail, N. H., & Rasol, N. E. (2022). The
547   antibacterial effect of *Plectranthus scutellarioides* (L.) R.Br. leaves extract against
548   bacteria associated with peri-implantitis. *Journal of Traditional and Complementary
549   Medicine*, 12(6), 556-566. https://doi.org/https://doi.org/10.1016/j.jtcme.2022.07.002
550 Böhle, S., Röhner, E., Zippelius, T., Jacob, B., Matziolis, G., & Rohe, S. (2022). Cytotoxic effect
551   of sodium hypochlorite (Lavanox 0.08%) and chlorhexidine gluconate (Irrisept 0.05%)
552   on human osteoblasts. *Eur J Orthop Surg Traumatol*, 32(1), 81-89.
553   https://doi.org/10.1007/s00590-021-02907-3
554 Bostanci, N., & Belibasakis, G. N. (2012). *Porphyromonas gingivalis*: An invasive and evasive
555   opportunistic oral pathogen. *FEMS microbiology letters*, 333(1), 1-9.
556 Bustamante, M., Oomah, B. D., Mosi-Roa, Y., Rubilar, M., & Burgos-Díaz, C. (2020).
557   Probiotics as an adjunct therapy for the treatment of halitosis, dental caries and
558   periodontitis. *Probiotics Antimicrob Proteins*, 12(2), 325-334.
559   https://doi.org/10.1007/s12602-019-9521-4

560 Clinical and Laboratory Standard Institute (CLSI). (2020). Performance standards for anti-
561   microbial susceptibility testing. 30th Edition, M100.
562   https://clsi.org/media/3481/m100ed30_sample.pdf

563 Fadare, O. S., Singh, V., Enabulele, O. I., Shittu, O. H., & Pradhan, D. (2022). *In vitro*
564   evaluation of the synbiotic effect of probiotic *Lactobacillus* strains and garlic extract
565   against *Salmonella* species. *LWT*, 153, 112439.
566   https://doi.org/https://doi.org/10.1016/j.lwt.2021.112439

567 Fivenson, E. M., Rohs, P. D. A., Vettiger, A., Sardis, M. F., Torres, G., Forchoh, A., &
568   Bernhardt, T. G. (2023). A role for the Gram-negative outer membrane in bacterial shape
569   determination. *BioRxiv*. https://doi.org/10.1101/2023.02.03.527047

570 He, Z., Zhang, X., Song, Z., Li, L., Chang, H., Li, S., & Zhou, W. (2020). Quercetin inhibits
571   virulence properties of *Porphyromas gingivalis* in periodontal disease. *Scientific reports*,
572   10, 18313. https://doi.org/10.1038/s41598-020-74977-y

573 Howard, K. C., Gonzalez, O. A., & Garneau-Tsodikova, S. (2021). *Porphyromonas gingivalis*:
574   Where do we stand in our battle against this oral pathogen? *RSC Med Chem*, 12(5), 666-
575   704. https://doi.org/10.1039/d0md00424c

576 Kamal, L. Z. M., Adam, M. A. A., Shahpudin, S. N. M., Shuib, A. N., Sandai, R., Hassan, N. M.,
577   Tabana, Y., Basri, D. F., Than, L. T. L., & Sandai, D. (2021). Identification of alkaloid
578   compounds arborinine and graveoline from *Ruta angustifolia* (L.) Pers for their
579   antifungal potential against Isocitrate lyase (ICL1) gene of *Candida albicans*.
580   *Mycopathologia*, 186(2), 221-236. https://doi.org/10.1007/s11046-020-00523-z

581 Kobatake, E., Kobayashi, R., Kabuki, T., & Kurita-Ochiai, T. (2019). *Lactobacillus helveticus*
582   SBT2171 upregulates the expression of β-defensin and ameliorates periodontal disease
583   caused by *Porphyromonas gingivalis*. *Microbiol Immunol*, 63(8), 293-302.
584   https://doi.org/10.1111/1348-0421.12719

585 Koszelewski, D., Kowalczyk, P., Brodzka, A., Hrunyk, A., Kramkowski, K., & Ostaszewski, R.
586   (2023). Enzymatic synthesis of a novel coumarin aminophosphonates: Antibacterial
587   effects and oxidative stress modulation on selected *E. coli* strains. *Int J Mol Sci*, 24(8).
588   https://doi.org/10.3390/ijms24087609

589 Lu, L., Hu, W., Tian, Z., Yuan, D., Yi, G., Zhou, Y., Cheng, Q., Zhu, J., & Li, M. (2019).
590   Developing natural products as potential anti-biofilm agents. *Chinese Medicine*, 14(1),
591   11. https://doi.org/10.1186/s13020-019-0232-2

592 Luan, C., Yan, J., Jiang, N., Zhang, C., Geng, X., Li, Z., & Li, C. (2022). *Leuconostoc*
593   *mesenteroides* LVBH107 antibacterial activity against *Porphyromonas gingivalis* and
594   anti-inflammatory activity against *P. gingivalis* Lipopolysaccharide-Stimulated RAW
595   264.7 Cells. *Nutrients*, 14(13), 2584. https://www.mdpi.com/2072-6643/14/13/2584

596 https://mdpi-res.com/d_attachment/nutrients/nutrients-14-02584/article_deploy/nutrients-14-
597   02584.pdf?version=1655908756

598 Madhloom, A. F., Bashir Hashim Al-Taweel, F., Sha, A. M., & Raad Abdulbaqi, H. (2022).
599   Antimicrobial effect of *Moringa oleifera* L. and red pomegranate against clinically
600   isolated *Porphyromonas gingivalis*: *In vitro* study. *Arch Razi Inst*, 77(4), 1405-1419.
601   https://doi.org/10.22092/ari.2022.357513.2051

602 Michaylova, M., Yungareva, T., Urshev, Z., Dermendzieva, Y., Yaneva, B., & Dobrev, I. (2021).
603   Probiotic candidates among dairy Lactobacilli and *Streptococcus thermophiles* strains for
604   control of the oral pathogen *Porphyromonas gingivalis*. *Folia Med (Plovdiv)*, 63(5), 720-
605   725. https://doi.org/10.3897/folmed.63.e56551

606 Mitchell, J. (2011). *Streptococcus mitis*: Walking the line between commensalism and
607   pathogenesis. *Molecular oral microbiology*, 26(2), 89-98.
608 Nahar, L., El-Seedi, H. R., Khalifa, S. A. M., Mohammadhosseini, M., & Sarker, S. D. (2021).
609   Ruta essential oils: Composition and bioactivities. *Molecules*, 26(16).
610   https://doi.org/10.3390/molecules26164766
611 Noer, S., Abinawanto, B. M. B., & Bowolaksono, A. (2021). Inhibition of *Candida albicans* and
612   *Streptococcus mutans* (single and mix species) biofilms by crude extract of *Ruta*
613   *angustifolia*. *Natural Volatiles & Essential Oils*, 8(4), 10533-10543.
614   https://www.nveo.org/index.php/journal/article/view/2170
615 Nordin, M. A., Wan Harun, W. H., & Abdul Razak, F. (2013). Antifungal susceptibility and
616   growth inhibitory response of oral Candida species to *Brucea javanica Linn.* extract.
617   *BMC Complement Altern Med*, 13, 342. https://doi.org/10.1186/1472-6882-13-342
618 Paranagama, M. P., Piyarathne, N. S., Nandasena, T. L., Jayatilake, S., Navaratne, A., Galhena,
619   B. P., Williams, S., Rajapakse, J., & Kita, K. (2020). The *Porphyromonas gingivalis*
620   inhibitory effects, antioxidant effects and the safety of a Sri Lankan traditional betel quid
621   - An *in vitro* study. *BMC Complementary Medicine and Therapies*, 20(1), 259.
622   https://doi.org/10.1186/s12906-020-03048-6
623 Pavão, F., Castilho, M., Pupo, M. T., Dias, R., Correa, A., Fernandes, J., da Silva, M. d. G.,
624   Mafezoli, J., Vieira, P., & Oliva, G. (2002). Structure of *Trypanosoma cruzi* glycosomal
625   glyceraldehyde-3-phosphate dehydrogenase complexed with chalepin, a natural product
626   inhibitor, at 1.95 Å resolution. *FEBS letters*, 520(1-3), 13-17.
627 Richardson, J. S., Sethi, G., Lee, G. S., & Malek, S. N. (2016). Chalepin: isolated from *Ruta*
628   *angustifolia* L. Pers induces mitochondrial mediated apoptosis in lung carcinoma cells.
629   *BMC Complement Altern Med*, 16(1), 389. https://doi.org/10.1186/s12906-016-1368-6
630 Richardson, J. S. M., Aminudin, N., & Abd Malek, S. N. (2017). Chalepin: A compound from
631   *Ruta angustifolia* L. Pers exhibits cell cycle arrest at S phase, suppresses Nuclear Factor-
632   Kappa B (NF-κB) pathway, Signal Transducer and Activation of Transcription 3
633   (STAT3) phosphorylation and extrinsic apoptotic pathway in non-small cell lung cancer
634   carcinoma (A549). *Pharmacogn Mag*, 13(Suppl 3), S489-s498.
635   https://doi.org/10.4103/pm.pm_13_17
636 Santhosh, V. N., Ankola, A., Sankeshwari, R., Jalihal, S., Varghese, A. S., Chavan, P., & Shah,
637   M. A. (2023). Impact of dental caries and periodontal disease on oral health-related
638   quality of life among the Karnataka State Reserve Police Personnel in Belagavi, India. *J*
639   *Prev Med Hyg*, 64(1), E78-e86. https://doi.org/10.15167/2421-4248/jpmh2023.64.1.2900
640 Saquib, S. A., AlQahtani, N. A., Ahmad, I., Kader, M. A., Al Shahrani, S. S., & Asiri, E. A.
641   (2019). Evaluation and comparison of antibacterial efficacy of herbal extracts in
642   combination with antibiotics on periodontal pathobionts: An *in vitro* microbiological
643   study. *Antibiotics*, 8(3), 89. https://www.mdpi.com/2079-6382/8/3/89
644 Seidel, V. (2012). Initial and bulk extraction of natural products isolation. *Methods Mol Biol*,
645   864, 27-41. https://doi.org/10.1007/978-1-61779-624-1_2
646 Sharma, D., & Saharan, B. S. (2016). Functional characterization of biomedical potential of
647   biosurfactant produced by *Lactobacillus helveticus*. *Biotechnology Reports*, 11, 27-35.
648   https://doi.org/https://doi.org/10.1016/j.btre.2016.05.001
649 Siddiqui, D. A., Fidai, A. B., Natarajan, S. G., & Rodrigues, D. C. (2022). Succession of oral
650   bacterial colonizers on dental implant materials: An *in vitro* biofilm model. *Dent Mater*,
651   38(2), 384-396. https://doi.org/10.1016/j.dental.2021.12.021

652 Siripipatthanakul, S., & Sixl-Daniell, K. (2021). Strategic management in oral care product
653   market: A case study of colgate-palmolive (Thailand) limited of the creative commons
654   attribution license (CC BY 4.0).
655 Suhaimi, S. A., Hong, S. L., & Abdul Malek, S. N. (2017). Rutamarin, an active constituent from
656   *Ruta angustifolia* Pers., induced apoptotic cell death in the HT29 colon adenocarcinoma
657   cell line. *Pharmacogn Mag*, 13(Suppl 2), S179-s188.
658   https://doi.org/10.4103/pm.pm_432_16
659 Tsuchiya, H., & Iinuma, M. (2000). Reduction of membrane fluidity by antibacterial
660   sophoraflavanone G isolated from *Sophora exigua*. *Phytomedicine*, 7(2), 161-165.
661 Wahyuni, T. S., Widyawaruyanti, A., Lusida, M. I., Fuad, A., Soetjipto, Fuchino, H., Kawahara,
662   N., Hayashi, Y., Aoki, C., & Hotta, H. (2014). Inhibition of hepatitis C virus replication
663   by chalepin and pseudane IX isolated from *Ruta angustifolia* leaves. *Fitoterapia*, 99, 276-
664   283. https://doi.org/10.1016/j.fitote.2014.10.011
665 Widyarman, A. S., Bachtiar, E. W., Bachtiar, B. M., & Seneviratne, C. J. (2019). Inhibitory
666   effect of probiotic lactobacilli against *Streptococcus mutans* and *Porphyromonas
667   gingivalis* biofilms. *Scientific Dental Journal*, 3(2), 50.
668 Wu, T.-S., Wang, J.-J., Iou, S.-C., Chang, H.-C., Chen, Y.-P., Kuo, Y.-H., Chang, Y.-L., & Teng,
669   C.-M. (2003). Cytotoxic and antiplatelet aggregation principles of *Ruta Graveolens*.
670   *Journal of the Chinese Chemical Society*, 50. https://doi.org/10.1002/jccs.200300024
671 Xu, W., Zhou, W., Wang, H., & Liang, S. (2020). Roles of *Porphyromonas gingivalis* and its
672   virulence factors in periodontitis. *Adv Protein Chem Struct Biol*, 120, 45-84.
673   https://doi.org/10.1016/bs.apcsb.2019.12.001
674 Yan, Y., Li, X., Zhang, C., Lv, L., Gao, B., & Li, M. (2021). Research progress on antibacterial
675   activities and mechanisms of natural alkaloids: A Review. *Antibiotics (Basel)*, 10(3).
676   https://doi.org/10.3390/antibiotics10030318
677 Yan, Y., Xia, X., Fatima, A., Zhang, L., Yuan, G., Lian, F., & Wang, Y. (2024). Antibacterial
678   activity and mechanisms of plant flavonoids against Gram-Negative bacteria based on the
679   antibacterial statistical model. *Pharmaceuticals*, 17(3), 292. https://www.mdpi.com/1424-
680   8247/17/3/292
681 Zou, P., Cao, P., Liu, J., Li, P., & Luan, Q. (2022). Comparisons of the killing effect of direct
682   current partially mediated by reactive oxygen species on *Porphyromonas gingivalis* and
683   *Prevotella intermedia* in planktonic state and biofilm state – An *in vitro* study. *Journal of
684   Dental Sciences*, 17(1), 459-467. https://doi.org/https://doi.org/10.1016/j.jds.2021.07.025

685

Figure 1

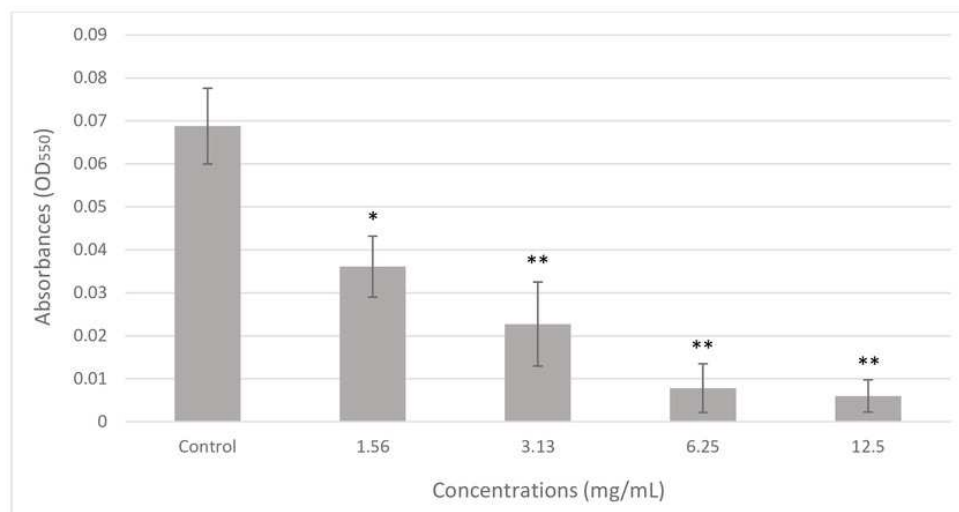Antimicrobial effect of *R. angustifolia* extracts against *P. gingivalis*.

Bacterial cells were treated with 50 and 100 mg/mL of (A) crude methanol extract, (B) hexane and (C) chloroform fractionated extract, respectively. The representative images display the measured inhibition zone (in mm) of the extracts; 0.12% CHX (used as a positive control); and 1% DMSO (served as the negative control). Larger zones indicate greater inhibition.
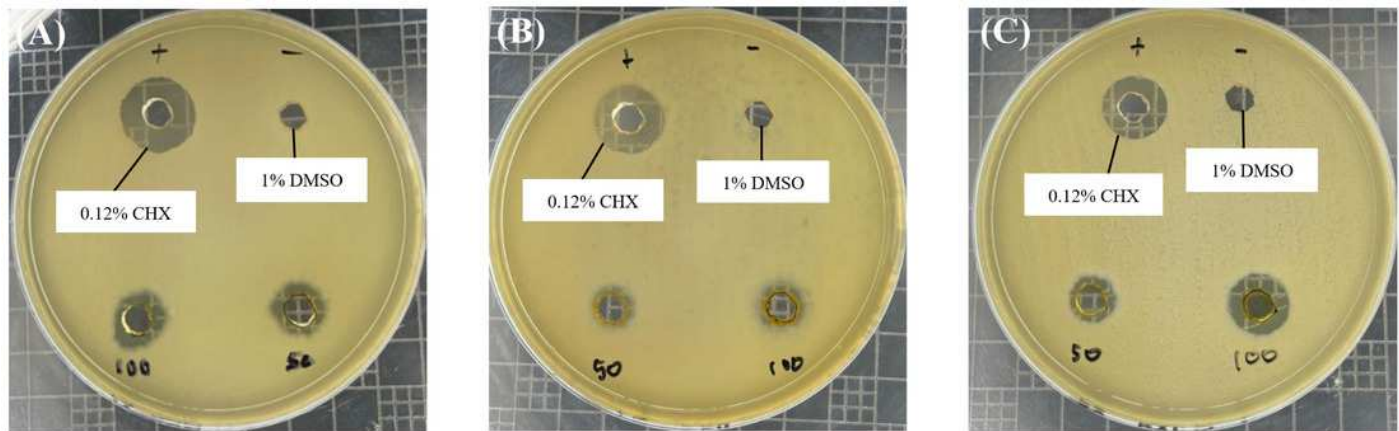
Figure 2

Effect of chloroform fractionated extract of *R. angustifolia* against *P. gingivalis* biofilm via the crystal violet assay.

The untreated *P. gingivalis* biofilm, is the negative control. Experiments were conducted in triplicate and values are represented as mean ± standard deviation. Statistically significant with ANOVA (p-value < 0.05) and Tukey's HSD post hoc analysis (*p < 0.05, **p < 0.001 versus control according to analysis of variance).
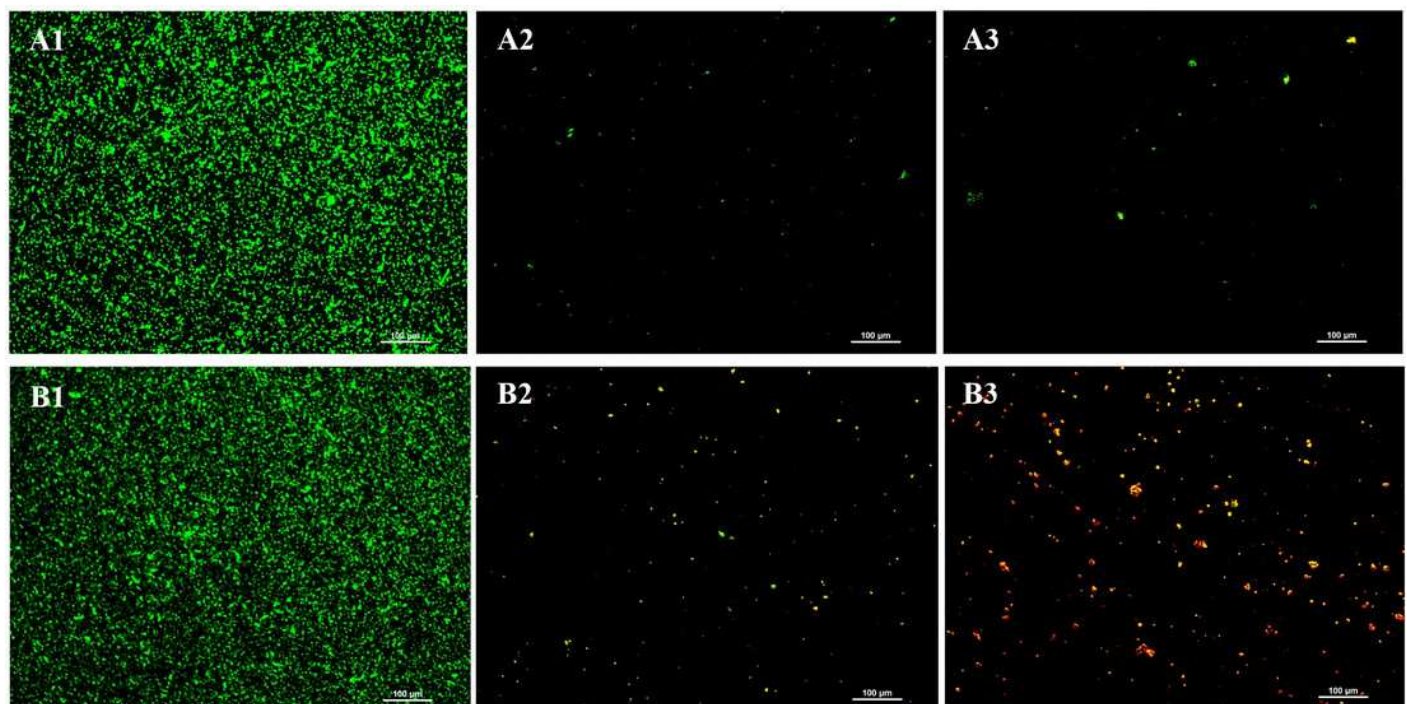
Figure 2: Effect of chloroform fractionated extract of *R. angustifolia* against *P. gingivalis* biofilm via the crystal violet assay. The untreated *P. gingivalis* biofilm, is the negative control. Experiments were conducted in triplicate and values are represented as mean ± standard deviation. Statistically significant with ANOVA (p-value < 0.05) and Tukey's HSD post hoc analysis (*p < 0.05, **p < 0.001 versus control according to analysis of variance).

Figure 3

Representative images from LIVE/DEAD® BacLight™ viability staining of untreated and treated *P. gingivalis* with chloroform fractionated extract of *R. angustifolia.*

Biofilm images at (A) 24 hours and (B) 48 hours incubation time. *P. gingivalis* biofilm (1) without any treatment, *P. gingivalis* biofilm after treated with (2) 3.13 mg/mL and (3) 6.25 mg/mL of chloroform fractionated extract of *R. angustifolia.* Bacteria that are viable/alive cells were stained green and non-viable/dead cells were stained red. (Magnification: 20x, scale bar: 100 μm).

Figure 4

FE-SEM images of (A) non-treated and (B) treated *P. gingivalis* with 6.25 mg/mL of chloroform fractionated extract of *R. angustifolia*.

Red arrows: Swollen rods, Blue arrow: Cells ruptured to form deep craters. Magnification: 80kx (left), 15kx (right).
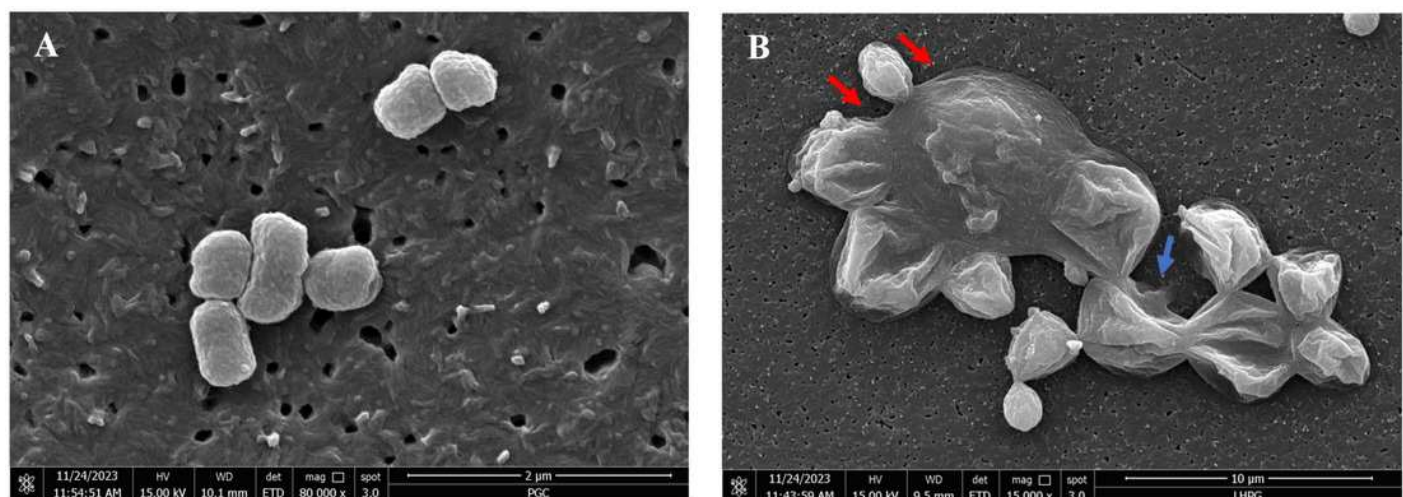
Table 1(on next page)

The inhibition zone diameters of *R. angustifolia* extracts against *P. gingivalis*.

[a]0.12% CHX served as the positive control, whilst 1% DMSO as the negative control against *P. gingivalis*. Experiments were conducted in triplicate and tabulated values are represented as mean ± standard deviation. Statistically significant with Kruskal-Wallis (p-value < 0.05) and Dunn's post-hoc analysis (*p < 0.05 versus control according to analysis of variance).

1   **Table 1:** The inhibition zone diameters of *R. angustifolia* extracts against *P. gingivalis.*

Oral microorganism		Inhibition zone diameter (mm)		
		R. angustifolia extracts		
		Crude methanol	Hexane	Chloroform
P. gingivalis	50 mg/mL	8.12 ± 0.77*	6.98 ± 0.26*	8.32 ± 1.08*
	100 mg/mL	9.30 ± 0.26	8.19 ± 0.27*	11.65 ± 0.62
	0.12% CHX[a]	13.79 ± 0.71	13.45 ± 0.79	13.34 ± 0.73

2   [a]0.12% CHX served as the positive control, whilst 1% DMSO as the negative control against *P.*
3   *gingivalis*. Experiments were conducted in triplicate and tabulated values are represented as mean
4   ± standard deviation. Statistically significant with Kruskal-Wallis (p-value < 0.05) and Dunn's
5   post-hoc analysis (*p < 0.05 versus control according to analysis of variance).

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Table 2(on next page)

MIC & MBC of chloroform fractionated extract of *R. angustifolia* against *P. gingivalis*.

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

1 **Table 2:** MIC & MBC of chloroform fractionated extract of *R. angustifolia* against *P. gingivalis.*

Microorganism	Chloroform fractionated extract of *R. angustifolia* (mg/mL)	
	MIC	MBC
P. gingivalis	6.25	6.25

2 MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

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